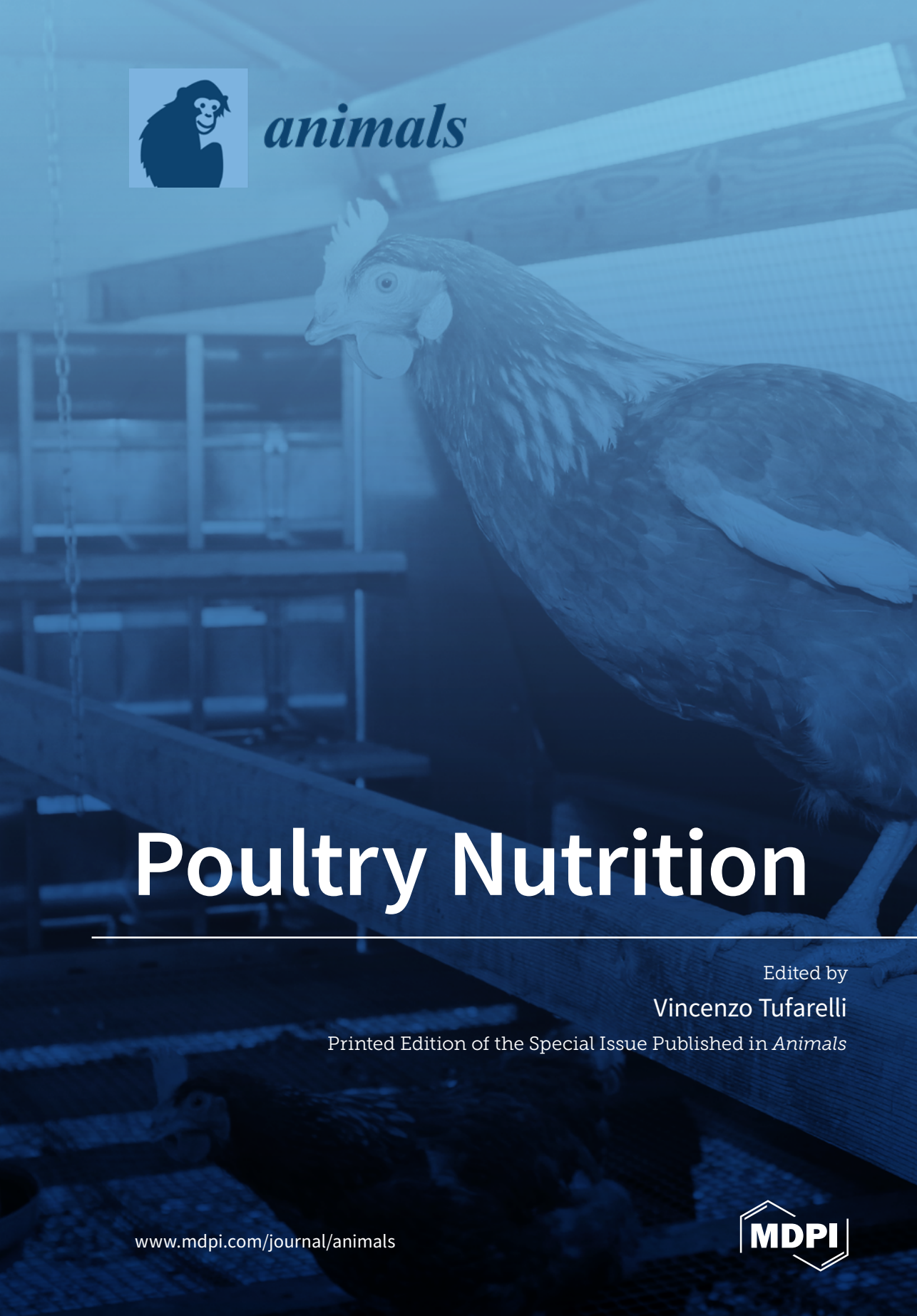

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Vincenzo Tufarelli

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Cover image courtesy of Vincenzo Tufarelli.

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About the Editor

Vincenzo Tufarelli is an Associate Professor in Animal Nutrition at the Department of Emergency and Organ Transplants (DETO), Section of Veterinary Science and Animal Production of the University of Bari Aldo Moro, Italy. He has considerable experience in animal and poultry science, with a particular interest in nutrition and feed technology. He is involved in many research collaborations, even with international institutions, in the field of animal science and feed quality. He serves as an editorial board member and peer reviewer for many indexed journals and he is the author of more than 180 scientific papers published in international journals and proceedings of national and international conferences.

Preface to "Poultry Nutrition"

Nutrition is defined in a range of ways, but is frequently inadequately understood. It is a simple concept, yet encompasses much complexity. In recent years, advances in poultry production, introduction of new and alternative products, and the development of new dietary management approaches have made it possible to increase poultry performance. However, to realize this, there must be further focus on diet quality. Producing suitable poultry products requires knowing the factors affecting quality, then exercising management accordingly. This book presents cross-discipline studies covering many aspects, ranging from poultry production and nutrition to alternative feeding systems, with the aim of disseminating information suitable for improving poultry health and products quality. Moreover, the purpose of this book is also to provide information about feed quality and alternative ingredients testing that can be used to improve poultry performance and producers' returns.

Vincenzo Tufarelli
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Article

Activity and Anti-Aflatoxigenic Effect of Indigenously Characterized Probiotic Lactobacilli against *Aspergillus flavus*—A Common Poultry Feed Contaminant

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Simple Summary: Mycotoxicosis in poultry has been seriously damaging the poultry production in Pakistan, resulting in economic losses to the country. The present study may act as a preliminary step for exploring the effect of indigenously characterized potential probiotic lactobacilli on aflatoxin production by *Aspergillus flavus*. The present study explored anti-fungal *Lactobacillus* strains. Further investigations revealed their in vitro aflatoxin binding and anti-aflatoxigenic capabilities. These findings demonstrated *L. gallinarum* PL 149 to be an effective binder of aflatoxin B1 which may be used as a biocontrol agent against *A. flavus* and aflatoxin B1 production. It may be further employed for aflatoxin binding in poultry gut after in vivo evaluations.

Abstract: Aflatoxin contamination in human food and animal feed is a threat to public safety. Aflatoxin B1 (AFB1) can be especially damaging to poultry production and consequently economic development of Pakistan. The present study assessed the in vitro binding of AFB1 by indigenously characterized probiotic lactobacilli. Six isolates (*Lactobacillus gallinarum* PDP 10, *Lactobacillus reuetri* FYP 38, *Lactobacillus fermentum* PDP 24, *Lactobacillus gallinarum* PL 53, *Lactobacillus paracasei* PL 120, and *Lactobacillus gallinarum* PL 149) were tested for activity against toxigenic *Aspergillus flavus* W-7.1 (AFB1 producer) by well diffusion assay. Only three isolates (PL 53, PL 120, and PL 149) had activity against *A. flavus* W-7.1. The ameliorative effect of these probiotic isolates on AFB1 production was determined by co-culturing fungus with lactobacilli for 12 days, followed by aflatoxin quantification by high-performance liquid chromatography. In vitro AFB1 binding capacities of lactobacilli were determined by their incubation with a standard amount of AFB1 in phosphate buffer saline at 37 °C for 2 h. AFB1 binding capacities of isolates ranged from 28–65%. Four isolates (PDP 10, PDP 24, PL 120, and PL 149) also ceased aflatoxin production completely, whereas PL 53 showed 55% reduction in AFB1 production as compared to control. The present study demonstrated *Lactobacillus gallinarum* PL 149 to be an effective candidate AFB1 binding agent against *Aspergillus flavus*. These findings further support the binding ability of lactic acid bacteria for dietary contaminants.

Keywords: Aflatoxin B1; *Lactobacillus*; anti-fungal; *Aspergillus flavus*; in vitro; poultry

1. Introduction

Poultry is one of the major sectors playing a role in the enhanced economic activity of Pakistan but still it faces a lot of problems, including mycotoxicosis. Mycotoxins are toxic secondary metabolites of fungal origin, which can cause various diseases and death in animals and humans. Ergot alkaloids, fumonisins, patulin, aflatoxin, citrinin, trichothecenes, ochratoxin A, and zearalenone are all examples of some different mycotoxins. Aflatoxins, produced by *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus nomius*, are of great importance because of their biological and biochemical effects on living systems [1]. Aflatoxin-producing molds are globally and can flourish on a variety of food and feed commodities during production, processing, storage, and transportation procedures [1–3]. These molds can infect crops, especially in hot and humid conditions, resulting in economic loss and adverse effects on consumers' health.

Aflatoxin is a potent carcinogen, mutagen, contains hepatotoxic and immunosuppressant effects and inhibit several metabolic systems resulting in liver and kidney damage [1,4]. Aflatoxin and citrinin cause increased fragility of the vascular system and produce hemorrhages in body tissues. Among aflatoxins, aflatoxin B1 is the most potent, and it is categorized among class 1 human carcinogens. Different factors including pH, temperature, water activity, available nutrients, and competitive inhibition by other microorganisms can affect aflatoxin production in feed [3]. Appropriate harvesting and storage conditions of crops and feed play important roles in aflatoxin reduction.

Various methods have been employed for the removal or inactivation of aflatoxins, including physical, biological, and chemical methods. Chemical treatments may include roasting, ammoniation, and other solvent extraction techniques. Many aflatoxin binders, like activated carbon and various mineral clays, are commercially available and act as sequestering agents and tightly bind aflatoxin; the resulting binding complex is then excreted from the animal's body [5]. These toxin binders can restore the nutritional value of the feed, but these chemical methods are unsafe, unhealthy, and expensive [6]. Toxin removal by microorganisms is a promising and economical method for decontaminating raw materials and food [7]. Numerous investigations have reported the inhibitory effects of microbes including actinomycetes, yeast, mold, and bacteria on mold growth and aflatoxin production [3]. Thus, beneficial microorganisms may serve as an alternative therapy for mycotoxicosis.

Anti-mutagenic lactic acid bacteria can remove mutagens from food by physical means [8]. Toxin binding by bacteria occurs through cell wall components, namely polysaccharides or polypeptides. Many researchers have studied this binding mechanism, but the exact mechanism of binding is still unknown [9].

Researchers are paying more attention towards preventing the absorption of aflatoxins in the gastrointestinal tracts of users by the aid of probiotic bacterial supplements in food and feed [10]. According to the World Health Organization (WHO), probiotics are defined as live microorganisms which when administered in adequate amounts exert healthy effects to host [11]. *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Saccharomyces*, and *Bacillus* may serve as probiotics.

Lactobacilli can efficiently remove aflatoxins from contaminated broth. The toxin removal mechanism involves sequestration by binding the toxin to the cell wall instead of metabolic degradation [12]. The present study may act as a preliminary step for studying the effect of indigenously characterized potential probiotic lactobacilli on aflatoxin production by *Aspergillus flavus*, so that lactobacilli can be used as biocontrol agents. The present study also assessed the in vitro AFB1 binding capacity of *Lactobacillus* spp., so that these probiotic strains can be employed as toxin binders in place of chemicals in animal feed and thereby the harmful effects of chemical toxin binders can be avoided.

2. Materials and Methods

2.1. Identification of Isolates

Previously characterized probiotic lactobacilli (n = 6) of poultry and fermented food origin [13] and toxigenic *Aspergillus flavus* W-7.1 were procured from the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, as listed in Table 1. Lactobacilli were revived using De Man, Rogosa, and Sharpe (MRS) agar and identified as describe previously [14]. Fungal strain was cultured on Sabouraud Dextrose Agar (SDA) medium incubated at 37 °C for 5–6 days. Culture and microscopic characters were observed for identification as described previously [15].

Table 1. Antifungal activity of cell free supernatants of lactobacilli.

Isolates	GenBank Accession #	Zones of Inhibition (mm)	
		pH 4	pH 7
L. gallinarum PDP 10	MF980924	NZ	NZ
L. reuteri PDP 24	MF980925	NZ	NZ
L. fermentum FYP 38	MF980923	NZ	NZ
L. gallinarum PL 53	MK182967	13	12
L. paracasei PL 120	MK182968	16	14
L. gallinarum PL 149	MK182969	17	15

NZ: No zone of inhibition.

2.2. Antifungal Activity of Lactobacilli

Antifungal activity of lactobacilli (n = 6) was determined by well diffusion assay as described elsewhere [16]. Briefly, SDA medium seeded with fungal spores (10^7 spores/mL) was poured into sterile Petri dishes and allowed to solidify. Wells were punctured in the medium which were then sealed with sterile molten agar. Cell free supernatant (100 µL) of each lactobacilli strain was added into the respective wells. After 3–4 days incubation at 28 °C aerobically, the diameter of zones of inhibition (mm) was measured.

2.3. Effect of Lactobacilli on Aflatoxin Production

The effect of lactobacilli on aflatoxin production by *Aspergillus flavus* was observed by inoculating 1 mL bacterial suspension (1 McFarland) in yeast extract sucrose broth (YESB) supplemented with a standard amount of fungal spores (10^7 spores/mL), followed by incubation at 28 °C and 100 rpm for 10 days. YESB media supplemented with known fungal spores and plain YESB media without any inoculation were also incubated as positive and negative controls, respectively. After incubation, medium containing lactobacilli and fungus was filtered through Whatman filter paper no 1 and aflatoxin B1 quantity in filtrate was measured by high-performance liquid chromatography (HPLC) and compared with controls [6]. Aflatoxin B1 was detected by HPLC and quantified using the following formulae:

$$\text{Quantity of Aflatoxins}\left(\frac{\text{ng}}{\text{mL}}\right) = \frac{\text{peak area of sample}}{\text{peak area of standard}} \times 100 \quad (1)$$

$$\%\text{ age reduction} = \frac{1 - (\text{Peak area of AFB1 in treatment})}{(\text{Peak area of AFB1 in control})} \quad (2)$$

2.4. Aflatoxin B1 Extraction

For toxin extraction, a previously established protocol was used with modifications [17]. Briefly, broth culture of *Aspergillus flavus* was autoclaved at 121 °C and 15 psi and then homogenized using homogenizer. Twenty-five grams of homogenate was treated with chloroform (90 mL), methanol (10 mL), NaCl (5 g), and distilled water (10 mL) and incubated at 37 °C with continuous shaking

(150–160 rpm) for 30 min. Filtration was carried out using Whatman filter paper #4 and filtrate was concentrated in a water bath at 50 °C. Concentrate was ground to fine powder and reconstituted in 3 mL chloroform volume and stored at 4 °C.

2.5. Toxin Binding Assay

Standard aflatoxin B1 solution was prepared by the method described elsewhere [18]. Prepared standard aflatoxin solution was then added to sterile phosphate buffer saline (PBS) containing lactobacilli culture (1 McFarland). After 2 h of incubation, cells with bound toxin were separated by centrifugation at 10,000 rpm for 5 min and unbound aflatoxin in supernatant was quantified by HPLC.

2.6. High Performance Liquid Chromatography (HPLC)

Aflatoxins were quantified by Agilent HPLC system, 1100 series (Agilent, Santa Clara, CA, USA) as described previously [19]. A mixture of acetonitrile, water, and methanol was used as mobile phase at a flow rate of 1 mL per minute. Mobile phase was firstly purified using a filtration assembly and then sonicated for 10 min at 20 °C in order to avoid gas bubbles. Next, 20 µL samples were injected using a micro-syringe. After 15 min, ultra violet (UV) absorbance was recorded at 254 nm. Sample peaks were analyzed and compared with standard UV absorption data of secondary metabolites at various retention times. Limit of detection (LOD) and limit of quantification (LOQ) of standard aflatoxin were 0.01 ng/mL–100 µg/mL and 0.1 ng/mL–100 µg/mL, respectively.

2.7. Statistical Analysis

Mitigation of aflatoxin production and toxin binding capacity of lactobacilli was compared by one-way ANOVA (analysis of variance) followed by Turkey's multiple comparison test using Graph pad prism 5.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

A total of six potential probiotic lactobacilli, including *Lactobacillus gallinarum* PDP 10, *Lactobacillus reuteri* PDP 24, *Lactobacillus fermentum* FYP 38, *Lactobacillus gallinarum* PL 53, *Lactobacillus paracasei* PL 120, and *Lactobacillus gallinarum* PL 149, were procured from the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan. All isolates were Gram-positive rods and catalase negative.

Only three isolates (PL 53, PL 120, and PL 149) had antifungal activity observed by well diffusion assay, as illustrated in Table 1 and Figure 1.

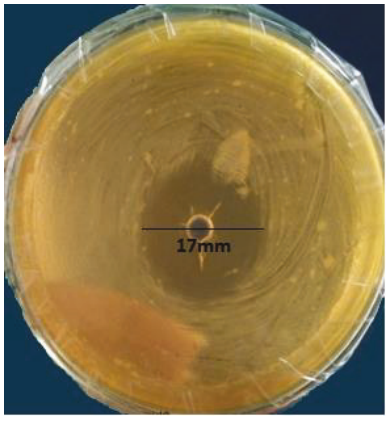

Figure 1. Activity of cell free supernatant of *Lactobacillus gallinarum* PL 149 against *Aspergillus flavus.*

Four isolates (PDP 10, PDP 24, PL 120, and PL 149) showed 100% removal of AFB1, PL 53 caused 55.2% reduction, while FYP 38 showed an enhancing effect on aflatoxin B1 production, as described in Table 2. All isolates showed a varied degree of toxin binding capacities, as described in Table 3 and Figure 2. PL 149 was the most effective binder of aflatoxin B1, with 65% capacity.

Table 2. Effect of lactobacilli on aflatoxin B1 production.

Isolates	Peak Areas	Quantity of AFB1 (ng/mL)	% Age Reduction
Standard	120.205	100	-
Control	0.58439	0.4	-
L. gallinarum PDP 10	ND	ND	100%
L. fermentum FYP 38	0.815847	0.6	−39.6%
L. reuteri PDP 24	ND	ND	100%
L. gallinarum PL 53	0.26124	0.2	55.2%
L. paracasei PL 120	ND	ND	100%
L. gallinarum PL 149	ND	ND	100%

AFB1: Aflatoxin B1; ND: Not detected.

Table 3. Aflatoxin B1 binding capacity of probiotic lactobacilli.

Isolates	Peak Areas	Quantity of AFB1 Bound (ng/mL)	% Age Reduction (Binding Capacity)
Standard	108.246	100	-
Control	927.763	857	-
L. gallinarum PDP 10	451.63	417.2	51.3%
L. fermentum FYP 38	407.553	376.5	56%
L. reuteri PDP 24	909.624	840	2%
L. gallinarum PL 53	546.523	504.8	42%
L. paracasei PL 120	676.472	624.9	28%
L. gallinarum PL 149	326.775	301.8	65%

AFB1: Aflatoxin B1.

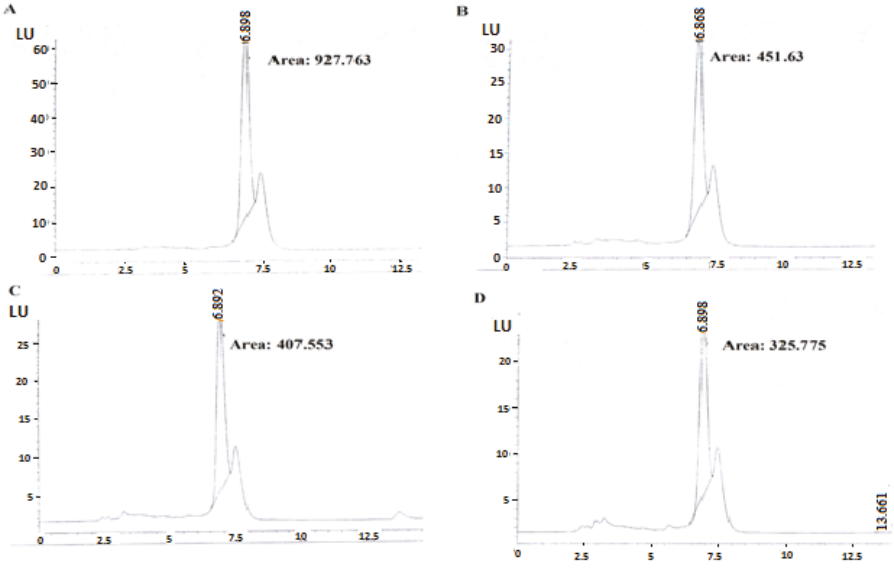

Figure 2. High-performance liquid chromatography chromatograms of aflatoxin B1 present in control and suspension after treatment with lactobacilli: (**a**) Control; (**b**) PDP 10; (**c**) FYP 38; (**d**) PL 149.

4. Discussion

Aflatoxins represent a group of fungal secondary metabolites that are of great health and economic importance. In developing countries, greater than five billion people are at risk of chronic exposure to aflatoxins, which are capable of causing liver cancer [4]. Consequently, there is an increasing demand for novel preventive and controlling strategies for aflatoxin contaminations in food and feed. Recent studies have revealed the aflatoxin binding ability of lactobacilli. Many bacteria have been reported as aflatoxin binders, including *Flavobacterium aurantiacum*, *L. plantarum*, *L. pentosus*, and *L. beveris* [20–22]. Likewise, *Lactobacillus casei psuedoplantarum* 371, obtained from silage inoculum, inhibited aflatoxin B1 and G1 synthesis by *Aspergillus flavus* subsp. *parasiticus* NRRL 2999 in liquid medium [23]. In a previous study, a mixture of lactobacilli was found to reduce mold growth, germination of spores, and production of aflatoxins by *Aspergillus flavus* subsp. *parasiticus* [24]. A large number of such studies have been reported worldwide, but few related studies have been reported in Pakistan.

The present study can act as a preliminary step in a multistep study to investigate the anti-fungal, anti-aflatoxigenic, and in vitro AFB1 binding capacities of previously characterized indigenous phytase-solubilizing probiotic lactobacilli spp. of poultry and fermented food origin [13] against toxigenic *Aspergillus flavus*. This study identified three probiotic lactobacilli isolates (*Lactobacillus gallinarum* PL 53, *Lactobacillus paracasei* PL 120, and *Lactobacillus gallinarum* PL 149) as antifungal agents. Such inhibitory effects may be a result of lactic acid production or physical interaction of lactobacilli with mold. Similar inhibitory effects of *L. acidophilus* ATCC 4495 and *L. brevis* were also demonstrated previously against *Aspergillus flavus* and *Aspergillus parasiticus*, respectively [25,26].

Four isolates (PDP 10, PDP 24, PL 120, PL 149) in the present study ceased aflatoxin production completely, whereas PL 53 showed 55% reduction. On the other hand, FYP 38 showed an enhancing effect on aflatoxin B1 production. These variable results may depict different bacterial cell wall structures. Many other investigators have reported similar results, in which various lactic acid-producing bacteria, including *Lactobacillus*, were capable of inhibiting aflatoxin production, whereas some lactic acid bacteria, like *Lactococcus lactis*, stimulated aflatoxin biosynthesis [27]. Cell wall polysaccharides and peptidoglycans have been considered bacterial tools for mycotoxin binding [28]. Extracellular metabolites of *Lactobacillus casei* KC 324 has been reported to mitigate mold growth and aflatoxin production of *Aspergillus flavus* ATCC 15517 [29]. Commercial silage was once reported to contain inhibitory lactobacilli against aflatoxin B1 and G1 production [30]. *L. plantarum* ATCC 4008, *L. plantarum* 12006, *Lactobacillus plantarum* 299V, *L. paracasei* subsp. *paracasei* LMG 13552, and *L. rhamnosus* VT1 reduced aflatoxin production by 85–92% to 96.3–98.3% [31], whereas in the present study a 100% reduction in AFB1 production by *L. gallinarum* PDP 10 and PL 149, *L. reuteri* PDP 24, and *L. paracasei* PL 120 was observed. It may also be a result of very low aflatoxin production in control conditions as well. Yeast can also act as an effective biocontrol agent against aflatoxins. *S. boulardii* and *S. cerevisiae* individually reduced aflatoxin production by 72.8% and 65.8%, respectively, while their combinations reduced aflatoxin production from 71.1% to 96.1%. Supplementation of peanut grains with combinations of *S. boulardii* plus *L. delbrueckii*, *S. boulardii* and *S. cerevisiae*, *L. delbrueckii* and *S. cerevisiae* showed reduction by 96.1%, 66.7%, and 71.1%, respectively [32]. *Lactobacillus fermentum* PTCC 1744 and *Bifidobacterium bifidum* PTCC 1644 were also reported to reduce aflatoxin production by more than 99% in comparison with controls [6], although this report is contradictory to the present research which revealed the enhancing effect of *Lactobacillus fermentum* on AFB1 production by *A. flavus*.

In the present study, *Lactobacillus gallinarum* PDP 10, *Lactobacillus fermentum* FYP 38, Lactobacillus reuteri PDP 24, *Lactobacillus gallinarum* PL 53, *Lactobacillus paracasei* PL 120, and *Lactobacillus gallinarum* PL 149 showed aflatoxin binding capacities of 51.3%, 56%, 2%, 42%, 28%, and 65%, respectively. These results were quite similar with that of Fazeli et al. [33]. In a previous study, the aflatoxin B1 binding capacities of *Lactobacillus* and *Bifidobacterium* strains were assessed, which were found to range from 5.8% to 31.3% [12]. On the other hand, the present study reported up to 65% AFB1 binding abilities of probiotic lactobacilli. A previous study reported that *Lactobacillus casei* had a 20% AFB1 binding capacity [34], which is less than that of *L. paracasei* PL 120 (28%), whereas *Lactobacillus delbrueckii*

subsp. *lactis* was reported to have the maximum antifungal (67.43% reduction) and anti-aflatoxigenic (94.33% reduction) activity against *A. flavus* [35]. Another previous study reported 43.9–64.2% aflatoxin degrading ability of lactobacilli strains [36]. Past investigations revealed similar responses of non-viable and viable cells of *Enterococcus faecium* strains, whose binding abilities were insignificant statistically. Hence, it was hypothesized that AFB1 detoxification of *Enterococcus faecium* is a result of aflatoxin binding to bacterial cell wall; a similar mechanism has been also described by other relevant studies [37]. An in vivo experiment revealed the neutralizing capability of *Lactobacillus casei Shirota* on AFB1 toxicity on the intestine and body weight of host via binding processes [38]. Thus, lactic acid bacteria have been declared as good candidates to prevent aflatoxicosis in farm animals and poultry [9].

5. Conclusions

The present study reported the anti-fungal, anti-aflatoxigenic, and AFB1 binding capacity of six indigenously characterized probiotic strains. It was concluded that *L. gallinarum* PL 149 may inhibit the AFB1 production by *A. flavus* and also bind AFB1. *L. gallinarum* PL 149 may be employed for aflatoxin binding in poultry gut after in vivo evaluations.

Author Contributions: Conceptualization: M.N. and A.A.A.; methodology: M.N., A.A.A., N.A., and S.S. (Shagufta Saeed); software: M.N.; validation: M.N., A.A.A., M.R.Y., S.S. (Shagufta Saeed), and S.S. (Saba Sana); formal analysis: M.N., N.A., M.R.Y., A.M., and S.S. (Saba Sana); investigation: N.A., A.M., and S.S. (Shagufta Saeed); resources: M.N. and A.A.A.; data curation: N.A. and A.M.; writing—original draft preparation: A.M. and N.A.; writing—review and editing: A.M., N.A., S.S. (Saba Sana), M.R.Y., and M.N.; visualization: M.N., A.A.A., S.S. (Shagufta Saeed), and N.A.; supervision: M.N., A.A.A., S.S. (Shagufta Saeed), and S.S. (Saba Sana); project administration: M.N. and A.A.A.; funding acquisition: M.N. and A.A.A.

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Evaluation of the Dietary Supplementation of a Formulation Containing Ascorbic Acid and a Solid Dispersion of Curcumin with Boric Acid against *Salmonella* Enteritidis and Necrotic Enteritis in Broiler Chickens

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Simple Summary: Prophylactic or therapeutic administration of a 0.1% mixture containing ascorbic acid (AA) and a solid dispersion of curcumin (CUR) with polyvinylpyrrolidone (PVP) and boric acid (BA) (AA-CUR/PVP-BA) significantly reduced the concentration of *Salmonella* Enteritidis in broiler chickens and had a positive effect in slightly diminishing the negative impact of necrotic enteritis (NE).

Abstract: Two experiments were conducted to evaluate the effect of the prophylactic or therapeutic administration of a 0.1% mixture containing ascorbic acid and a solid dispersion of curcumin with polyvinylpyrrolidone and boric acid (AA-CUR/PVP-BA) against *Salmonella* Enteritidis (*S*. Enteritidis) in broiler chickens. A third experiment was conducted to evaluate the impact of the dietary administration of 0.1% AA-CUR/PVP-BA in a necrotic enteritis (NE) model in broiler chickens. The prophylactic administration of 0.1% AA-CUR/PVP-BA significantly decreased *S*. Enteritidis colonization in cecal tonsils (CT) when compared to the positive control group (PC, $p < 0.05$). The therapeutic administration of 0.1% AA-CUR/PVP-BA significantly reduced the concentration of *S*. Enteritidis by 2.05 and 2.71 log in crop and CT, respectively, when compared with the PC on day 10 post-*S*. Enteritidis challenge. Furthermore, the serum FITC-d concentration and total intestinal IgA levels were also significantly lower in chickens that received 0.1% AA-CUR/PVP-BA. Contrary, the PC group showed significantly higher total intestinal IgA levels compared to the negative control or AA-CUR/PVP-BA groups in the NE model. However, 0.1% AA-CUR/PVP-BA showed a better effect in reducing the concentration of *S*. Enteritidis when compared to the NE model. Further studies

with higher concentration of AA-CUR/PVP-BA into the feed to extend these preliminary results are currently being evaluated.

Keywords: chickens; ascorbic acid; curcumin; boric acid; necrotic enteritis; *Salmonella* Enteritidis

1. Introduction

In the poultry industry, enteric bacterial pathogens pose a threat to intestinal health and can contribute to the transmission of zoonotic diseases [1,2], increased mortality in poultry flocks, reduced feed efficiency, decreased rate of body weight gain and, therefore, increase in total production costs [3,4]. *Salmonella* infection and necrotic enteritis (NE) produced by *Clostridium perfringens* (CP) are two significant bacterial diseases in poultry [5,6]. Each year, millions of foodborne salmonellosis cases are reported, resulting in an estimated 155,000 deaths [6]. The most common route of transmission from animals to humans is through contaminated food such as meat, eggs and meat-based products [7,8]. It has also been reported that the presence of salmonellosis has caused significant economic losses in poultry production due to the reduction in overall performance and high mortality in affected flocks [4,9]. Another economically significant disease affecting chicken production is NE induced by CP and occurs in two forms. In its acute clinical form, NE can cause significant flock mortality [10–12] for several days, whereas the subclinical and chronic form can significantly impair performance [13,14]. The economic impact of NE on the worldwide poultry industry was estimated at over five to six billion dollars per year [15]. Thus, controlling enteric bacterial disease in poultry is essential to maintain efficient production and improve food safety [2].

Restrictions on the use of antimicrobials at sub-therapeutic doses in animal production [16] have pressured the poultry industry to look for alternatives to reduce the problems of bacterial resistance and also continue to provide performance benefits, eliminating foodborne pathogens as *Salmonella*, and reducing the NE incidence. Some of these alternatives include probiotics (yeasts or bacteria), plant derivatives such as essential oils or extracts, organic acids, enzymes and lysozymes [2,17,18].

A recent in vitro study published by our laboratory demonstrated the capability of 1% ascorbic acid (AA) to significantly reduce the concentration of *Salmonella* Enteritidis (*S*. Enteritidis) in the compartment that simulates the crop, derived from its acidification capacity, but not in the intestinal compartment since it degrades as the pH increases [19]. Another study showed that broiler chickens supplemented with 0.1% of a solid dispersion of curcumin (CUR) with polyvinylpyrrolidone (PVP) and boric acid (BA, CUR/PVP-BA) resulted in a lower *S*. Enteritidis recovery in both crop and cecal tonsils (CT) because of a possible synergistic effect between them [20]. Therefore, the purposes of the present study were to evaluate the effect of the prophylactic or therapeutic administration of a 0.1% mixture containing AA and a solid dispersion of CUR with PVP and BA (AA-CUR/PVP-BA) in broiler chickens infected with *S*. Enteritidis, as well as the impact of the dietary administration of 0.1% AA-CUR/PVP-BA in broilers using a laboratory NE challenge model.

2. Materials and Methods

2.1. Preparation of Treatments and Diets

The mixture containing AA and CUR/PVP-BA (AA-CUR/PVP-BA) were prepared in two steps. The first step involved the preparation of the solid dispersion of CUR/PVP-BA (1:1 ratio) as previously described [19]. Subsequently, a mixture of 90% of AA and 10% of microcrystalline cellulose (MCC) pH 10.2 was granulated, dried, sieved and finally associated with the solid dispersion of CUR/PVP-BA. The proportion of each component was 33.3% (1:1:1, AA:CUR/PVP:BA) and the particle size obtained was around 700 µm. The AA-CUR/PVP-BA mixture was mixed into the feed for 15 min using a rotary mixer to obtain the experimental diet with a final concentration of 0.1% (1 g/kg of feed).

The starter feed used in this study was formulated to approximate the nutritional requirements for broiler chickens as recommended by the National Research Council [21], and adjusted to the breeder's recommendations [22]. No antibiotics, coccidiostats or enzymes were added to the feed (Table 1). All animal handling procedures complied with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville (protocol #15006).

Table 1. Ingredient composition and nutrient content of a basal starter diet used in the experiments on as-is basis.

Item	Starter Diet
Ingredients (g/kg)	
Corn	574.5
Soybean meal	346.6
Poultry fat [1]	34.5
Dicalcium phosphate	18.6
Calcium carbonate	9.9
Salt	3.8
DL-Methionine	3.3
L-Lysine HCL	3.1
Threonine	1.2
Choline chloride 60%	2.0
Vitamin premix [2]	1.0
Mineral premix [3]	1.0
Antioxidant [4]	0.5
Calculated analysis	
Metabolizable energy (MJ/kg)	12.7
Crude protein (g/kg)	221.5

[1] Poultry fat West Coast Reduction LTD is primarily obtained from the tissue of poultry in the commercial process of rendering or extracting. This finished product was used as an energy source for animal and aquaculture feed. [2] Vitamin premix supplied per kg of diet: Retinol, 6 mg; cholecalciferol, 150 µg; dl-α-tocopherol, 67.5 mg; menadione, 9 mg; thiamine, 3 mg; riboflavin, 12 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.3 mg; cyanocobalamin, 0.4 mg. [3] Mineral premix supplied per kg of diet: Mn, 120 mg; Zn, 100 mg; Fe, 120 mg; copper, 10 mg to 15 mg; iodine, 0.7 mg; selenium, 0.2 mg; and cobalt, 0.2 mg. [4] Ethoxyquin.

2.2. Salmonella Strain and Culture Conditions

A primary poultry isolate of *Salmonella enterica* serovar Enteritidis (*S*. Enteritidis) bacteriophage type 13A, was obtained from the USDA National Veterinary Services Laboratory (Ames, IA, USA). This strain is resistant to 25 µg/mL of novobiocin (NO, catalog no. N-1628, Sigma, St. Louis, MO, USA) and was selected due to its resistance to 20 µg/mL of nalidixic acid (NA, catalog no. N-4382, Sigma, St. Louis, MO, USA) in our laboratory. In the present study, 100 µL of *S*. Enteritidis from a frozen aliquot were added to 10 mL of tryptic soy broth (TSB, Catalog No. 22092, Sigma, St. Louis, MO, USA) and incubated at 37 °C for 8 h, and passed three times every 8 h to ensure that all bacteria were in log phase as previously described [23]. Post-incubation, bacterial cells were washed three times with sterile 0.9% saline by centrifugation at 1864× *g* for 10 min, reconstituted in saline, quantified by densitometry with a spectrophotometer (Spectronic 20DC, Spectronic Instruments Thermo Scientific, Rochester, NY, USA) and finally diluted to an approximate concentration of 4×10^4 cfu/mL and 4×10^7 cfu/mL. Concentrations of *S*. Enteritidis were further verified by serial dilutions and plated on brilliant green agar (BGA, Catalog No. 70134, Sigma, St. Louis, MO, USA) with NO and NA for enumeration of actual cfu used in the experiment.

2.3. Experiment 1

Two independent trials were conducted to evaluate the prophylactic administration of 0.1% AA-CUR/PVP-BA in reducing the incidence of *S*. Enteritidis in broiler chickens. In each trial, 30 day-of-hatch male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were randomly allocated

to one of two groups (n = 15 chickens): (1) Group challenged with *S*. Enteritidis (positive control group, PC) and (2) 0.1% (w/w) AA-CUR/PVP-BA into feed and challenged with *S*. Enteritidis (AA-CUR/PVP-BA group). Chicks were housed in brooder battery cages, provided with their respective diet and water ad libitum, and maintained at an age-appropriate temperature during the seven days of the experiment. On day six of age, all chicks were orally challenged with 1×10^7 cfu of *S*. Enteritidis per bird. Chicks were euthanized by CO_2 inhalation 24 h post-*S*. Enteritidis challenge (Day 7), and samples of crop and CT were taken for *S*. Enteritidis recovery.

2.4. Experiment 2

The purpose of experiment 2 was to evaluate the effectiveness of the therapeutic administration of 0.1% AA-CUR/PVP-BA in broiler chickens infected with *S*. Enteritidis during three and 10 days of treatment. For this, an experiment with 60 one-day-old male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were challenged with 1×10^4 *S*. Enteritidis cfu per bird and randomly allocated to one of two groups (n = 30 chickens): (1) Positive control group (PC); (2) 0.1% (w/w) AA-CUR/PVP-BA into the feed (AA-CUR/PVP-BA group). Chicks were housed in brooder battery cages, provided with their respective diet and water ad libitum, and maintained at an age-appropriate temperature during the 10 days of the experiment. On days three and 10 post-*S*. Enteritidis challenge, 15 chicks from each group were euthanized by CO_2 inhalation, and the crop and CT from 12 birds per group were aseptically collected to evaluate *S*. Enteritidis recovery. Blood samples were collected from the femoral vein and centrifuged (1000× g for 15 min) to separate the serum for the determination of fluorescein isothiocyanate-dextran (FITC-d) concentration on day 10 post-*S*. Enteritidis challenge. The concentration of FITC-d administered was calculated based on group body weight at day 9 post-*S*. Enteritidis challenge. Furthermore, intestinal samples for total intestinal IgA levels were also collected.

2.5. Salmonella Recovery

In experiment 1 and 2, the crop and CT were homogenized and diluted with saline (1:4 w/v), and 10-fold dilutions were plated on BGA with NO and NA, incubated at 37 °C for 24 h to enumerate total *S*. Enteritidis colony forming units. Subsequently, the crop and CT samples were enriched in 2 × concentrated tetrathionate enrichment broth and further incubated at 37 °C for 24 h. Enrichment samples were streaked onto Xylose Lysine Tergitol-4 (XLT-4, Catalog No. 223410, BD DifcoTM) selective media for confirmation of *Salmonella* presence.

2.6. Experiment 3

This experiment was conducted to evaluate the impact of the dietary administration of 0.1% AA-CUR/PVP-BA on growth performance, intestinal barrier integrity and ileum lesions in broiler chickens using a laboratory necrotic enteritis (NE) challenge model. One hundred and twenty day-of-hatch male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were randomly assigned to three different groups of four replicates each with ten broiler chickens (n = 40/group): (1) Non-challenged control (negative control group, NC), (2) challenged control (positive control group, PC) and, (3) challenge control + 0.1% AA-CUR/PVP-BA into the feed (AA-CUR/PVP-BA group). All chicks were raised in floor pens (300 cm × 150 cm) for 21 days, provided with their respective diet and water ad libitum, and maintained at an age-appropriate temperature protocol during the experiment. On day 21, broiler chicks were euthanized by CO_2 inhalation, and the right half of the liver from 12 broiler chickens was aseptically collected in sterile sample bags (Nasco, Fort Atkinson, WI, USA) to evaluate bacterial translocation. Additionally, blood samples were collected from the femoral vein and centrifuged (1000× g for 15 min) to separate the serum for FITC-d estimation. The concentration of FITC-d administered was calculated based on group body weight at 20-day-old. Likewise, intestinal samples for the measurement of total intestinal IgA levels were also collected. Ileum NE lesion scores (ILS, n = 25 broiler chickens/group) were evaluated as recommended by Hofacre [24]: 0 = No lesions; 1 = thin-walled and friable intestines; 2 = focal necrosis, gas production and ulceration; 3 = extensive

necrosis, hemorrhage and gas-filled intestines; and 4 = generalized necrosis typical of field case, marked hemorrhage. Finally, body weight (BW) and body weight gain (BWG) were evaluated on a weekly basis. Feed intake (FI) and feed conversion ratio (FCR) were obtained at 21-d of age.

2.7. NE Model: Challenge or Ganisms

NE was induced in the broiler chickens as previously described [25,26] with slight modifications. Briefly, day-old broiler chickens were challenged with a concentration of 1×10^8 cfu of *Salmonella* Typhimurium (ST) per bird by oral gavage. This organism was isolated from poultry and obtained from the USDA National Veterinary Services Laboratory (Ames, IA, USA). The isolate was resistant to novobiocin (25 μg/mL of NO, catalog no. N-1628, Sigma) and was selected for resistance to nalidixic acid (20 μg/mL of NA, catalog no. N-4382, Sigma) in our laboratory. ST culture was performed in the same way as described above for *S*. Enteritidis. However, ST suspension was diluted to an approximate concentration of 4×10^8 cfu/mL. The concentration of ST was further verified by serial dilution and plated on brilliant green agar (BGA, Catalog no. 70134, Sigma) with NO and NA for enumeration of actual cfu used in the experiment. Subsequently, at day 13 of age, broiler chickens were challenged with a dose of 2×10^4 sporulated oocysts of *Eimeria maxima* (EM) per bird by oral gavage. Oocysts were propagated in vivo, according to previously published methods [27,28] and a preliminary dose titration study was carried out, offset by one week, to determine the EM challenge selection for the present study. At day 18 of age, chickens were challenged with a concentration of 1×10^9 cfu of a mixture of two *Clostridium perfringens* (CP) isolates per bird by oral gavage. Dr. Jack McReynolds (USDA-ARS, College Station, TX, USA) kindly donated the first strain of CP previously described in an NE challenge model [29]. The second strain was isolated from a separate *Eimeria* challenge experiment in our laboratory with an inadvertent resulting NE (four weeks of age). Then, a single aliquot of each isolate was individually amplified in TSB with thioglycolate (Catalog no. 212081, Becton Dickinson, Sparks, MD, USA) overnight and subsequently mixed. Plating 10-fold dilutions confirmed the concentration of CP on phenylethyl alcohol agar plates (PEA, Becton Dickinson, Sparks, MD, USA) with 5% sheep blood (Remel, Lenexa, KS, USA).

2.8. Liver Bacterial Translocation (BT)

Briefly, liver samples were homogenized, weighed and diluted 1:4 w/v with sterile 0.9% saline enriched with sodium thioglycolate. Then, 10-fold dilutions were plated on tryptic soy agar (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD, USA) with thioglycolate for anaerobic bacteria (AB) recovery. Plates were then incubated anaerobically at 37 °C for 24 h to enumerate entire AB colony forming units per g of tissue.

2.9. Serum Determination of FITC-d Leakage

FITC-d (MW 3–5 kDa; Sigma-Aldrich Co., St. Louis, MO, USA) was used as a marker of paracellular transport and mucosal barrier dysfunction [30,31]. One hour before the chicks were euthanized by CO_2 inhalation, 12 or 20 broiler chickens from each group were given an oral gavage dose of FITC-d (8.32 mg/kg of body weight), and three or five broiler chickens per group were used as controls for the experiment 2 or 3, respectively. The concentrations of FITC-d from diluted sera were measured fluorometrically at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA) [32].

2.10. Total Intestinal Immunoglobulin A (Iga) Levels

Total IgA levels in experiments 2 and 3 were determined in 12 gut rinse samples each as previously described [33]. An intestinal section of 5 cm from the Meckel's diverticulum to the ileocecal junction was taken and rinsed three times with 5 mL of 0.9% saline; then the rinse was collected in a tube and centrifuged at 1864× *g* at 4 °C for 10 min. The supernatant was poured into a 96-microwell plate and stored at −20 °C until tested. A commercial indirect ELISA set was used to quantify IgA according to

the manufacturer's instructions (Catalog No. E30-103, Bethyl Laboratories Inc., Montgomery, TX 77356, USA). 96-well plates (Catalog No. 439454, Nunc MaxiSorp, Thermo Fisher Scientific, Rochester, NY, USA) were used, and samples diluted 1:100 were measured at 450 nm using an ELISA plate reader (Synergy HT, multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT, USA). Total intestinal IgA levels obtained were multiplied by the dilution factor (100) to determine the amount of chicken IgA in the undiluted samples.

2.11. Data and Statistical Analysis

Data from *S*. Enteritidis and AB counts (Log cfu/g), BW, BWG, FI, FCR, total IgA levels, serum FITC-d concentration and ileum NE lesion score were subjected to an analysis of variance (ANOVA) as a completely randomized design, using the general linear models procedure of Statistical Analysis System (SAS) [34]. The experimental unit for each variable is reported in each table respectively. Significant differences among the means were determined by Duncan's multiple range test at $p < 0.05$. Enrichment data were expressed as positive/total chickens (%), and the recovery percentage of AB and *S*. Enteritidis were compared using the chi-squared test of independence [35], testing all possible combinations to determine the significance ($p < 0.05$).

3. Results

The results of the prophylactic administration of 0.1% AA-CUR/PVP-BA on *S*. Enteritidis colonization in the crop and CT of broiler chickens in trials 1 and 2 (Exp 1) are summarized in Table 2. Although there was no reduction in *S*. Enteritidis colonization in the crop of chickens treated with 0.1% AA-CUR/PVP-BA into the feed in both trials, in CT, the concentration of *S*. Enteritidis significantly decreased by more than 1.6 log in comparison with the positive control (PC, $p < 0.05$). Furthermore, chickens receiving 0.1% of AA-CUR/PVP-BA had a significant reduction in the number of positive *S*. Enteritidis samples in CT compared to PC.

Table 2. Prophylactic administration of a 0.1% mixture containing ascorbic acid (AA) and a solid dispersion of curcumin with polyvinylpyrrolidone (CUR/PVP, ratio 1:9) and boric acid (BA) (AA-CUR/PVP-BA) on crop and cecal tonsils (CT) colonization of *Salmonella* Enteritidis (*S*. Enteritidis) [1] in broiler chickens (Experiment 1).

Treatments	Crop *S*. Enteritidis Log cfu/g	Crop *S*. Enteritidis Incidence	CT *S*. Enteritidis Log cfu/g	CT *S*. Enteritidis Incidence
	Trial 1			
Positive control	2.68 ± 0.47 [a]	9/12 (75%)	4.01 ± 0.29 [a]	12/12 (100 %)
AA-CUR/PVP-BA	2.60 ± 0.45 [a]	10/12 (83%)	2.32 ± 0.50 [b]	8/12 (67 %) *
	Trial 2			
Positive control	2.69 ± 0.48 [a]	9/12 (75%)	3.94 ± 0.22 [a]	12/12 (100 %)
AA-CUR/PVP-BA	2.57 ± 0.55 [a]	7/12 (58%)	2.28 ± 0.59 [b]	7/12 (58 %) **

Data are presented in Log cfu/g of tissue. Mean ± SE from 12 chickens. [1] Chickens were orally gavaged with 10^7 cfu of *S*. Enteritidis per chicken at six-day old, samples were collected 24 h later. [a,b] Values within treatment columns for each treatment with different superscripts differ significantly ($p < 0.05$). For *S*. Enteritidis incidence, data are presented as positive/total chickens (percentage). * $p < 0.05$; ** $p < 0.01$.

Table 3 summarizes the effect of the therapeutic administration of 0.1% AA-CUR/PVP-BA in broiler chickens on *S*. Enteritidis colonization in the crop and CT (experiment 2). On day three post-*S*. Enteritidis challenge, no significant differences were observed in the concentration of *S*. Enteritidis in the crop and CT when comparing the PC and treated group. However, a reduction of 2.05 log and 2.71 log in *S*. Enteritidis concentration was observed in the crop and CT of treated chickens when compared with control group at 10-day post-*S*. Enteritidis challenge, respectively (Table 3). Furthermore, a significant decrease in serum FITC-d concentration and significantly lower total

intestinal IgA levels were observed in broilers treated with 0.1% AA-CUR/PVP-BA when compared to PC on 10-day post-*S*. Enteritidis challenge (Table 4).

Table 3. Therapeutic administration of a 0.1% mixture containing ascorbic acid (AA) and a solid dispersion of curcumin with polyvinylpyrrolidone (CUR/PVP, ratio 1:9) and boric acid (BA) (AA-CUR/PVP-BA) on crop and cecal tonsils (CT) colonization of *Salmonella* Enteritidis (*S*. Enteritidis) [1] in broiler chickens at three and ten days post-*S*. Enteritidis challenge (experiment 2).

Treatments	Crop *S*. Enteritidis Log cfu/g	Crop *S*. Enteritidis Incidence	CT *S*. Enteritidis Log cfu/g	CT *S*. Enteritidis Incidence
	Three days post-*S*. Enteritidis challenge			
Positive control	3.18 ± 0.46 [a]	10/12 (83%)	6.44 ± 0.15 [a]	12/12 (100%)
AA-CUR/PVP-BA	2.21 ± 0.48 [a]	8/12 (67%)	5.33 ± 0.73 [a]	10/12 (83%)
	Ten days post-*S*. Enteritidis challenge			
Positive control	2.93 ± 0.65 [a]	7/12 (58%)	6.61 ± 0.21 [a]	12/12 (100%)
AA-CUR/PVP-BA	0.88 ± 0.46 [b]	3/12 (25%)	3.90 ± 0.86 [b]	8/12 (67%) *

Data are presented in Log cfu/g of tissue. Mean ± SE from 12 chickens. [1] Chickens were orally gavaged with 10^4 cfu of *S*. Enteritidis per chicken at 1-day old, samples were collected three and ten days post-*S*. Enteritidis challenge. [a,b] Values within treatment columns for each treatment with different superscripts differ significantly ($p < 0.05$). For *S*. Enteritidis incidence, data are presented as positive/total chickens (percentage). * $p < 0.05$.

Table 4. Therapeutic administration of a 0.1% mixture containing ascorbic acid (AA) and a solid dispersion of curcumin with polyvinylpyrrolidone (CUR/PVP, ratio 1:9) and boric acid (BA) (AA-CUR/PVP-BA), on serum concentration of fluorescein isothiocyanate-dextran (FITC-d), and total intestinal immunoglobulin A (IgA) levels in broiler chickens on day ten post *Salmonella* Enteritidis (*S*. Enteritidis) challenge [1] (experiment 2).

Treatments	FITC-d (μg/mL)	IgA (μg/mL)
Positive control	0.700 ± 0.020 [a]	14.34 ± 2.81 [a]
AA-BA-CUR/PVP	0.489 ± 0.026 [b]	7.38 ± 1.08 [b]

Data expressed as mean ± SE from 12 chickens. [1] Chickens were orally gavaged with 10^4 cfu of *S*. Enteritidis per chicken at 1-d old, samples were collected ten days post-*S*. Enteritidischallenge. [a,b] Values within columns with different superscripts differ significantly ($p < 0.05$).

The effect of the dietary inclusion of 0.1% AA-CUR/PVP-BA on growth performance of broiler chickens in the NE model is summarized in Table 5. Seven days post *Salmonella* Typhimurium (ST) challenge, body weight (BW) and body weight gain (BWG) of the PC and AA-CUR/PVP-BA groups were significantly reduced (≈11 g in both cases) as compared to the negative control (NC) group. However, there were no significant differences in BW and BWG between the NC and AA-CUR/PVP-BA groups in the second week (7–14 d). Although PC was the group with the highest BW and BWG during the second week, the inclusion of 0.1% AA-CUR/PVP-BA into the feed resulted in a numerical increase in BWG (4.7 g) after the *Eimeria maxima* (EM) challenge (14–18 d) as compared to PC group. After the *Clostridium perfringens* (CP) challenge (day 18), 0.1% AA-CUR/PVP-BA allowed the chickens to gain 2.88 g from day 18 to day 21, meanwhile PC group reduced its BW in 11.16 g in the same period. During the last week of the trial (14–21 d), a numerical increase in BWG (≈17 g) was observed in the group supplemented with 0.1% AA-CUR/PVP-BA in comparison with the PC group. Interestingly, the feed intake (FI) accumulated (0–21 d) was significantly lower in the AA-CUR/PVP-BA group when compared to the NC and PC groups (Table 5). In the case of the feed conversion ratio (FCR), the PC group (0–21 d) had a significant and numerically lower efficiency ratio compared to the NC and AA-CUR/PVP-BA groups, respectively. Furthermore, the NC group clearly showed significant lower values in ileum lesion scores (ILS), bacterial translocation (BT) and serum FITC-d when compared to the PC or AA-CUR/PVP-BA groups (Table 6). Broilers supplemented with 0.1% AA-CUR/PVP-BA

tended to have a reduction in ILS, BT and serum FITC-d concentration, when compared to the PC group ($p = 0.07$). Interestingly, the PC group showed a significant increase in total intestinal IgA levels when compared to the NC or AA-CUR/PVP-BA groups (Table 6).

Table 5. Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) in broiler chickens consuming a diet supplemented with or without a 0.1% mixture containing ascorbic acid (AA) and a solid dispersion of curcumin with polyvinylpyrrolidone (CUR/PVP, ratio 1:9) and boric acid (BA) (AA-CUR/PVP-BA) on a Necrotic enteritis challenge model [1] (Experiment 3).

Item	Negative Control	Positive Control	AA-CUR/PVP-BA
	BW, g/broiler		
d 0	46.88 ± 0.64 [a]	46.54 ± 0.64 [a]	47.24 ± 0.66 [a]
d 7	127.14 ± 2.90 [a]	115.58 ± 3.27 [b]	115.69 ± 3.15 [b]
d 14	273.80 ± 11.02 [a]	295.78 ± 12.10 [a]	264.60 ± 10.91 [a]
d 18	457.79 ± 18.97 [a]	456.32 ± 19.39 [a]	436.14 ± 16.41 [a]
d 21	603.81 ± 24.32 [a]	445.16 ± 18.50 [b]	438.91 ± 17.79 [b]
	BWG, g/broiler		
d 0–7	80.39 ± 3.06 [a]	67.74 ± 3.24 [b]	68.46 ± 3.18 [b]
d 7–14	147.01 ± 9.51 [b]	182.60 ± 9.48 [a]	149.89 ± 8.83 [b]
d 14–18	183.99 ± 9.85 [a]	160.55 ± 9.02 [a]	165.25 ± 6.72 [a]
d 14–21	325.78 ± 15.58 [a]	152.13 ± 9.67 [b]	169.11 ± 9.78 [b]
d 0–21	552.72 ± 24.35 [a]	399.42 ± 19.79 [b]	395.12 ± 17.46 [b]
	FI, g/broiler		
d 0–21	808.21 ± 29.86 [a]	772.34 ± 10.66 [a]	685.05 ± 25.21 [b]
	FCR		
d 0–21	1.46 ± 0.04 [b]	1.93 ± 0.10 [a]	1.73 ± 0.15 [a]

[1] Data expressed as mean ± SE from 40 chickens. [a,b] Values within columns with different superscripts differ significantly ($p < 0.05$).

Table 6. Evaluation of a 0.1% mixture containing ascorbic acid (AA) and a solid dispersion of curcumin with polyvinylpyrrolidone (CUR/PVP, ratio 1:9) and boric acid (BA) (AA-CUR/PVP-BA) on ileum NE lesion scores (ILS), bacterial translocation (BT) to the liver, serum concentration of fluorescein isothiocyanate–dextran (FITC-d) and immunoglobulin A (IgA) levels in broiler chickens [1].

Treatments	ILS [2]	BT Log_{10} cfu/g [3]	FITC-d (μg/mL) [4]	IgA (μg/mL) [5]
Negative Control	0.33 ± 0.12 [b]	1.52 ± 0.46 [b]	0.312 ± 0.048 [b]	36.14 ± 3.79 [b]
Positive Control	2.04 ± 0.18 [a]	3.34 ± 0.46 [a]	0.692 ± 0.050 [a]	50.85 ± 4.48 [a]
AA-CUR/PVP-BA	1.92 ± 0.13 [a]	3.09 ± 0.54 [a]	0.553 ± 0.056 [a]	35.35 ± 2.07 [b]

[1] Data expressed as mean ± SE. [2] ILS was evaluated in 25 broiler chickens. [3] BT was expressed in Log_{10} cfu /g of tissue from 12 chickens. [4] FITC-d concentration of 20 serum samples. [5] IgA levels determined in 12 intestine samples. [a,b] Values within treatment columns for each treatment with different superscripts differ significantly ($p < 0.05$).

4. Discussions

Foodborne pathogens and control of avian diseases as NE, remain high priority topics in the poultry industry [36]. However, due to the ban of antibiotic growth promoters by the spread of bacterial resistance to common antibiotics and the incidence of NE, this industry has been actively looking for other equally active molecules to avoid these problems [37,38].

In experiment 1, prophylactic administration of 0.1% AA-CUR/PVP-BA (1:1:1) in broiler chickens was capable to significantly reduce the concentration of *S*. Enteritidis in CT but not in the crop (Table 2). Previous in vitro and in vivo studies performed in our laboratory showed that 1% AA had the best antimicrobial properties against *S*. Enteritidis in the compartment that simulates the crop, but not

in the intestinal compartment [19], whereas the administration of 0.1% CUR/PVP-BA into the feed significantly decreased the concentration of *S*. Enteritidis in broiler chickens [20]. Therefore, reduction in *S*. Enteritidis concentration in CT could only be related to the antimicrobial effect of CUR/PVP-BA derived from the synergistic effect between CUR/PVP and BA.

In the second experiment, the concentration of *S*. Enteritidis in the crop and CT of broiler chickens treated with 0.1% AA-CUR/PVP-BA for 10 days post-*S*. Enteritidis challenge was significantly reduced. Probably, the decrease of *S*. Enteritidis in the crop was due to the combination of the acidifying effect of AA (0.033% in the mixture) given the release of protons in the medium (pKa = 4.1 and 11.6) [8] and the antimicrobial effect of CUR/PVP-BA (0.066% in the mixture). However, the decrease of *S*. Enteritidis in CT was closely related to the antimicrobial effect of CUR/PVP-BA since it has been reported that AA is not capable of acidifying the intestine of chicks, even when administered at 1% into the feed [39] and it is also unstable at a neutral pH [40]. The effectiveness of CUR/PVP-BA in *S*. Enteritidis reduction could be due to the improvement in the solubility and stability of CUR for its association with PVP compared to CUR alone and its interaction with BA to form complexes with better antimicrobial properties [20,41], as well as to the higher residence time in the intestine.

Gut integrity is essential to maintain health and performance of animals [42]. *Salmonella* infections are associated with inflammation and alterations in gut permeability [43–45]. The results in Table 4 show that chickens treated with 0.1% AA-CUR/PVP-BA had both lower serum FITC-d concentration and total intestinal IgA levels compared to PC on day 10 post-*S*. Enteritidis challenge. FITC-d is a marker for evaluating intestinal permeability since it is a high molecular weight molecule (3–5 kDa) that is not permeable under standard conditions [32,46]. The increase in the secretion of IgA provides a critical mucosal immunity [47]. Therefore, these results confirm the decrease in the severity of *S*. Enteritidis infection given the antimicrobial activity of 0.1% AA-CUR/PVP-BA. Total intestinal IgA levels were not evaluated in experiment 1 since it has been reported that in early phases of *S*. Enteritidis infection, there are no significant differences in the secretion of intestinal IgA between infected and uninfected chickens [48,49].

Considering that the prophylactic or therapeutic administration of 0.1% AA-CUR/PVP-BA significantly reduced the concentration of *S*. Enteritidis in CT of broilers, in the third experiment the impact of the dietary administration of 0.1% AA-CUR/PVP-BA using a NE model in broiler chickens was evaluated. Many predisposing factors in NE, such as the ST and EM infection, increased the colonization and proliferation of CP, and the subsequent release of protein toxins that cause intestinal damage [25,50–52]. During the first seven days after the ST challenge, BW of PC and AA-CUR/PVP-BA groups was significantly reduced ($p < 0.05$) as compared to NC (Table 4), confirming that ST had a negative impact in BW [25]. However, in the second week, there were no significant differences in BW and BWG when comparing NC and AA-CUR/PVP-BA groups. Probably, the concentration of ST decreased due to the acidifying effect of AA and the antimicrobial effect of CUR/PVP-BA as described in experiment 2.

Although the *Eimeria* and CP challenges had adverse effects on performance parameters in PC group and in broiler chickens treated with 0.1% AA-CUR/PVP-BA in comparison with NC group, dietary administration of 0.1% AA-CUR/PVP-BA numerically improved FCR (0–21 d) when compared to PC group. Furthermore, broilers supplemented with 0.1% AA-CUR/PVP-BA did not show a reduction in BW after CP challenge as the PC group did, and tended to have a reduction on ILS, BT and serum FITC-d concentration, when compared to the PC group ($p = 0.07$). It has been reported that CUR has anticoccidial properties [53–56]. However, the anticoccidial mechanism of CUR has remained, but it has been proposed that it involves the induction of oxidative stress in coccidia, as well as neutralization of reactive oxygen species [2,57]. Despite that CUR/PVP was associated with BA (CUR/PVP-BA), the anticoccidial effect of CUR is not lowered since the boron molecules interact with the keto-enol groups of CUR [37,58,59] without affecting its phenolic groups, which are responsible for its anticoccidial properties [60]. Therefore, the results obtained in this experiment suggest that

0.1% AA-CUR/PVP-BA into the feed had a positive effect in slightly diminishing the effects of coccidia, a well-documented predisposing factor in NE.

Interestingly, chickens of the PC group showed a significant increase in total intestinal IgA levels when compared to NC and AA-CUR/PVP-BA groups (Table 4). Secretory IgA (SIgA) is an essential part of the adaptive humoral immune system and the primary immunoglobulin that neutralizes pathogens on external mucosal surfaces [33,61,62]. Hence, the significant decrease of IgA levels in the group supplemented with 0.1% AA-CUR/PVP-BA could be related to the anti-inflammatory properties of CUR and BA. While CUR reduces the inflammatory responses by regulating the production of some proinflammatory cytokines [56,63], BA has the ability to reduce levels of inflammatory biomarkers as TNF-α and IL-6 [64,65].

5. Conclusions

In conclusion, prophylactic or therapeutic administration of 0.1% AA-CUR/PVP-BA significantly reduced the concentration of *S*. Enteritidis in broiler chickens. Furthermore, dietary administration of 0.1% AA-CUR/PVP-BA had a positive effect in slightly diminishing the negative impact of NE. However, 0.1% AA-CUR/PVP-BA showed a better effect in reducing the concentration of *S*. Enteritidis when compared to the NE model. Further studies with a higher concentration of AA-CUR/PVP-BA into the feed to extend these preliminary results are currently being evaluated.

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Ameliorative Effect of *Bacillus subtilis* on Growth Performance and Intestinal Architecture in Broiler Infected with Salmonella

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Simple Summary: Salmonellosis is a dangerous disease in broilers that causes huge economic losses. We assumed that instead of antibiotics, a Bacillus-based probiotic may serve as an alternative to alleviate the negative effects of *Salmonella* infection. A control group with no feed additive, a positive control supplemented with a standard antibiotic and two groups that were supplemented with different strains and levels of *Bacillus subtilis* were the experimental animals of the present study. It was revealed that supplementation of probiotic bacteria induced similar results in terms of feed intake, body weight gain and feed efficiency in comparison with the group treated with antibiotics. In addition, the dimensions of intestinal villi were also improved in the probiotic-treated birds. As concluded from the results of the present study, probiotic bacteria could be used as an alternative to antibiotics against *Salmonella* in broilers.

Abstract: A total of 600 day-old broiler chicks (Ross 308) confirmed for the absence of *Salmonella* were randomly allocated to five treatments each with 10 replicates: negative control (basal diet only); positive control (basal diet) + infected with *Salmonella*; T1, *Salmonella* infected + avilamycin; T2, *Salmonella* infected + *Bacillus subtilis* (ATCC PTA-6737; 2×10^7 CFU/g) and T3, *Salmonella* infected + *B. subtilis* (DSM 172999; 1.2×10^6 CFU/g). The results revealed that feed intake (FI) and body weight (BW) were significantly ($p < 0.01$) lower in T1 compared to T2. The feed conversion ratio (FCR) was significantly ($p < 0.01$) lower in T2 and T3 compared to other treatments. Similarly, the performance efficiency factor (PEF) was also significantly ($p < 0.01$) higher in T2 and T3 compared to positive control. Villus height was significantly ($p < 0.01$) higher in T2 compared to all other treatments. However, villus width and surface area were significantly ($p < 0.01$) higher in T1. In conclusion, dietary supplementation with *B. subtilis* improved growth and intestinal health by reversing the negative effects of Salmonellosis.

Keywords: broiler; growth; intestinal villi; *Salmonella*

1. Introduction

In the modern broiler industry, antimicrobials used as growth promoters are among the most popular synthetic agents used in poultry production for the improvement of feed efficiency and the reduction of microbial pathogenesis [1,2]. Antimicrobials as an additive have produced promising results; however, their regular use has caused drug resistance and residues in eggs and meat [3].

Under such circumstances, many countries are considering a strict ban or have already banned (European Union) the use of Antimicrobial growth promoters (AGPs) [4–6]. Therefore, there is a necessity to find suitable alternatives that could replace AGPs. Recently natural products have gained special interest, since they improve growth performance and reduce mortality rates as an effect of infectious agents [7–9].

Salmonella is one of the most important poultry diseases causing heavy economic losses through stunted growth and increased mortality rates [6,8]. Incidences of *Salmonella* are most frequent during the starter phase, since the immune system of the chick is not well developed [9]. Chickens are frequently exposed to *Salmonella* during their life and micro-organisms can be readily transferred to humans through the consumption of poultry meat, causing severe gastrointestinal symptoms [10]. A number of practices are used to reverse the symptoms of salmonellosis in broilers including the use of probiotics [9,11]. Probiotics are used in poultry production due to the wide range of their positive effects [11]. Probiotics are now considered an alternative to antibiotics and added in the animals' diet as a microbial supplement [12]. It has been reported that probiotics enhance growth, provide protection against a wide range of pathogens and improve immunity [11–13]. *Bacillus subtilis* is naturally isolated from the gut of chickens and it is known to produce antimicrobial substances such as surfactants [11]. Recently, it was reported that *B. subtilis* improved the growth and antioxidant status in broilers exposed to *Salmonella* [8]. In the literature, positive effects of the two strains of *B. subtilis* have been reported; however, their comparative effects have not been described. The aim of the present study was to evaluate the effects of two different strains of *B. subtilis* on the performance and intestinal health of *Salmonella*-infected broilers during the starter phase.

2. Methods

2.1. Animals and Feeding Practices and Randomization

A total of 600 day-old broiler chicks (Ross 308) were randomly divided into five treatments (10 replicates and 12 birds per replicate). On arrival, all chicks were confirmed for the absence of *Salmonella*. The experiment was carried out in an environmentally controlled closed poultry unit. Straw was used as bedding material on the concrete floor. Initially the temperature was set to 31 °C and gradually decreased to a thermoneutral temperature of 22–24 °C and a relative humidity of 70%. An automated exhaust fan drew outside air in at 45.8 m^3/min. The photoperiod was maintained at 23:1 L:D at the intensity rate of 20 lux using cool light fluorescent tubes. The stocking density was maintained at 50 kg/m^2.

Broiler chicks were raised according to the recommendations of the Ross guide. A standard starter (0–15) diet with isocaloric and isonitrogenous contents was offered in a mash form based on corn-SBM and was formulated to meet the requirements of the broilers (Table 1). On day 1, chicks received one of five treatments randomly as follows: negative control (basal diet); positive control (basal diet) + infected with *Salmonella enterica*, subspecies *typhimurium*; T1, infected with *Salmonella* + avilamycin (0.2 g/kg); T2, infected + probiotics that have viable spores of *B. subtilis* (ATCC PTA-6737; (2×10^7 CFU/g) and T3, infected + *B. subtilis* (DSM 17299; 1.2×10^6 CFU/g).

Table 1. Dietary composition of broiler chicken starter diets.

Ingredient (%)	Starter Phase
Yellow corn	57.39
Soybean meal	27.00
Palm oil	2.20
Corn gluten meal	8.80
Dicalcium phosphate	2.30
Ground limestone	0.70
Choline chloride	0.05
DL-methionine	0.10

Table 1. *Cont.*

Ingredient (%)	Starter Phase
L-lysine	0.39
Salt	0.40
Threonine	0.17
V-M vitamins-minerals premix [1]	0.50
Analyses	
ME Metabolizable energy, kcal/kg	3000
Crude protein, %	23.0
Non phytate P, %	0.48
Calcium, %	0.96
D. Lysine, %	1.28
D. Methionine, %	0.60
Sulfur amino acids, %	0.95
Threonine, %	0.86

[1] Vitamin-mineral premix contains the following per kg: vitamin A, 2,400,000 IU; vitamin D, 1,000,000 IU; vitamin E, 16,000 IU; vitamin K, 800 mg; vitamin B1, 600 mg; vitamin B_2, 1600 mg; vitamin B_6, 1000 mg; vitamin B_{12}, 6 mg; niacin, 8000 mg; folic acid, 400 mg; pantothenic acid, 3000 mg; biotin 40 mg; antioxidant, 3000 mg; cobalt, 80 mg; copper, 2000 mg; iodine, 400; iron, 1200 mg; manganese, 18,000 mg; selenium, 60 mg; zinc, 14,000 mg.

2.2. Challenge Inoculum

On the second day, all groups apart from the negative control were orally administered with a 3×10^9 live culture of *Salmonella enterica* subspecies *typhimurium* as described by Abudabos et al. [9].

2.3. Growth Performance

Growth performance parameters such as body weight (BW), feed intake (FI) and feed conversion rate (FCR) were recorded at five-day intervals. The BW was measured on an electronic digital balance with a sensitivity of 0.1 g (Berkley, Columbia, SC, USA). The production efficiency factor (PEF) was calculated as described by Abudabos et al. [6].

2.4. Sitophological Measurements

Histological study of intestinal tissue was conducted as described by Rahman et al. [14]. On day 15, 10 birds per treatment were randomly selected. For histological studies, about 2×2 cm^2 long samples from the proximal portion of the ileum were collected, fixed and then embedded in paraffin. The tissues were sectioned to roughly 5 mm small pieces and stained (hematoxylin and eosin). Ten well-oriented villi per sample were selected and measured using a simple microscope (Olympus) connected to an image analysis system.

2.5. Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System [15]. Means were compared by the method described by Steel and Torrie [16] and a significant level was obtained by the Duncan multiple-range test [17].

2.6. Ethical Approval

The study was approved by the Committee on Care and Use of Animals, King Saud University, Saudi Arabia (1127/18/DAP).

3. Results

3.1. Growth Performance

The results of growth performance for the control and experimental groups are provided in Table 2. Feed intake was significantly ($p < 0.01$) higher in T2 compared to T1. Similarly, BW was significantly ($p < 0.01$) higher in T2 compared to T1 and the positive control. As a result, FCR was significantly ($p < 0.01$) lower in T2 and T3 compared to the positive control. Similarly, PEF was also significantly ($p < 0.01$) higher in T2 and T3 compared to the positive control. Although PEF in T1 was not significantly different from T2 and T3, values were slightly better in T2 and T3 compared to T1.

Table 2. Means ± SE of feed intake (FI), body weight (BW), feed conversion ratio (FCR), body weight (BW) and performance efficiency factor (PEF) for the cumulative starter period (0 to 15 days of age).

Treatment	FI (g)	BW (g)	FCR (g:g)	PFE
Negative control	437.0 ^{a,b}	346.9 ^{a}	1.259 ^{d}	196.6 ^{a}
Positive control	426.8 ^{a}	282.9 ^{c}	1.511 ^{a}	134.6 ^{d}
T1	391.4 ^{b}	281.9 ^{c}	1.390 ^{b}	150.5 ^{b,c,d}
T2	440.8 ^{a}	321.5 ^{a,b}	1.374 ^{b,c}	171.3 ^{b}
T3	416.3 ^{a,b}	314.0 ^{b}	1.329^{c}	165.8 ^{b,c}
SEM±	11.46	9.12	0.019	8.19
p value	0.0005	0.0001	0.0001	0.0001

^{a,b,c} Means within a column differ significantly ($p < 0.01$). T1, infected + avilamycin at a rate of 0.2 g/kg; T2, infected + probiotics that have viable spores (2×10^7 CFU/g) of *Bacillus subtilis* (ATCC PTA-6737); T3: infected + *B. subtilis* (DSM 17299;1.2×10^6 CFU/g).

3.2. Intestinal Histology

The effects of *B. subtilis* on histological structures of broiler chickens are presented in Table 3. Villus height was significantly ($p < 0.01$) higher in T2 compared to all other treatments. However, villus width and surface area were significantly ($p < 0.01$) higher in T1 compared to the positive control group. Villus width was not statistically significant between the negative control and T1 groups.

Table 3. Means ± SE of villi height (L), width (W) and villi total area (TA) of ileum in broiler chickens at 15 days.

Treatment	Villus Height (μm)	Villus Width (μm)	Total Area (mm^2)
Negative control	439 ^{c}	76.7 ^{a}	0.100 ^{b,c}
Positive control	425 ^{c}	64.17 ^{b}	0.085 ^{d}
T1	544 ^{b}	73.9 ^{a}	0.124 ^{a}
T2	614 ^{a}	57.6 ^{b,c}	0.110 ^{b}
T3	562 ^{b}	61.1 ^{b,c}	0.108 ^{b,c}
SEM±	11.96	2.79	0.004
p value	0.0001	0.0001	0.0001

^{a,b,c} Means within a column differ significantly ($p < 0.01$). T1, infected + avilamycin at the rate of 0.2 g/kg; T2, infected + probiotics that have viable spores (2×107 CFU/g) of *B. subtilis* (ATCC PTA-6737); T3: T3, infected + *B. subtilis* (DSM 17299 1.2×10^6 CFU/g).

4. Discussion

In the current study, growth performance and intestinal histological parameters were improved in the probiotic-treated birds infected with *Salmonella*. The results were similar to those of the antibiotic-treated birds. Probiotics are considered one of the viable alternatives to antibiotics, particularly in view of the recent ban of regular use of AGPs in poultry diet [6]. In the present study, the growth performance and intestinal architecture were significantly deteriorated in the *Salmonella*-infected birds. Reduced growth and lesion in the intestinal villi are some of the most prominent signs of salmonellosis, leading to heavy economic losses [8].

Interestingly, the results of the probiotic-treated birds were comparable to those of the birds fed antibiotic supplements. The improved growth performance of broilers infected with different kinds of pathogens such as *Clostridium* and *Salmonella* in response to supplementation of *B. subtilis* or phytogentics has been published recently [8,9]. The positive effects of probiotics are well documented, e.g., improved performance (body weight gain and feed conversion rate); enhanced immune response and healthy intestine [11]. The effects of the two types of probiotics on the performance of the birds were not significantly different. The efficacy of probiotic use is linked to genetics, nutritional status, frequency, dose, specificity of the strain, survival and stability [11]. A number of mechanisms through which probiotics produce positive effects are involved, such as the reduction of intestinal pH, production of volatile fatty acids and suppression of pathogenetic bacteria through competitive exclusion [18].

As indicated in the current study, villus dimensions were restored as an effect of probiotic supplementation. Dietary probiotics have been shown to enhance the intestinal microbiome in a positive direction [8,9]. The intestinal villi secrete different kinds of defensive mucins such as MUC2 and MUC3 from the goblet cells [11]. In addition, probiotic bacteria improve the humoral and cellular immunity through increased production of delayed hypersensitivity, respiratory burst of macrophages, antibody production, natural killer cells, interleukins, cytokines, antibody-secreting cells and T lymphocytes [11,18].

5. Conclusions

Dietary supplementation with *B. subtilis* improved the growth performance and gut health of *Salmonella*-infected broiler chickens.

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Effect of Soybean Isoflavones on Growth Performance, Immune Function, and Viral Protein 5 mRNA Expression in Broiler Chickens Challenged with Infectious Bursal Disease Virus

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Simple Summary: Infectious bursal disease virus (IBDV) is characterized by inflammation and subsequent atrophy of the bursa of Fabricius and immune suppression. However, nutritional strategies are able to ameliorate the negative effects of viral infections. Therefore, the aim of the present study was to determine the effect of different levels of soybean isoflavones (SI) on broiler chickens challenged with IBDV. Based on the findings, supplemental 10~20 mg/kg SI may have a positive effect on broiler chickens infected with IBDV, probably because SI decrease the severity of bursa lesions and viral protein 5 mRNA expression, and have strong antioxidant activity.

Abstract: A total of 200 one-day-old male broilers were assigned to five groups, and each group consisted of four replicates with 10 birds per replicate. Chicks were fed the basal diet with 0 (non-infected control), 0 (infected control), 10, 20, and 40 mg/kg soybean isoflavones (SI) for 42 days. At 21 days of age, chickens were inoculated with an infectious bursal dose (causing 50% morbidity) of the infectious bursal disease virus (IBDV) BC 6/85 strain by the eye-drop and nasal route (except for the non-infected group). Average daily gain (ADG) and average daily feed intake (ADFI) decreased ($p < 0.05$) in broilers infected with infectious bursal disease virus (IBDV) from 22 to 42 days. However, infected broilers fed 10 and 20 mg SI/kg had the maximum ($p < 0.05$) ADG and ADFI from 1 to 42 days. Body weight (BW) increased ($p < 0.05$) in infected broilers fed the 10 and 20 mg SI /kg diet. The bursa weight at 7 days post-infection (dpi) was increased ($p < 0.05$) by the supplemental 10 mg SI/kg diet. Infected broilers showed the highest ($p < 0.05$) bursa lesions, with an average score of 4.0 ± 0.0, while the severity of bursa lesions was decreased ($p < 0.05$) at 3 dpi and 7 dpi by the supplemental 20 mg SI/kg diet. Supplemental SI at 20 mg/kg decreased ($p < 0.05$) the viral protein 5 (VP5) mRNA expression at 3 dpi and 7 dpi. The level of interferon gamma (IFNγ) was elevated ($p < 0.05$) in the infected group at 3 dpi and 7 dpi as compared with the control group, while its level was decreased ($p < 0.05$) by supplemental 10 mg/kg SI at 3 dpi. The level of nuclear factor κB in the bursal tissue showed the lowest value ($p < 0.05$) with supplemental 10 and 20 mg SI/kg diet at 7 dpi. Supplemental 10, 20, 40 mg/kg SI improved ($p < 0.05$) the serum total antioxidant

activity (T-AOC) in infected broilers at 3 dpi. In addition, the serum level of malondialdehyde (MDA) decreased ($p < 0.05$) in the group fed 20 mg/kg SI at 7 dpi. In conclusion, supplemental 10~20 mg/kg SI may have a positive effect on broiler chickens infected with IBDV, probably because SI decrease the severity of bursa lesions and viral protein 5 mRNA expression, and have strong antioxidant activity.

Keywords: broilers; IBDV; soybean isoflavones; immune function; viral protein 5 mRNA expression

1. Introduction

Infectious bursal disease (IBD), or Gumboro disease, is an acute, highly contagious disease of young chickens caused by the infectious bursal disease virus (IBDV), characterized by inflammation and subsequent atrophy of the bursa of Fabricius, and immune suppression and mortality, generally at 3 to 6 weeks of age [1–4]. It has been shown that IBDV induces suboptimal feed conversion and weight gain [5]. In addition, immune dysfunction decreases the growth performance and increases carcass condemnation rates, but increases the rate of mortality and morbidity due to secondary viral and bacterial infections [6].

Recently, bioactive compounds in feedstuffs or feed additives are considered as an important strategy to boost immunity in modern poultry production [7–10]. Isoflavones are natural molecules available in edible plants, particularly in soybeans, red clover, and kudzu root [11,12]. Isoflavones, as phenolic compounds, are the main phytoestrogens of soybeans [13]. Isoflavones, including genistein, daidzein, and glycitein, are similar in structure to 17-β-estradiol. Soy isoflavones are used as a supplement to improve growth performance, antioxidant activity, and immune function [14–19]. These reasons caused us to hypothesize that supplemental soy isoflavones may improve the performance and immune function of broilers chickens infected with IBDV.

This study was conducted to investigate the ability of isoflavones in the amelioration of oxidative stress and immune function of broilers chickens challenged with IBDV.

2. Materials and Methods

The experimental protocol was reviewed and approved by the Institute of Animal Science, Guangdong Academy of Agricultural Sciences, China (GAASISA-2015-03).

2.1. Birds, Virus, and Diets

A total of 200 one-day-old Lingnan yellow-feathered male broilers were obtained from a commercial hatchery Guangdong Wiz Agricultural Science and Technology Co., Guangzhou, China) and raised under standard conditions with free access to water and feed. The strain of IBDV, BC 6/85, is a classic strain of virulent IBDV used as a standard challenge strain in China and was purchased from the China Institute of Veterinary Drug Control (Haidian District, Beijing, China). Nutrient levels of the diets were based on the National Research Council [20] recommended nutrient requirements for broiler chickens (Table 1)

Table 1. Ingredient and composition of the basal diets for Chinese yellow-feathered broilers at 1–21 and 22–42 days of age (as fed-basis).

Ingredients	Composition, g/kg	
	1–21 Days of Age	22–42 Days of Age
Maize	584.0	608.0
Wheat bran	43.0	38.0
Fish meal	22.0	10.0
Soybean meal	264.0	220.0
Maize gluten meal	20.0	30.0
Soybean oil	13.0	29.0
Lysine	0.0	1.0
Methionine	1.0	0.8
Limestone	12.7	12.0
Dicalcium phosphate	15.1	14.5
Salt	2.5	2.5
Zeolite	12.7	24.2
Vitamin-mineral premix [1]	10.0	10.0
Total	1000.0	1000.0
Chemical composition, g/kg [2]		
Crude protein	199.3	187.4
Lysine	10.5	9.8
Methionine	4.6	4.0
Calcium	10.0	9.0
Non-phytate P	4.5	4.0
Metabolizable Energy, MJ/kg	12.13	12.55

[1] Supplied per kilogram of diet: vitamin A, 14,700 IU; vitamin D_3, 3300 IU; vitamin E, 20 IU; vitamin K_3, 3.9 mg; vitamin B_1, 3 mg; vitamin B_2, 9.6 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.03 mg; nicotinic acid, 60 mg; pantothenic acid, 18 mg; folic acid, 1.5 mg; biotin, 0.36 mg; $FeSO_4 \cdot 7H_2O$, 80 mg; $CuSO_4 \cdot 5H_2O$, 8 mg; MnO, 80 mg; KI, 0.38 mg; and $NaSeO_3$, 0.44 mg. The carrier was zeolite. [2] Values were calculated from data provided by Feed Database in China (2016) except that crude protein was analysed.

2.2. Experimental Design

On the first day of the experiment, 200 one-day-old yellow-feathered male broiler chickens were weighed and allotted randomly to five treatment groups, each of which included four replicates of 10 birds. Broilers were placed in floor pens (1 × 2 m). The litter thickness was 5 cm of sawdust. All birds were fed the same basal diet, supplemented with 0 (non-infected control group), 0 (infected control), 10, 20, or 40 mg/kg soybean isoflavones (SI) (supplied by Newland Feed Science and Technology Co., Guangdong, China).These treatments are described as non-infected control, IBDV (0 SI), IBDV (10 SI), IBDV (20 SI), and IBDV (40 SI), respectively (Table 2).

Table 2. The design of the experimental study.

Treatments	Control	IBDV (0 SI)	IBDV (10 SI)	IBDV (20 SI)	IBDV (40 SI)
SI, mg/kg	0	0	10	20	40
IBDV	−	+	+	+	+

SI: synthetic soybean isoflavones; IBDV: infectious bursal disease virus.

At 21 days of age, chickens were inoculated with the bursal infectious dose causing 50% morbidity of the IBDV BC 6/85 strain by the eye-drop and nasal route, except for the non-infected control group.

A pre-experiment had been conducted to titrate the optimal dose of the inoculation. By administering the chosen dose, visible pathological changes were visible on the bursa of Fabricius at 5 days post-infection (dpi) without evident mortality. During the experiment, which lasted 42 days, the infected and non-infected groups of chickens were housed in equivalent but separate places.

2.3. Growth Performance

Broiler chickens were weighed at 1, 21, and 42 days of age. Average daily feed intake was determined on a per pen basis. The feed conversion ratio (FCR) was calculated. Mortality and health status were recorded daily.

2.4. Blood Sampling and Laboratory Analyses

Eight broilers per treatment group (two birds per replicate) were selected randomly and bled into tubes (5 mL per bird) from a wing vein at 3 and 7 dpi to collect the serum, and then the broilers were slaughtered and bursa of Fabricius was collected and weighed from each broiler. The half of bursa was fixed in 4% buffered formaldehyde. Another half was snap-frozen with liquid nitrogen and stored at −80°C to analyze viral protein 5 (VP5) mRNA expression.

2.5. Histology of Bursa of Fabricius

The collected tissues of bursa of Fabricius were fixed with 4% formaldehyde solution for 24 h. Serial sections were cut at 5 μm were dehydrated, cleared, embedded in paraffin, deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin. Three sections were made for each sample to observe the bursal lesion and to measure the degree of damage of the bursal follicle using light microscope. To observe the bursal lesion and to measure the degree of damage of the bursal follicle, the histopathological changes of the bursa of Fabricius were scored according to the methods of Sharma et al. [21] and Kim et al. [22]: 0 = normal bursa of Fabricius; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% of follicles showing cellular depletion.

2.6. Viral Protein 5 (VP5) mRNA Expression

Total RNAs was extracted from the Bursal homogenates using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Gene-specific primers for viral protein 5 (VP5) and the endogenous reference gene (β-actin) are shown in Table 3. Briefly, the 50-μL reaction mixture contained 10 μL of extracted RNA, 10 μL of 5· RT-PCR buffer, 2 μL primer F, 2 μL primer R, 2 μL dNTP mix containing 400 μM each of dATP, dGTP, dCTP, and dTTP, and 2 μL of Qiagen One Step Enzyme Mix. A fragment of 94 bp of the 5′ noncoding region was amplified using a PCR reaction with the SYBR Premix PCR kit (Takara, Dalian, China). The PCR program was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60 °C for 60 s. The standard curve was generated using pooled samples and efficiency was calculated from standard curves. Each sample was run in duplicate and a no-template control was included. Specificity of the amplification was verified via melting curve analysis and the specificity of the product was confirmed by electrophoresis on a 1.2% agarose gel, with purification using a DNA purification kit (Takara), and sequencing (Shanghai Sangon Biotech Co. Ltd., Shanghai, China). The difference of the cycle threshold (Ct) value for the 18s rRNA was less than 0.5 across all treatments, and therefore was considered to be an appropriate endogenous control. Average gene expression relative to the endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method [23]. The calibrator for each studied gene was the average $^{\Delta}Ct$ value of the control group.

Table 3. Sequences of primers for the real-time PCR.

Gene name	Primer Sequence	Amplicon Size [bp]
VP5 [1]	F:5′-GAGCCTTCTGATGCCAACAAC-3′ R:5′-CAAATTGTAGGTCGAGGTCTCTGA-3′	94 bp
β-actin	F:5′-TGGCATTGCTGACAGGAT-3′ R:5′-CTGCTTGCTGATCCACAT-3′	160 bp

[1] viral protein 5(encodes a 17-kDa non structural polypeptide).

2.7. Determination of Antioxidant Capacity in the Serum

Serum total antioxidant activity (T-AOC) and malonaldehyde (MDA) were measured spectrophotometrically. A total antioxidant capacity assay kit (A015–1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used according manufacturer's instructions and was expressed in U/mL. The MDA levels were assayed using the thiobarbituric acid method [24], reading the absorbance at 532 nm with the spectrometer.

2.8. Determination of Bursal Immunologic Indices

Bursa of Fabricius samples were thawed at 4°C and homogenized in 10 volumes of cold normal saline. The homogenates were then centrifuged at 20,000× *g* for 20 min at 4 °C and the supernatant was collected for analyses. Diluent solution and standard samples were added at 100 μL per well in duplicate wells. The plate was incubated for 2 h at 37 °C, followed by three washings with wash solution. The immunological indicators of interleukin-2 (IL-2), interleukin-6 (IL-6), interferon gamma) IFNγ), and nuclear factor κB were (NF-κB) determined by ELISA kits. The kits were purchased from Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China), and the specific operation was carried out according to the instructions.

2.9. Statistical Analyses

The replicate was the experimental unit. The effects of SI supplementation levels were examined by one-way ANOVA using the general linear model GLM procedures of SAS software (v9.2, SAS Institute, Cary, NC, USA). In the absence of SI, IBDV-infected and non-infected controls were compared by *t*-tests. Significance was declared at $p < 0.05$. All data are expressed as means ± SE.

3. Results and Discussions

Some of the main strategies during stress periods such as viral infections are to boost the immune function, maximize antioxidant ability, and minimize lipid peroxidation. Therefore, this study was conducted to investigate the ability of isoflavones in the amelioration of oxidative stress and in the immune function of broilers chickens challenged with IBDV.

3.1. Bursa of Fabricius

The effect of SI supplementation on bursa of Fabricius weight and index of broilers challenged with infectious bursal disease virus is shown in Table 4. In addition, the bursa lesion score is shown in Table 5.

Table 4. Effect of adding soy isoflavones on bursa development of IBDV-challenged broilers [1].

Indices		Treatments				
		Control	IBDV (0 SI)	IBDV (10 SI)	IBDV (20 SI)	IBDV (40 SI)
Bursa weight, g	3days PI	2.45 ± 0.15 ^{A}	1.60 ± 0.05 ^{B}	1.88 ± 0.18	1.88 ± 0.17	1.80 ± 0.10
	7days PI	2.25 ± 0.22 ^{A}	0.58 ± 0.06 ^{Bb}	0.87 ± 0.05 ^{a}	0.63 ± 0.08 ^{b}	0.59 ± 0.05 ^{b}
Bursa index, %	3days PI	0.46 ± 0.03 ^{A}	0.32±0.02 ^{B}	0.34 ± 0.04	0.36 ± 0.04	0.35 ± 0.02
	7days PI	0.32 ± 0.02 ^{A}	0.10±0.01 ^{B}	0.14 ± 0.00	0.13 ± 0.02	0.10 ± 0.01

[1] Data are means of eight broilers chickens per treatment (two broilers/replicate). Capital letters indicate statistically significant ($p < 0.05$) differences between control group and IBDV group by Student's *t*-test; small letters indicate statistically significant ($p < 0.05$) differences between IBDV (0 SI), IBDV (10 SI), IBDV (20 SI) and IBDV (40 SI).

Table 5. Effect of adding soy isoflavones on bursa lesion score of IBDV-challenged broilers [1].

Indices		Treatments				
		Control	IBDV (0 SI)	IBDV (10 SI)	IBDV (20 SI)	IBDV (40 SI)
Bursa Score	3days PI	0.00±0.00 ^{B}	4.00 ± 0.00 ^{Aa}	3.63 ± 0.26 ^{ab}	3.13 ± 0.30 ^{b}	3.75 ± 0.16 ^{ab}
	7days PI	0.00±0.00 ^{B}	3.88 ± 0.13 ^{Aa}	4.00 ± 0.00^{a}	3.13 ± 0.30 ^{b}	3.86 ± 0.14 ^{a}

[1] Data are means of eight broilers chickens per treatment (two broilers/replicate). Capital letters indicate statistically significant ($p < 0.05$) differences between the control group and IBDV group by Student's *t*-test; small letters indicate statistically significant ($p < 0.05$) differences between IBDV (0 SI), IBDV (10 SI), IBDV (20 SI) and IBDV (40 SI).Note: Histopathological score of bursa of Fabricius: 0 = normal bursa of Fabricius; 1 = 1–25%; 2 = 26–50%;3 = 51–75%; and 4 = 76–100% of follicles showing cellular depletion.

The weight (g) and the index of bursa of Fabricius (%) were reduced significantly ($p < 0.05$) in broiler chicks infected with IBDV as compared with those of the control group (non-infected) at 3 dpi and 7 dpi. However, supplemental 10 mg of SI increased the bursa weight significantly ($p < 0.05$) at 7 dpi. Our finding is in agreement with the findings of Li et al. [25]. They reported that a significant decrease in the bursa to body weight ratios (B/BW) had appeared at 7 dpi. Bursa lesions in infected broiler had an average score of 4.0 ± 0.0 compared with non-infected control ($p < 0.05$). However, the severity of bursa lesions was decreased ($p < 0.05$) at 3 dpi and 7 dpi by the supplemental 20 mg SI/kg diet. This is in agreement with the result of Li et al. [25]. They found that bursa lesion score was 4.0 ± 0.0 at 3 dpi in IBDV-infected group.

In terms of bursal damage, the control group (non-infected) had no signs of bursal damage (Figure 1), while all infected broilers had bursal damage at 3 dpi and 7 dpi (Figures 2 and 3). This finding is in agreement with that of Li et al. [25], who demonstrated a depletion of lymphoid cells in bursal follicles was observed microscopically. In the present study, infected broilers fed a 20 mg SI/kg diet had the lowest amount of bursal damage. As shown in Figure 3, the architecture is almost clear between the follicles, the lining epithelium was less corrugated, there was less necrosis and heterophil invasion, and fewer fibrous tissues were observed. It has been shown that IBDV infection induced a temporary or permanent destruction of the bursa of Fabricius and other lymphoid organs [26,27]. Destruction of B cells contributes to IBDV-induced immune suppression [28].

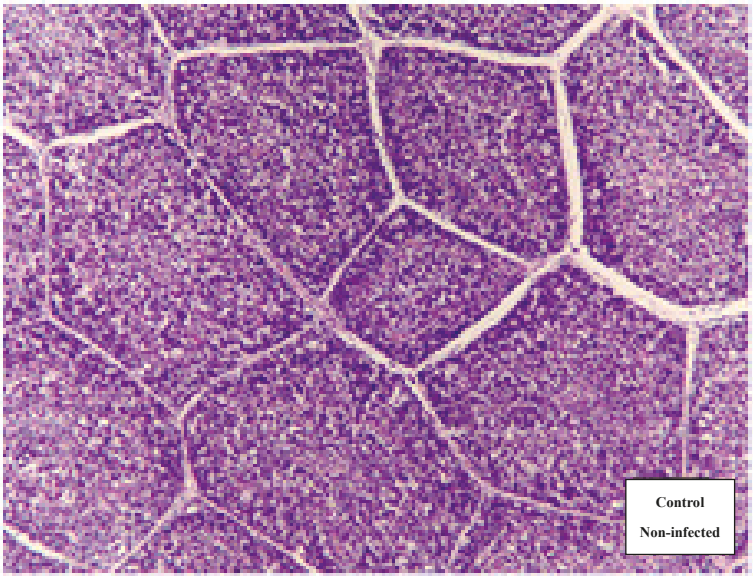

Figure 1. Normal bursal tissue section stained with hematoxylin and eosin (H&E) in the non-infected group.

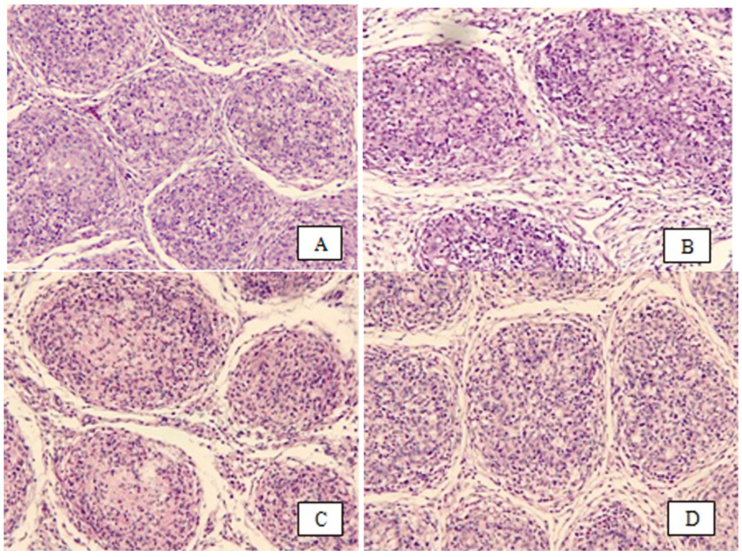

Figure 2. Bursal tissue section stained with hematoxylin and eosin (H&E) of each group at 3 dpi (200×). (**A**) is from the IBDV (0 SI); (**B**) is from the IBDV (10 SI); (**C**) is from the IBDV (20 SI); and (**D**) is from the IBDV (40 SI).

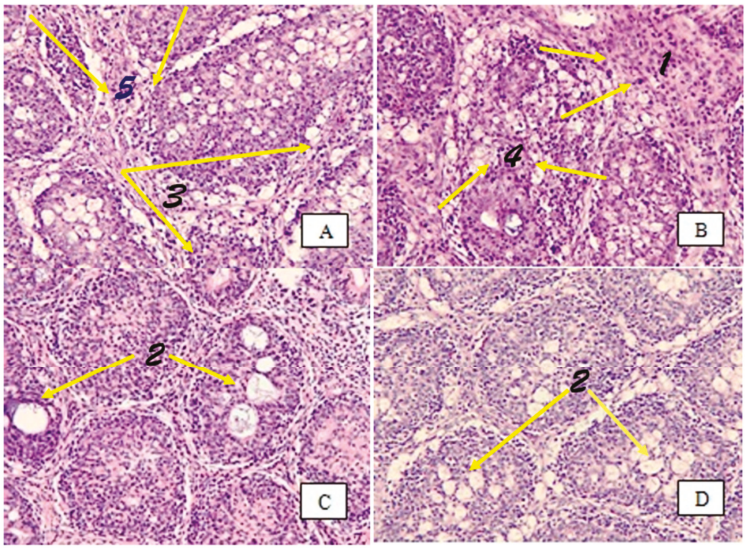

Figure 3. Bursal tissue section stained with hematoxylin and eosin (H&E) of each infected group at 7 dpi (200×). (**A**) is from the IBDV (0 SI); (**B**) is from the IBDV (10 SI); (**C**) is from the IBDV (20 SI); and (**D**) is from the IBDV (40 SI). (1) Bursa microscopically revealed complete loss of architecture. (2) Bursa with infectious bursitis presenting necrosis and heterophil invasion. (3) The lining epithelium was highly corrugated. (4) There was no intact lymphoid follicle. (5) The entire area was filled up by fibrous tissue. The IBDV+20 SI group was the best among the infected groups (the architecture is almost clear between follicles, the lining epithelium was less corrugated, and there was less necrosis, heterophil invasion, and fibrous tissue).

3.2. Viral Protein 5 (VP5) mRNA Expression

Viral protein 5 (VP5) expression was higher ($p < 0.05$) in broilers infected with IBDV as compared with those of the control group at 3 dpi and 7 dpi (Table 6). However, supplemental 20 mg/kg of SI reduced VP5expression at 3 dpi and 7 dpi. It has been indicated that RT-PCR was a sensitive test to detect the IBDV [2,29,30]. According to our knowledge, no previous study investigated the effects of SI on VP5. VP5 is one protein employed by IBDV to induce the programmed cell death process [31]. It has been suggested that VP5 might play an important role as anti-apoptotic protein at an early stage of IBDV infection [32]. In addition, Qin and Zheng [33] suggested that VP5 as an anti-apoptotic protein is an important factor to support viral replication at the early stage of IBDV infection. The anti-apoptotic activity of VP5 was noticed at 8 or 12 h post-infection [34], while VP5 induced apoptosis were found after 24 h post-infection [35].

Table 6. Effect of adding soy isoflavones on the IBDV mRNA expression of bursal tissue in IBDV—challenged broilers [1].

Indices		Treatments				
		Control	IBDV (0 SI)	IBDV (10 SI)	IBDV (20 SI)	IBDV (40 SI)
viral protein 5 [VP5]	3days PI	0.00 ± 0.00 ^{B}	0.0554 ± 0.0211 ^{Aa}	0.0492 ± 0.0080 ^{a}	0.0052 ± 0.0026 ^{b}	0.0548 ± 0.0197 ^{a}
	7days PI	0.00 ± 0.00 ^{B}	0.0025 ± 0.0005 ^{Aa}	0.0012 ± 0.0003 ^{b}	0.0010 ± 0.0004 ^{b}	0.0034 ± 0.0004 ^{a}

[1] Data are means of eight broilers chickens per treatment (two broilers/replicate). Capital letters indicate statistically significant ($p < 0.05$) differences between control group and IBDV group by Student's *t*-test; small letters indicate statistically significant ($p < 0.05$) differences between IBDV (0 SI), IBDV (10 SI), IBDV (20 SI), and IBDV (40 SI).

3.3. Immune Function

Effect of adding soy isoflavones on immunity of IBDV-challenged broilers is presented in Table 7.

Table 7. Effect of adding soy isoflavones on bursal immune response of IBDV-challenged broilers [1].

Indices		Treatments				
		Control	IBDV (0 SI)	IBDV (10 SI)	IBDV (20 SI)	IBDV (40 SI)
IL-2, pg/mL	3d PI	106.52 ± 5.55	99.90 ± 3.34 ^{b}	104.05 ± 3.91 ^{ab}	111.90 ± 2.99 ^{a}	113.32 ± 3.43 ^{a}
	7d PI	216.07 ± 14.27	201.13 ± 9.86 ^{b}	220.39 ± 15.57 ^{b}	195.21 ± 9.47^{b}	270.04 ± 24.25 ^{a}
IL-6, pg/mL	3d PI	50.22 ± 5.62	40.24 ± 3.14 ^{b}	53.61 ± 4.78 ^{a}	48.29 ± 2.78 ^{ab}	47.41 ± 2.53 ^{ab}
	7d PI	33.71 ± 4.47 ^{B}	56.78 ± 6.02 ^{A}	47.58 ± 5.09	51.43 ± 2.87	55.31 ± 3.24
IFNγ, ng/mL	3d PI	7.95 ± 1.05 ^{B}	12.94 ± 0.85 ^{Aa}	9.69 ± 0.52 ^{b}	10.34 ± 1.34 ^{ab}	9.24 ± 1.06 ^{b}
	7d PI	5.58 ± 0.24 ^{B}	7.64 ± 0.96 ^{A}	8.53 ± 0.38	6.49 ± 0.89	6.38 ± 1.14
NF-κB, pg/mL	3d PI	54.26 ± 8.67	50.64 ± 7.29	58.39 ± 9.80	67.52 ± 7.29	50.50 ± 6.90
	7d PI	58.91 ± 1.11 ^{B}	113.08 ± 9.50 ^{Aa}	68.93 ± 6.42 ^{b}	80.56 ± 13.31 ^{b}	92.85 ± 8.93 ^{ab}

[1] Data are the means of eight broilers chickens per treatment (two broilers/replicate). IL-2; interleukin 2; IL-6: interleukin 6; IFNγ: interferon gamma; NF-κB: nuclear factorκB. Capital letters indicate statistically significant ($p < 0.05$) differences between the control group and IBDV group by Student's *t*-test; small letters indicate statistically significant ($p < 0.05$) differences between IBDV (0 SI), IBDV (10 SI), IBDV (20 SI) and IBDV (40 SI).

The bursal concentration of interleukin 2 (IL-2) was lower ($p < 0.05$) in challenged broilers, while supplemental 20 mg of SI increased its level ($p < 0.05$) at 3 dpi, which is considered an acute stage of IBDV infection. IL-2 is a cytokine secreted by activated T lymphocytes, which has an important role in regulation of host response to pathogenic challenge [36]. In addition, the bursal concentration of interleukin 6(IL-6) was lower ($p < 0.05$) in the infected group, while supplemental 10 mg of SI increased its level ($p < 0.05$) at 3 dpi.

Long et al. [37] reported that IBDV infection increased IFN-γ mRNA relative expression in the bursa of Fabricius. Interferon-γ is one of the proinflammatory Th1 cytokines [38]. The level of interferon gamma (IFNγ) in the bursa of Fabricius was elevated ($p < 0.05$) in infected broilers with IBDV at 3 dpi and 7 dpi as compared with the control group, while its level was decreased ($p < 0.05$) by supplemental 10 mg/kg SI at 3 dpi. In the current study, the effect of dietary SI in immune function was significant

during early stages of infection, and it was obviously significant at 10 and 20 mg SI/kg diet. It is well known that infectious bursal disease (IBD) disease peaks between 2 to 5 day post infection and is practically cleared by day 7 [39].

The bursal level of nuclear factorκB (NF-κB) was higher in infected broilers with IBDV at 7 dpi, while its level was decreased ($p < 0.05$) by supplemental 10 and 20 mg SI at 3 dpi and 7 dpi (Table 8). It has been shown that SI are associated with cell survival, cell cycle, inflammation, and apoptosis, and they suppress nuclear factor (NF)-κB and other signaling pathways [40]. In addition, it has been reported that NF-κB activity was blunted more efficiently by genistein, probably due to its additional antioxidant effect [41]. In the present study, supplemental 10, 20, and 40 mg/kg SI significantly ($p < 0.05$) improved the serum T-AOC in infected broilers at 3 dpi (Table 8). In addition, the serum level of MDA was decreased ($p < 0.05$) in the group fed 20 mg/kg SI at 7 dpi.

Table 8. Effect of adding soy isoflavones on serum antioxidant index of IBDV-challenged broilers [1].

Indices [2]		Treatments				
		Control	IBDV (0 SI)	IBDV (10 SI)	IBDV (20 SI)	IBDV (40 SI)
TAOC, U/mL	3days PI	9.86 ± 0.36 ^{A}	4.20 ± 0.20 ^{Bb}	7.30 ± 0.33 ^{a}	6.63 ± 0.54 ^{a}	6.84 ± 0.45 ^{a}
	7days PI	7.70 ± 0.63	7.60 ± 0.61	7.24 ± 0.49	7.30 ± 0.43	6.66 ± 0.52
MDA, mol/mL	3days PI	5.19 ± 0.57	4.12 ± 0.55	5.21 ± 0.81	4.40 ± 0.98	3.18 ± 0.68
	7days PI	4.33 ± 0.21	4.45 ± 0.75 ^{a}	3.37 ± 0.57 ^{ab}	2.45 ± 0.30 ^{b}	2.07 ± 0.26 ^{b}

[1] Data are means of eight broilers chickens per treatment (two broilers/replicate). [2] Total antioxidant activity (T-AOC), malonaldehyde (MDA). Capital letters indicate statistically significant ($p < 0.05$) differences between control group and IBDV group by Student's *t*-test; small letters indicate statistically significant ($p < 0.05$) differences between IBDV (0 SI), IBDV (10 SI), IBDV (20 SI), and IBDV (40 SI).

Huang et al. [19] reported that SI improved the immune function in young piglets fed oxidized fish oil. Moreover, Lv et al. [42] reported that genistein (GEN boosted the anti-viral capacity of broilers chickens. They reported that the Newcastle disease (ND) and IBD antibody titers in the GEN group were higher ($p < 0.05$) than broilers in the control group.

3.4. Antioxidant Capacity and Oxidative Stress

In the present experiment, supplemental 10, 20, and 40 mg/kg SI significantly ($p < 0.05$) improved the serum T-AOC in infected broilers at 3 dpi (Table 8). It has been reported that SI have antioxidant properties via detoxifying free radical species and up-regulating antioxidant genes [40].The serum level of MDA was decreased($p < 0.05$) in the group fed 20 mg/kg SI at 7 dpi. The level of MDA can be a marker of the level of lipid peroxidation endogenously, which is the result of diminished antioxidant protection as levels of reactive oxygen species and antioxidants ROS increase or there is weak antioxidant activity.

3.5. Growth Performance

Infected broilers with IBDV had decreased ($p < 0.05$) average daily gain (ADG) and average daily feed intake (ADFI) from 22 to 42 days. However, infected broilers fed 10 and 20 mg/kg had the maximum ($p < 0.05$) ADG and ADFI from 1 to 42 days. In addition, body weight (BW) was increased ($p < 0.05$) in infected broilers fed 10 and 20 mg/kg (Table 9).

Table 9. Effect of adding soy isoflavones on growth performance of IBDV-challenged broilers [1].

Indices		Treatments				
		Control	IBDV (0 SI)	IBDV (10 SI)	IBDV (20 SI)	IBDV (40 SI)
BW, g	1	39.55 ± 0.19	39.75 ± 0.17	40.00 ± 0.07	39.93 ± 0.20	39.70 ± 0.14
	21	455.00 ± 2.04	452.50 ± 14.22 ^{ab}	473.75 ± 5.54 ^{a}	460.00 ± 2.89 ^{ab}	436.25 ± 9.87 ^{b}
	42	1351.50 ± 16.05	1288.04 ± 27.34 ^{ab}	1320.58 ± 13.98 ^{a}	1332.06 ± 23.64 ^{a}	1228.58 ± 40.72 ^{b}
Average daily gain, g	1–21	20.77 ± 0.10	20.64 ± 0.71 ^{ab}	21.69 ± 0.28 ^{a}	21.09 ± 0.11 ^{ab}	19.83 ± 0.49 ^{b}
	22–42	40.75 ± 0.79 ^{A}	37.98 ± 0.73 ^{B}	38.49 ± 0.66	39.57 ± 1.04	36.02 ± 1.69
	1–42	31.23 ± 0.39	29.72 ± 0.65 ^{ab}	30.49 ± 0.33 ^{a}	30.76 ± 0.56 ^{a}	28.31 ± 0.97 ^{b}
ADFI, g	1–21	34.05 ± 0.32	34.26 ± 0.37 ^{b}	35.87 ± 0.43 ^{a}	34.78 ± 0.22 ^{b}	32.38 ± 0.24 ^{c}
	22–42	77.55 ± 1.57 ^{A}	73.23 ± 0.42 ^{Bb}	77.11 ± 1.21 ^{a}	77.90 ± 1.43 ^{a}	71.79 ± 1.11 ^{b}
	1–42	56.84 ± 0.74	54.67 ± 0.80 ^{ab}	57.47 ± 0.56 ^{a}	57.37 ± 0.66 ^{a}	52.18 ± 1.28 ^{b}
FCR, g:g	1–21	1.63 ± 0.00	1.67 ± 0.06	1.66 ± 0.01	1.66 ± 0.01	1.64 ± 0.03
	22–42	1.92 ± 0.00	1.93 ± 0.04	2.01 ± 0.03	1.95 ± 0.02	2.00 ± 0.06
	1–42	1.83 ± 0.00	1.84 ± 0.01	1.89 ± 0.02	1.85 ± 0.02	1.85 ± 0.02

[1] Data are means of four replications per treatments, with 10 broilers per replicate. Capital letters indicate statistically significant ($p < 0.05$) differences between the control group and IBDV group by Student's *t*-test; small letters indicate statistically significant ($p < 0.05$) differences between IBDV (0 SI), IBDV (10 SI), IBDV (20 SI), and IBDV (40 SI). ADFI: average daily feed intake; FCR: feed conversion ratio.

It has been reported that IBDV decreased weight gain and feed efficiency [5]. Recently, Wang and Wu [43] reported that SI alleviated the growth suppression induced by dextran sulfate sodium in mice. In addition, Greiner et al. [44,45] reported that soybean genistein (200 mg/kg) and daidzein (200 or 400 mg/kg) could improve growth in virally challenged pigs. There is a positive effect of SI on infected broiler chickens with IBDV, probably because SI decrease the severity of bursa lesions and viral protein 5 mRNA expression, and have strong antioxidant activity.

In the present study, no broilers died due to IBDV infection. These findings are in agreement with other studies [46,47].

4. Conclusions

Supplemental 10~20 mg/kg SI may have a positive effect on broiler chickens infected with IBDV, probably because SI decrease the severity of bursa lesions andviral protein 5 mRNA expression, and have strong antioxidant activity.

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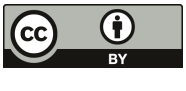

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Effects of Phytase Supplementation to Diets with or without Zinc Addition on Growth Performance and Zinc Utilization of White Pekin Ducks

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Simple Summary: The environment sustainability of farms is extremely important for the future of the world. In this context, the lowering of the pollution from intensive poultry farms is necessary. Due to its low levels and low digestibility in feeds, Zn is often overdosed in poultry feed, and its excess in the excreta can accumulate in the soil, inhibiting the growth of soil microorganism as well as altering their morphology and metabolism, thus reducing the crop yield and quality. Enzymes, such as phytase, can breakdown the linkage of Zn with phytic acid in vegetable feeds, thus increase the Zn availability for animal digestion. In this way, very low supplementation of Zn to the diets can meet the requirement of poultry.

Abstract: The effect of phytase and inorganic Zn supplementation was studied in 180 male White Pekin ducks (WPD) from 1 to 56 days of age. The birds were divided into four groups fed the same basal diet (containing 26 ppm of Zn from raw materials): the control group did not receive Zn supplementation; the second group was supplemented with 30 ppm of Zn oxide; and the third and fourth groups were fed the control and the 30 ppm diets, respectively, both supplemented with 500 U of *E. coli* phytase. Each group contained five replicates of nine ducks. The body weight and feed intake were recorded at 1, 28 and 56 days of age. At 56 days of age, five birds/group were used to measure feed digestibility and five other birds/group were slaughtered. Zn at 30 ppm increased the body weight gain (BWG, $p < 0.01$) and feed intake ($p < 0.05$) and improved the feed conversion (FCR, $p < 0.05$) of the growing ducks. The Zn retention and Zn level in the excreta increased ($p < 0.01$) due to Zn supplementation. The addition of phytase improved BWG ($p < 0.01$) and FCR ($p < 0.05$) of growing ducks. The use of phytase reduced ($p < 0.01$) the level of Zn in duck excreta. Phytase supplementation to the basal diet at 30 ppm seems to be adequate to meet Zn requirements for ducks without further Zn additions.

Keywords: ducks; zinc oxide; phytase; growth performance; zinc utilization

1. Introduction

Zinc (Zn) is an essential trace mineral with several roles in animal metabolism, acting as a functional component of more than 200 enzymes [1,2]. In the NRC guidelines [3], Zn requirements for ducks are not provided; therefore, the dietary requirement of Zn for ducks is based on those for other bird species.

In poultry nutrition, Zn is required for eggshell deposition [4]; inadequate amount of Zn negatively affects the feed intake, growth rate and feed conversion ratio of broilers [5]. In addition, abnormalities in the immune responses, as well as reproduction, skeletal and skin disorders can be tied to the deficiency of Zn in poultry diets [6].

In general, the level of Zn in feedstuffs is low [3] and in vegetable products this element is also poorly available for digestion because its chelation to the phytic acid [7]. Thus, the addition of Zn to poultry diets is a common practice. A comparison between NRC [3] recommendation and modern commercial strains of broilers suggests that industries often use a big safety margin of Zn in feed formulation [5], also considering that amount of Zn up to 2000 mg/kg in poultry diets does not negatively affect the bird performance [8]. However, high amount of Zn in the diets is responsible of the high excretion of this trace element into the environment [9] as fecal Zn content linearly increases with Zn dietary levels [10]. Thus, the European Commission has recently established a maximum limit for the total Zn content, including the supplemental premix, of poultry diets at 100 ppm [11]. Therefore, the knowledge of the specific Zn requirements can reduce its supplementation in poultry diets, without affecting animal health, welfare and productivity [2].

A possible solution could be the use of enzymes associated with vegetables. Phytase is a useful additive that improves the nutritive value of feedstuffs rich in phytic acid and also reduces environmental pollution related to nitrogen, and several metals (Cu, Zn, Fe, and Mg) by improving their availability to the animal and decreasing their excretion into the environment [12–16]. Yu et al. [17] indicated that phytate reduces the Zn absorption in the broiler intestinal tract; therefore, it can be hypothesized that adding the phytase to the diets, the amount of Zn available for poultry digestion can be increased.

The objective of this study was to investigate the effects of phytase supplementation to diets with or without Zn addition on productive performance and physiological traits of growing ducks. The addition of phytase to the diet with Zn oxide aimed to verify if only the supplementation of inorganic Zn is enough to sustain animal performance or if more Zn can provide further improvements.

2. Materials and Methods

2.1. Experimental Design, Birds, Diets, and Husbandry

All procedures were approved by the Animal and Poultry Production Department, Faculty of Agriculture, Damanhour University (Egypt) that recommends animal rights and welfare.

One hundred eighty one-day-old male White Pekin ducks (*Anas platyrhynchos* domestica, WPD) were homogeneously distributed into four groups fed the same starter and finisher diets (basal diets). The groups were subjected to four dietary treatments as follow: the first group (control) was fed basal diets unsupplemented with Zn oxide or phytase; the second group was fed the basal diets supplemented with 30 ppm of Zn oxide (72% Zn); the third group was fed the basal diets supplemented with 500 U of *E. coli* phytase (*E. coli* 6-phytase, 500 U/kg diet; Danisco Animal Nutrition, England); and the fourth group was fed the basal diets supplemented with 30 ppm of Zn oxide and 500 U of *E. coli* phytase. The basal (starter and finisher) diets were obtained by using a Zn-free trace mineral mixture and contained 26 ppm of Zn from raw materials without Zn supplementation, as measured by Atomic Absorption Spectrometry analysis. The starter diet (fed from 1 to 35 days of age) and the finisher diet (36–56 days of age) were formulated according to NRC [3] recommendations and their ingredients and chemical-nutritional characteristics [18] are reported in Table 1.

Table 1. Ingredients and chemical-nutritional characteristics of the basal diets fed to White Pekin ducks during the starter (1–35 days of age) and the finisher (36–56 days) periods.

Ingredients (kg/ton)	Starter Diets		Finisher Diets	
Yellow corn	564.0	564.0	680.0	680.0
Soybean meal, 44%	383.0	383.0	267.0	267.0
Dicalcium phosphate	20.0	20.0	20.0	20.0
Sunflower oil	15.0	15.0	15.0	15.0
Limestone	10.0	9.58	10.0	9.58
Zinc Oxide	0.0	0.420	0.0	0.420
Salt	3.0	3.0	3.0	3.0
Vit+Min premix *	3.0	3.0	3.0	3.0
DL-methionine	1.0	1.0	1.0	1.0
Antifungal	1.0	1.0	1.0	1.0
Composition (calculated values)				
Metabolizable energy, MJ/kg	12.05	12.05	12.60	12.60
Methionine, g/kg	4.4	4.4	3.9	3.9
Methionine + Cysteine (SSA), g/kg	7.9	7.9	6.9	6.9
Lysine g/kg	11.8	11.8	9.0	9.0
Calcium g/kg	9.5	9.5	9.2	9.2
Available phosphorous g/kg	4.5	4.5	4.3	4.3
Composition (measured values)				
Dry matter, g/kg	894.1	894.1	897.5	897.5
Crude protein, g/kg	212.6	212.6	173.6	173.6
Ether extract, g/kg	42.1	42.1	43.8	43.8
Crude fiber, g/kg	46.1	46.1	46.2	46.2
Ash, g/kg	77.0	77.0	78.4	78.4
Nitrogen free extract, g/kg	593.3	593.3	633.9	633.9
Zn, ppm	26.0	26.0	26.0	26.0

* Vit + Min Premix provides the following (per kg of diet): Vitamin A, 1800 mg retinol; Vitamin E, 6.67 mg d-alpha-tocopherol; menadione, 2.5 mg; Vit D3, 50 mcg cholecalciferol; riboflavin, 2.5 mg; Ca pantothenate, 10 mg; nicotinic acid, 12 mg; choline chloride, 300 mg; vitamin B12, 4 mcg; vitamin B6, 5 mg; thiamine, 3 mg; folic acid, 0.50 mg; biotin 0.2 mg; Mn, 80 mg; Fe, 40 mg; Cu, 4 mg; Se, 0.10 mg.

Each diet was fed to five replicates consisting of nine male WPD each. Each replicate was housed in floor pens (1.0 m × 2 m) furnished with wood shavings. The brooding temperature was 34, 32, 30 and 28 °C during Weeks 1, 2, 3 and 4, respectively, and thereafter the temperature inside the house was about 27 °C. The light program provided 24 h of light on the first day; and then the lighting was gradually reduced to 10 h/day at 21 days of age. The light was supplied continuously. Water and mash form of feed were offered ad libitum.

2.2. Data Collection

The ducks were individually weighed at 1, 28, and 56 days of age in the morning, before offering feed. The remaining, scattered and consumed feed were measured during the periods 1–28, 28–56 and 1–56 days for each replicate; thus, the average feed intake per bird was calculated as the ratio between feed intake and the number of ducks per replicate. The feed conversion ratio (FCR) was calculated as units of feed intake required to produce one unit of gain in live body weight in the periods 1–28, 28–56 and 1–56 days. The mortality rate was recorded along the entire experimental period. At the end of the trial (56 days of age), five birds per treatment were randomly chosen, weighed after being fasted overnight, and slaughtered according to the Islamic guidelines. Feathers were plucked, the inedible parts (head, feet, and inedible viscera) were removed and the remaining (dressed) carcass was weighed. The feathers, liver, spleen, gizzard, heart, pancreas, and abdominal fat were separated and individually weighed. The percentage carcass yield and the percentages of internal organ weights relative to live body weight were calculated. A 50/50 (*w*/*w*) sample of skinless breast and thigh meat

was weighed and kept in an electric drying oven at 70 °C until a constant weight was reached. The dried flesh was finely ground through a suitable mixer, passed through a sieve (1 mm^2), and then carefully mixed and stored in tightly sealed glass containers for subsequent analysis. The physical characteristics of a sample mixture of breast and thigh meats were evaluated. The ability of meat to hold water (WHC) and meat tenderness were measured according to the methods of Volvoinskaia and Kelman [19]. The pH was measured as described by Aitken et al. [20]. The color intensity (optical density) of meat was determined according to the method of Husani et al. [21].

At 56 days of age, five ducks per group were housed in individual cages and used to evaluate the nutrient digestibility of the experimental diets. The birds were housed in individual cages. The methodology involved a four-day adaptation period followed by a three-days excreta collection period. After each day of collection, the excreta samples were dried to come to equilibrium with the atmosphere, weighed, ground and, finally, mixed together and stored in screw-top glass jars until analysis. The proximate chemical composition of the feed and excreta was according to the official methods of Association of Official Analytical Chemists (AOAC) [18].

The Zinc content was determined after ashing of the samples with 10 mL of concentrated sulfuric acid. Three drops of bichloric acid were added and the samples were incubated at room temperature for 2 h. Zinc concentration in the diets, liver, bones, excreta, and plasma were determined by atomic absorption spectroscopy (GBC Avanta Z, GBC Scientific Equipment, Braeside, Australia) using a standard curve. The apparent retention of Zn was calculated by dividing the difference between the amount consumed and that excreted by the amount consumed.

Blood samples were collected from wing vein from five ducks per treatment and placed into heparinized tubes. The plasma was separated by centrifugation at 1500× *g* for 15 min and stored at −18 °C until analysis. The plasma levels of Zn and Cu were determined by atomic absorption spectrometry after processing the samples as previously described.

2.3. Statistical Analysis

The data were analyzed using a two-way ANOVA of the General Linear Model (GLM) procedure of SAS [22] in which Zn and phytase supplementations were the main effects. The potential interactions between the effects were also evaluated. A probability of less or equal to 0.05 was considered significant, based on the Student Newman–Keuls Test of mean differences among treatments [22]. The data are reported based on the main effects and significant interactions. The differences among mortality rate were analyzed by chi-square test.

3. Results

The grower and the finisher basal diets used in the trial contained 26 ppm of Zn from raw materials (Table 1) as determined by atomic absorption spectrometry. The data on in vivo performance are reported in Table 2.

The mortality rate was not statistically different among the experimental groups. The addition of 30 ppm of Zn to the basal diets increased the body weight gain ($p < 0.01$) and feed intake ($p < 0.05$), and improved the FCR ($p < 0.05$) of ducks considering the entire period of the trial. The supplementation of phytase also improved ($p < 0.01$) BWG and FCR from 1 to 56 days, but the feed intake was not different from the control group. Except for the feed intake, the interaction between the two tested factors was significant: when no Zn was included in the diet, the addition of phytase improved both FCR and BWG; however, when 30 ppm of Zn were added to the basal diet, the addition of phytase did not improve the duck performance.

The addition of Zn to the diets reduced ($p < 0.01$) Zn retention (Table 3) and increased the level of Zn in tibia ($p < 0.01$), liver ($p < 0.05$) and excreta ($p < 0.01$).

Table 2. Effect of zinc supplementation, with and without phytase addition, on body weight gain, feed intake and feed conversion ratio of ducks *.

Treatment Group	BWG (g/Bird/Period)			Feed Intake (g/Bird/Period)			FCR (g/Bird/Period)			Dead (*n*)
	1–28 Day	29–56 Day	1–56 Day	1–28 Day	29–56 Day	1–56 Day	1–28 Day	29–56 Day	1–56 Day	
Zn addition										
0 ppm	1010 ^{b}	916 ^{b}	1926 ^{b}	1931 ^{b}	4182 ^{b}	6114 ^{b}	1.91	4.59	3.18 ^{a}	3
30 ppm	1079 ^{a}	965 ^{a}	2044 ^{a}	1991 ^{a}	4307 ^{a}	6297 ^{a}	1.85	4.46	3.08 ^{b}	1
Phytase inclusion										
0 U/kg diet	1032	910 ^{b}	1942 ^{b}	1961	4247	6209	1.91	4.67^{a}	3.20 ^{a}	2
500 U/kg diet	1056	970 ^{a}	2026 ^{a}	1960	4242	6202	1.86	4.38^{b}	3.06 ^{b}	2
Interaction between Zn and phytase										
0 ppm Zn + 0 U phytase	974 ^{d}	855 ^{b}	1830 ^{b}	1937	4189	6126	1.99 ^{a}	4.90 ^{a}	3.35 ^{a}	2
0 ppm Zn + 500 U phytase	1045 ^{c}	976 ^{a}	2021 ^{a}	1924	4175	6100	1.84 ^{b}	4.28 ^{b}	3.02 ^{b}	1
30 ppm Zn + 0 U phytase	1089 ^{a}	966 ^{a}	2054 ^{a}	1985	4305	6291	1.82 ^{b}	4.46 ^{b}	3.06 ^{b}	0
30 ppm Zn +500 U phytase	1068 ^{b}	965 ^{a}	2032 ^{a}	1995	4308	6303	1.87 ^{b}	4.47 ^{b}	3.10 ^{a,b}	1
p value										
Zn addition	0.0003	0.0023	0.0004	0.0295	0.0364	0.0336	0.1071	0.0711	0.0471	NS
Phytase inclusion	0.1191	0.0005	0.0007	0.9529	0.9221	0.9323	0.2344	0.0021	0.0077	NS
Interaction	0.0069	0.004	0.0002	0.6578	0.8849	0.8089	0.0289	0.0022	0.0012	NS
RMSE	33.15	30.6	45.17	55.23	121.6	176.5	0.094	0.149	0.105	0.94

* n = 180 bird as 45 bird per treatment group for body weight gain and n = 20 replicates as five replicates per each treatment for feed intake and feed conversion ratio. RMSE, Root mean square error. $^{a–d}$ means with different superscripts in the same column in similar treatment group are significantly different; NS, not significant.

Table 3. Effect of zinc supplementation, with and without phytase addition, on Zn retention and tissue and excrement concentrations, and plasma Zn and Cu concentrations in White Pekin ducks *.

	Zn Retention (%)	Tibia Ash (%)	Tibia Zn (ppm)	Liver Zn (ppm)	Excrement Zn (ppm)	Plasma Zn (mg/100 mL)	Plasma Cu (mg/100 mL)
Zn addition							
0 ppm	37.67 [a]	44.65	161.3 [b]	61.3 [b]	72.15 [b]	160.1 [b]	6.61 [b]
30 ppm	35.17 [b]	45.13	165.4 [a]	63.4 [a]	160.0 [a]	189.3 [a]	7.22 [a]
Phytase inclusion							
0 U/kg diet	35.90	45.15	162.8 [b]	61.0 [b]	117.6 [a]	164.9 [b]	5.93 [b]
500 U/kg diet	36.96	44.63	164.0 [a]	63.7[a]	114.3 [b]	184.0 [a]	8.28[a]
Interaction between Zn and phytase							
0 ppm Zn + 0 U phytase	36.64	44.46 [b]	160.6	60.7	74.06	149.2 [d]	5.23[d]
0 ppm Zn + 500 U phytase	38.70	44.91 [a]	161.9	61.8	70.06	169.0 [c]	7.97 [b]
30 ppm Zn + 0 U phytase	35.12	45.91 [a]	165.0	61.4	161.2	180.7 [b]	6.63 [c]
30 ppm Zn +500 U phytase	35.22	44.36 [b]	165.7	65.5	158.4	197.6 [a]	8.60 [a]
p value							
Zn addition	0.0008	0.2795	0.0001	0.0367	0.0001	0.0001	0.0001
Phytase concentration	0.0918	0.2429	0.0458	0.0126	0.0001	0.0001	0.0001
Interaction	0.1232	0.0302	0.5068	0.1378	0.4466	0.0006	0.0001
RMSE	1.346	0.958	1.072	2.12	1.491	0.736	0.164

* $n = 20$ samples as five sample per each treatment. RMSE, Root mean square error. [a–d] means with different superscripts in the same column in similar treatment groups are significantly different; NS, not significant.

In addition, the level of Zn and Cu in the plasma increased ($p < 0.01$) due to Zn inclusion in the basal diets. The addition of phytase increased the level of Zn in tibia ($p < 0.05$) and liver ($p < 0.01$) as well as the concentration of Zn and Cu in plasma ($p < 0.01$) but decreased the Zn content in the excreta ($p < 0.01$). The interaction between Zn level and phytase was significant for tibia ash, plasma Zn and plasma Cu. The use of phytase significantly decreased the tibia ash when 30 ppm of Zn oxide were added to the diets, but it did not happen for the Zn-free diet. The addition of phytase to the basal diet increased Zn and Cu concentration by 13.4% and 34.4%, respectively, while the addition of phytase to 30 ppm Zn diets increased the Zn and Cu plasma levels by 9.4% and 29.7%, respectively.

The addition of Zn to the basal diet decreased ($p < 0.01$) the percentage of gizzard but the other carcass traits were unaffected (Table 4). The use of phytase decreased the percentage of liver ($p < 0.01$) and abdominal fat ($p < 0.01$). There was a significant interaction between Zn level and phytase supplementation on gizzard percentage. Results indicate that phytase increased gizzard percentage of ducks fed 30 ppm Zn diet but had no effect when added to the basal diets.

Table 4. Effect of zinc supplementation, with and without phytase addition, on the percentage weights of the dressed carcass, carcass parts, inner organs, and abdominal fat in White Pekin ducks *.

	Dressing (%)	Front Part (%)	Hind Part (%)	Pancreas (%)	Spleen (%)	Liver (%)	Gizzard (%)	Heart (%)	Abdominal Fat (%)
Zn addition									
0 ppm	65.4	41.4	24	0.333	0.061	2.25	3.79 [a]	0.66	0.618
30 ppm	65.5	41.7	23.8	0.388	0.050	2.17	3.05 [b]	0.616	0.641
Phytase concentration									
0 U/kg diet	66.6	42.3	24.2	0.352	0.055	2.39 [a]	3.42	0.638	0.790 [a]
500 U/kg diet	64.4	40.7	23.7	0.369	0.056	2.03 [b]	3.41	0.614	0.472 [b]
Interaction between Zn and phytase									
0 ppm Zn + 0 U phytase	67.5	43.1	24.4	0.373	0.058	2.31	4.02a	0.693	0.820
0 ppm Zn + 500 U phytase	63.4	39.7	23.7	0.291	0.064	2.18	3.56 [a,b]	0.626	0.413
30 ppm Zn + 0 U phytase	65.6	41.6	23.9	0.332	0.051	2.46	2.83 [c]	0.622	0.748
30 ppm Zn + 500 U phytase	65.4	41.7	23.7	0.446	0.048	1.88	3.26 [b]	0.602	0.530
p value									
Zn addition	0.9343	0.1353	0.7878	0.3446	0.075	0.5699	0.0001	0.5213	0.6869
Phytase concentration	0.0941	0.0729	0.4483	0.7761	0.8531	0.0123	0.9465	0.2596	0.0001
Interaction	0.1293	0.0602	0.7413	0.1188	0.4321	0.093	0.0288	0.5684	0.9421
RMSE	2.712	1.881	1.548	0.132	0.013	0.28	0.413	0.255	0.119

* $n = 20$ samples as five sample per each treatment; RMSE, Root mean square error. [a–d] means with different superscripts in the same column in similar treatment groups are significantly different; NS, not significant.

4. Discussion

The natural presence of Zn in the diets from the raw materials is not enough alone to adequately sustain the duck growth. In our trial, the addition of 30 ppm of Zn oxide to the basal diets improved the animal performance: the increase of feed intake was responsible for the increased body weight gain, giving a more favorable FCR. Cufadar and Bahtiyarca [23] indicated that 30 ppm of Zn was adequate for growth performance of male WPD. The live weight of the ducks at the end of the trial (56 days) was lower than the data recorded in the literature. However, as reported by Dodu [24], the imported breed of ducks, along the years, was mixed with local populations giving genetic lines differing for some growing characteristics from the original breed. In particular, Dodu [24] indicated that the body weight at 56 days of some Pekin ducks bred in Romania was around 2 kg. In a previous study, Attia et al. [25] found similar body weight for 56-day-old Pekin ducks breed in Egypt.

The supplementation of Zn to the basal diet strongly increased its amount in the excreta, also due to the lowering of the retention rate. In fact, the primary mechanism of trace minerals homeostasis is the modification of the trace minerals absorption and excretion in the gut [25–27]. Cao et al. [28] observed that bone and fecal Zn contents were significantly increased when the diets of chickens were supplemented with organic and inorganic sources of Zn. The significant increase of gizzard percentage due to the addition of Zn in the present trial could be justified by the increased feed intake which could play a physical effect on gizzard expansion.

The improved BWG and FCR in ducks supplemented with phytase diets were not due to an increase of feed intake: in fact, the percentage of gizzard was also unchanged between the groups. The positive effect of phytase on growth performance of WPD could be attributed to the increase in the availability of others inorganic and organic nutrients [14,29–31]. The positive effect of Zn on BWG was probably due to an improved activity of the copper-requiring metalloenzymes, such as ceruloplasmin, cupro-zinc superoxide dismutase and cytochrome c oxidase, which have a very important role as anti-oxidants in the metabolism [32]. In addition, looking at the interaction effect, the use of the basal diet without Zn supplementation induced lower growth rate than that with an addition of 500 U of phytase or 30 ppm of Zn. The increased growth rate due to phytase or Zn supplementation resulted in an improved FCR. The phytase improved Zn utilization, as evidenced by the increase of Zn in plasma and its decrease in the excreta, but the effect on Zn retention was weak. In the literature, the effect of phytase on Zn availability is contradictory: phytase is reported to increase the availability of dietary Zn [4,23] as well as the bone Zn content in pigs and chicks [33], but to have no significant effect on Zn digestibility and apparent absorption percentage of Zn, Fe, or Cu in chicks [34]. These differences could be ascribed to the differences in the metabolism among the species, the different dietary composition or Zn level in the basal diet. Dietary Zn at 800 ppm negatively affected phytate breakdown by phytase [35] as a result of a conformational change in the phytate moiety, thereby making it less accessible to phytase.

The effect of phytase on plasma Zn content was stronger in WPD fed the basal diet than in those fed diets supplemented with 30 ppm of Zn oxide (13.3% vs. 9.4%). These results are consistent with those reported by Mohana and Nys [36]. In addition, the value for Zn retention found herein agrees with those reported by other authors [10,36]. Similar to the present findings, Jondreville et al. [15] found that 100 FTU of phytase were equal to 1 ppm of Zn, and that the Zn excretion could be reduced by 10% if a corn–soybean diet were supplemented with 500 FTU phytase/kg diet.

In the present study, the phytase significantly increased plasma content of Cu, according to Attia et al. [37,38]. Revy et al. [30] reported a positive effect of phytase on Cu availability due to the effect of phytase on phytate-mineral complex formation. However, Jondreville et al. [15] reported that microbial phytase had a negative effect on the liver Cu content in chicks and pigs, likely because of the negative effect of Zn on Cu availability due to release of Zn by phytase [39].

The lower percentage of the abdominal fat and liver in WPD fed diets with phytase may be attributed to the reapportioning of nutrients towards growth rather than fat accumulation. Similar results were reported by Attia et al. [12,29]. Furthermore, Cufadar and Bahtiyarca [23] reported that

increasing dietary phytase at three dietary Zn levels increased the results for all carcass parameters, although the effects were not proportional to the level of dietary phytase; rather, phytase prevented the deleterious effects of dietary Zn on carcass traits. This might explain the positive effect of phytase on the growth and the decrease of the abdominal fat of WPD in the present study. Orban et al. [40], Attia et al. [12,29] and Cufadar and Bahtiyarca [23] found that phytase significantly increased the carcass weight, neck, thigh, back + breast and wings of broilers.

5. Conclusions

The natural presence of Zn in raw materials is not enough alone to satisfy the Zn requirements of the growing ducks. The addition of 30 ppm of Zn or 500 U of phytase to the basal diet increased the growth rate and improved the FCR of the ducks. However, the addition of 30 ppm of Zn oxide also increased the level of Zn in the excreta, while the addition of 500 U of phytase had an opposite effect and is more appropriate to reduce the potential risks for environmental pollution.

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Assessment of Residual Feed Intake and Its Relevant Measurements in Two Varieties of Japanese Quails (*Coturnixcoturnix japonica*) under High Environmental Temperature

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Simple Summary: Residual feed intake (RFI) is an important factor in improving poultry production and laying performance, particularly for poultry raised under heat stress. An experiment was conducted to assess RFI and its related measurements in Japanese quails (*Coturnixcoturnix japonica*) of two varieties (gray and white) reared under high environmental temperatures.The current results confirmed that raising gray quails for egg production under high environmental temperature is recommended. Multiple regression analysis clearly identified a significant effect of metabolic body weight and egg mass for the computation of expected feed intake rather than body weight gain in both varieties of Japanese quails.

Abstract: Three hundred and ten 12-week-old laying Japanese quails (*Coturnixcoturnix japonica*) from gray and white varieties (155 each) were randomly selected from the initial population and kept in individual battery cages. The measurements of growth and egg production were determined to derive residual feed intake (RFI). The relationship between RFI and egg quality, blood parameters, and carcass characteristics was also determined. The results indicated that the gray quails had significantly higher egg mass and lower broken eggs compared to the white quails. A significant increase of eggshell strength and shell percentage was found in eggs produced from gray quails compared to their white counterparts, although their shell thickness means weresimilar. The results of multiple regression analysis clearly identified a significant effect of metabolic body weight and egg mass for the computation of expected feed intake, rather than body weight gain, in both varieties of Japanese quails. A strong positive correlation between RFI and feed intake in both gray and white quail varieties was found. The same trend was also observed for feed conversion ratio (FCR). Therefore, including RFI in the selection criteria of Japanese quails in order to improve FCR under high environmental temperature is highly recommended.

Keywords: residual feed consumption; quail; high environmental temperature

1. Introduction

Feed expenses represent almost 70% of the gross cost of poultry production. Lowering costs of maintenance processes would leave more energy remaining for higher output. Minimizing residual feed intake (RFI) and, in turn, improving feed efficiency would be beneficial for more efficient quail hens, particularly under high environmental temperatures. However, a bird's ability to convert consumed feed to produce eggs and/or meat is greatly influenced by genotype and environmental factors. Birds that require less feed than expected for maintenance and production requirements have a negative RFI and are desirable in poultry breeding programs to reduce feed costs. RFI has increasingly become a critical factor for measuring feed efficiency and is commonly considered one of the target traits in animal breeding programs [1]. However, many selection programs take RFI into consideration to improve economic productive traits of synthetic or commercial strains. It has already been reported that RFI could be used in selection programs in laying hens and quails. Altan et al. [2] indicated that the selection for RFI in Japanese quails (*Coturnixcoturnix japonica*) might provide a tool to improve the efficiency of feed utilization without significant negative changes in egg production and egg quality traits and with a decreased susceptibility of the laying hens to stress. Most researchers concluded that a four-week recording period provides sufficient information for the genetic evaluation of residual feed intake in many species of poultry [3–7].

Direct selection for more efficient birds is becoming one of the primary goals in breeding programs of laying hens [7]. Improving feed efficiency is of great economic concern for commercial egg producers to maximize project outcomes. Traditionally, feed efficiency has been improved by selection for increased egg mass and decreased body weight and getting a correlated response in feed efficiency [2]. Identifying birds that require less feed for body maintenance could improve feed efficiency. However, selection for feed conversion ratio can lead to unfavorable changes in the component traits. Additionally, direct selection for feed efficiency requires measurement of individual feed intake, which is time consuming and very expensive. Well-designed feeders are also required to prevent feed wastage. On the other hand, additional criteria for feed utilization should be involved. Residual feed intake may be used as selection criteria to attain these goals [2]. To our knowledge, there are no previous reports on the residual feed intake of different lines or varieties of Japanese quails raised under hot ambient temperature [4]. Due to the need to adjust patterns of feed consumption according to ambient temperature, the present study was carried out to estimate RFI, as well as its relationship with productive traits, in two varieties of Japanese quails under high environmental temperature.

2. Materials and Methods

2.1. Birds, Housing, and Management

A total of 310 laying Japanese quails of two varieties (gray and white) (155 each) were randomly selected from an initial population of 800-day-old hens that were reared to point of lay and transferred to individual battery cages. Each hen was individually housed in a wire cage (20 × 20 × 20 cm) supplied with an individual feed trough in the front and a nipple drinker. The quail received a laying ration containing 18% crude protein (CP) and 2850 kcal/kg metabolizable energy (ME) during the experimental period. Throughout the experiment, feed and water were available ad libitum. Birds were exposed to a lighting period of 16 h per day.All quails received uniform care and management practices throughout the whole experimental period. The average high and low ambient temperatures recorded during the experimental period were 38.9 °C and 24.3 °C, respectively. No vaccination or medication was performed. The use and handling of quails were approved by the Ethical Committee of Qassim University.

2.2. Egg Production Parameters

Starting from 12weeks of age, egg production (weight and number), feed intake (FI), and body weight (initial and final) were determined for each hen over a four-week experimental period.

Egg production (%) was calculated as total laid eggs divided by the total number of days (28 days). Feed intake was measured on a cage basis and combined with egg production data to calculate feed conversion ratio (FCR).

2.3. Egg Quality Measurements

A total of 460 eggs (230 eggs from each variety) were collected at 16 weeks of age. External and internal egg quality measurements were assessed according to Fathi et al. [8]. Each egg was weighed to the nearest 0.1 g. Egg width (equatorial axis) and egg length (longitudinal axis) were measured using Vernier caliper to 0.1 mm. Egg shape was calculated according to the following formula:

$$\text{Egg shape} = (\text{egg width})/(\text{egg length}) \times 100 \quad (1)$$

The breaking strength for intact eggs was determined in Kg/cm^2 using Egg Force Reader^{TM}, Orka Food Technology Ltd. (Wanchai, Hong Kong). The liquid contents were put aside and the shell plus membranes were washed under running water to remove the adherent albumen. The wet eggshell was left for 24 h at room temperature for drying and then weighed to the nearest 0.01 g. The relative weight of dry eggshell was calculated on the basis of egg weight. To measure shell thickness, pieces from three different regions (two poles and equator) of each eggshell with intact membranes were measured with a dial gauge micrometer to the nearest 0.01 mm. The height of thick albumen and egg yolk was measured by placing the liquid content on a balanced surface using a tripod micrometer. Then, the yolk was separated and rolled on tissue papers to remove the residual albumen. Albumen weight was calculated by subtracting the yolk and shell weight from egg weight. The weight of eggshell, yolk, and albumen were expressed as a percentage of egg weight. Haugh units (HU) were calculated according to the following formula:

$$HU = 100 \log (H - 1.7W^{0.37} + 7.57) \quad (2)$$

where H is the albumen height (mm) and W is the egg weight (g). Yolk color was measured by comparing yolk color to the DSM yolk color fan.

2.4. Carcass and Internal Organs

At the end of the experiment, 50 quails (25 from each variety) were randomly assigned for carcass yield assessment. After a pre-slaughter fasting period of 4 h, the quails were weighedand slaughtered by cutting their jugular veins. Following a 2-min bleeding time, each quail was dipped in a hot water bath at 60 °C for 60 s. and manually defeathered. Head and feet were removed. The carcass was eviscerated manually and weighed. Upon evisceration, the weight of eviscerated carcass, liver, heart, and gizzard was recorded and expressed as a percentage of live body weight according to Fathi et al. [9]. To minimize variations in the carcass procedure, all dissections were carried out by the same person.

2.5. Blood Hematology and Plasma Biochemistry

During slaughter, 25 blood samples were collected from each variety in heparinized tubes. The hematological parameters were determined by using Automatic Fully Digital Hematology Analyzer (BC-3000 Plus, Shenzhen Mindray, Bio-Medical Electronics Co., LTD, Shenzhen, China). These parameters were total count of red blood cells (RBC), hemoglobin (HGB), hematocrit (HT), and thrombocytes. The collected blood samples were centrifuged at 4000× rpm for 15 min. The resulting plasma samples were frozen at −20 °C for further analysis. The plasma concentrations of total protein, albumen, total cholesterol, and triglycerides were spectrophotometrically determined according to Fathi et al. [10] using commercial reagent kits (Stanbio Laboratory, Boerne, TX, USA). The globulin was calculated as the difference between the total protein and albumen.

2.6. Calculation of Residual Feed Intake and Statistical Analysis

Expected feed intake was computed using mid-metabolic body weight ($BW^{0.75}$), body weight gain (ΔBW), and total egg mass (EM) for a given time considered by multiple regression analysis. Residual feed intake (RFI) was calculated as the difference between observed feed intake (OFI) and expected feed intake (EFI) for each experimental hen using the PROC REG procedure of JMP Ver. 11 (SAS, Cary, NC, USA) [11]. Each variety had its own partial regression coefficients according to the following multiple regression equation:

$$EFI = aBW_i^{0.75} + bEM_i + c\Delta BW_i + d \quad (3)$$

where EFI = expected feed intake of hen i (g); $BW_i^{0.75}$ = mean metabolic body weight of hen i ($g^{0.75}$); EM_i = egg mass production of hen i (g); ΔBW_i = body weight gain (g); a, b, and c = partial regression coefficients; d = intercept.

Student *t* test analysis was applied to separate between means. All data were presented as means and the pooled SEM. Correlation coefficient was computed between RFI and some studied traits within each variety using PROC CORR procedure.

3. Results and Discussion

Productive performance of two varieties of Japanese quails is shown in Table 1. No significant difference for body weight (initial and final), weight gain, FI, or FCR was identified between the varieties. Gray quails had significantly higher ($p < 0.02$) egg mass and egg production percentage than that of white quails. A superiority of egg production in brown variety Japanese quails compared to both gray and white ones was previously detected [12]. Broken eggs were significantly ($p < 0.05$) affected by the quail variety. The gray quails recorded the lower value (1.13%) compared to the white quails (2.06%). Mortality levels fell within the normal range and there was no significant difference between quail varieties.

Table 1. Productive performance of two varieties of Japanese quails (*Coturnixcoturnix japonica*).

Parameter	Variety		SEM	*p*-Value
	Gray	White		
Initial body weight, g	203.9	201.1	1.72	0.41
Final body weight, g	213.8	211.5	1.74	0.51
Body weight gain, g	9.8	10.4	1.32	0.84
Egg mass, g	289.5 ^{a}	277.8 ^{b}	2.43	0.02
FI, g	719.9	722.6	3.75	0.87
FCR	2.49	2.60	0.07	0.11
Egg production, %	89.9 ^{a}	86.4 ^{b}	0.73	0.02
Broken eggs, %	1.13 ^{b}	2.06 ^{a}	0.02	0.05
Egg weight, g	11.5	11.4	0.023	0.93

N = 155 quails/ variety; FI: feed intake; FCR: feed conversion ratio; ^{a,b} mean values in a raw without a common superscript are significantly different.

Internal and external egg quality characteristics are presented in Table 2. Shape index was significantly ($p < 0.01$) higher in eggs produced from white quails compared to their gray counterparts. Consistent with our results, Yilmaz et al. [13] and Sari et al. [14] reported that the egg shape index depended on the plumage color of the quails. They found that the mean shape index obtained from the gray plumage line was significantly lower than that of the white plumage line. In contrast to our results, Bagh et al. [10] did not find a significant difference between gray and white lines for all physical properties of egg quality. A numerical increase ($p = 0.08$) in HU was found in gray quails when compared with white quails. However, Bagh et al. [12] reported that there was no significance difference among the quail varieties for HU. In terms of yolk properties, there were no significant

differences between quail varieties for yolk color, yolk index, and yolk percentage. Significantly ($p < 0.04$) higher albumen percentage was foundin eggs produced from white variety compared to the gray variety. In regard to eggshell quality, a significant ($p < 0.01$) increase in eggshell breaking strength was found in eggs produced from gray quails (1.43 kg/cm^2) compared to the white quails (1.34 kg/cm^2). Also, gray quails had a significantly ($p < 0.01$) higher relative weight of eggshell (9.4%) compared to that of their white counterparts (9.0%). However, shell thickness did not exhibit a significant difference due to the effect of variety. This advantage in eggshell strength associated with gray quails may be attributed to better ultrastructural featuresin comparison to eggshells of the white variety. Changes in external and internal quality characteristics of eggs obtained from quails with different plumage colors have previously been reported [12–14]. However, literature on the external and internal quality characteristics of eggs obtained from quail varieties with different plumage color under high environmental conditions is very limited.

Table 2. Internal and external egg quality of two varieties of Japanese quails.

Parameter	Variety		SEM	*p*-Value
	Gray	White		
Shape index, %	75.95 [b]	76.90 [a]	0.15	<0.01
HU	88.7	88.2	0.13	0.08
Yolk color score [1]	6.96	6.98	0.03	0.70
Yolk index	47.67	47.31	0.12	0.15
Yolk, %	33.5	33.3	0.12	0.39
Albumen, %	57.1 [b]	57.7 [a]	0.14	0.04
Shell, %	9.4 [a]	9.0 [b]	0.05	<0.01
Shell thickness, µ	254.0	255.2	0.73	0.40
Breaking strength, kg/cm^2	1.43 [a]	1.34 [b]	0.01	<0.01

N = 230 intact eggs/variety; HU: Haugh units; [1] DSM yolk color fan. [a,b] mean values in a raw without a common superscript are significantly different.

Plasma biochemical and hematological parameters of gray and white feathered Japanese quails are summarized in Table 3. No significant effect on the blood biochemical variables was detected due to variety, except for cholesterol level. White quails had a significant ($p < 0.01$) increase in cholesterol level (198.5 mg/dL) compared to the gray variety (152.5 mg/dL). In terms of blood hematology, the white feathered quails had significantly higher levels of RBC, HGB, and HT compared to the gray quails. Generally, hematological parameters fell within the normal range for quails [15]. These results indicate that different genotypes in the present study were of normal physiological status.

Table 3. Biochemical and hematological blood parameters of two varieties of Japanese quails.

Parameter	Variety		SEM	*p*-Value
	Gray	White		
Total Protein, g/dL	5.34	5.43	0.17	0.82
Albumin, g/dL	3.13	3.42	0.09	0.27
Globulin, g/dL	2.21	2.01	0.18	0.69
Cholesterol, mg/dL	152.50 [b]	198.47 [a]	8.98	<0.01
Triglyceride, mg/dL	121.75	120.67	4.69	0.93
RBC	3.28 [b]	3.51 [a]	0.05	0.02
HGB	19.36 [b]	20.59 [a]	0.25	<0.01
HT	44.37 [b]	47.21 [a]	0.63	0.02
Thrombocytes	15.84	15.83	0.85	0.99

N = 25 quails/variety; RBC: red blood cells; HGB: hemoglobin; HT: hematocrit; [a,b] mean values in a raw without a common superscript are significantly different.

Results of carcass traits studied as affected by variety of quails are shown in Table 4. It was found that neither carcass percentage nor giblets (liver, heart, and gizzard) significantly differed due to variety effect. However, an insignificant increase ($p = 0.15$) in carcass % was found in white quails (63.3%) compared to their gray counterparts (62.4%). Similar to the present study, Charati and Esmailizadeh [16] found that genotype had no significant effect on carcass percentage in white and wild (gray) Japanese quails. In contrast, several previous studies reported that feather color had a significant effect on live weight and carcass characteristics in Japanese quails. The white feathered quails had less body weight than that of the wild-type [17–19]. Similarly, Vali et al. [20] found significant differences in two quail strains for carcass weight, carcass percentage, and the relative weight of breast and femur.

Table 4. Carcass traits of two varieties of Japanese quails.

Trait	Variety		SEM	*p*-Value
	Gray	White		
Live body weight, g	223.9	223.5	3.18	0.93
Dressed carcass, %	62.4	63.3	0.30	0.15
Liver, %	2.02	2.12	0.08	0.51
Heart, %	1.20	1.14	0.02	0.19
Gizzard, %	1.66	1.56	0.04	0.13

$N = 25$ quails/variety.

The results of the multiple regression analysis are listed in Table 5. Below the table, the prediction equations for the expected feed consumption for each variety have been provided. RFI is defined as the difference between the realized feed consumption and the expected feed consumption, which was estimated based on metabolic BW, body weight gain, and EM [3,21]. As shown, the partial regression coefficients for metabolic body weight and egg mass had significant effects in computing expected feed intake in both quail varieties. The intercept value also had a significant effect. On the other hand, body weight gain (Δ BWT) did not significantly affect the computation of RFI in either the gray variety ($p = 0.08$) or the white variety ($p = 0.63$). Estimates of regression coefficients in the models of gray and white quails were close. Similar to our findings, Badawe et al. [3] found thatthe prediction of feed intake and residual feed intakederived from multiple regression analysis was significantly affected by metabolic body weight and egg mass in laying chickens (*gallus gallus*).

Table 5. Partial regression coefficients for factors affecting expected feed intake of two varieties of Japanese quails.

Parameter Estimate	Partial Regression Coefficient		Prob.	
	Gray	White	Gray	White
Intercept	914.6 **	396.2 *	<0.001	0.02
Δ BWT	−0.96	−0.36	0.08	0.63
$(BWT)^{0.75}$	5.21 *	11.84 **	0.04	<0.001
Egg mass (EM)	−1.64 **	−1.11 **	<0.001	<0.001

$N = 155$ individual records/variety, $^{*}p < 0.05$, $^{**}p < 0.01$. Prediction equations: $Y = 914.6 - 0.96\ \Delta\ BWT + 5.21\ (BWT)^{0.75} - 1.64\ EM$ (Gray variety), $Y = 396.2 - 0.36\ \Delta\ BWT + 11.84\ (BWT)^{0.75} - 1.11\ EM$ (White variety); where Y stands for expected feed consumption, Δ BWT = body weight gain, $(BWT)^{0.75}$ = metabolic body weight, EM = total egg mass.

Phenotypic correlations between RFI and some studied traits are presented in Table 6. A strong positive correlation between RFI and FI in gray and white quail varieties (0.89 and 0.91, respectively) was found. Notably, the correlation between RFI and FI in our study was much higher than those estimated in previous works on laying chickens [7,22,23]. The selection for low RFI could reduce FI without significant changes in EM [7]. FCR is widely used but not a suitable selection trait because of its complex correlations with growth and production traits [6,7]. As shown in Table 6, a significantly high

correlation was recorded between RFI and FCR (0.55 and 0.49, respectively). These strong relationships have indicated that selection for negative RFI would genetically improve feed efficiency and reduce feed intake. These results are consistent with those of Zhang et al. [1], who found a high phenotypic correlation between RFI and FCR (0.55). Moreover, RFI was strongly correlated with FI (0.82) in a random population of Pekin duck (*anas paltyrhynchos*). Similarly, RFI was positively correlated with FI in laying hens of chickens [22–24] and Japanese quails [2]. It is worthy to note that there was a low or neglected correlation between RFI and both egg weight and egg production% for the quail varieties. A low correlation between RFI with body weight gain was found (close to zero) in both quail varieties. Our results are in accordance with the findings of Luiting and Urff [25] and Altan et al. [2], who also described that the phenotypic correlation of RFI with both egg mass and body weight was almost zero. Likewise, these results are in agreement with the findings of Varkoohi et al. [5], reflecting the fact that RFI is phenotypically independent of weight gain. On the other hand, phenotypic correlations between RFI and both blood parameters and carcass traits were found to be rather low and insignificant. No significant relationship was observed between RFI with live body weight and eviscerated carcass weight [26].

Table 6. Phenotypic correlations between RFI and some studied traits in Japanese quails.

Trait	Phenotypic Correlation	
	Gray	White
Δ Body wt	−0.003	0.002
Feed intake	0.89 **	0.91 **
FCR	0.55 *	0.49 *
Prod%	−0.07	−0.05
Egg wt	0.11	0.18
Total protein	−0.21	0.22
Albumin	−0.38	0.38
Globulin	0.01	0.03
Cholesterol	0.23	−0.15
Triglyceride	0.51	0.20
Hemoglobin	−0.18	−0.40
Red blood cells	0.32	−0.49
Hematocrit	0.20	−0.50
Thrombocytes	−0.15	−0.14
Carcass	0.09	0.09
Liver	0.04	−0.22
Heart	−0.38	−0.03
Gizzard	0.09	−0.22

* $p < 0.05$, ** $p < 0.01$.

4. Conclusions

The current results indicate that the egg mass significantly increased in the gray variety of Japanese quails compared to the white variety. Additionally, gray quails had a significantly lower percentage of broken eggs. Color variations in Japanese quails should be considered when selecting for type of production. We recommended raising gray quails for egg production under high environmental temperature, while the white variety may be more suitable for meat type. Results derived from multiple regression analysis clearly identified a significant effect of metabolic body weight and egg mass in computing expected feed intake rather than body weight gain in both varieties of Japanese quails. Including residual feed intake in the selection criteria of Japanese quailsin order to improve feed conversion ratio under high environmental temperature is highly recommended.

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animals

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Article

Intestinal Morphology in Broiler Chickens Supplemented with Propolis and Bee Pollen

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Simple Summary: Consumers are becoming more aware of the nutritional value of foods, and they want to consume food that provides health benefits beyond the provision of essential nutrients. Chicken meat could fulfil the above requirements due to its high nutrient content and relatively low caloric value, and it serves as an interesting basis for functional foods. In this study, we evaluated the effects of propolis and bee pollen, as potential additives, on the intestinal morphology and absorptive surface areas of broiler chickens. The results of this study showed that supplementation of broilers with propolis and/or bee pollen has a profoundly beneficial effect on intestinal morphology and absorptive surface areas. Thus, these natural additives could be used as alternative additives in modern broiler production, while chicken meat can be even more beneficial for human health.

Abstract: The aim of this study was to determine the influence of dietary supplementation with propolis and bee pollen on the intestinal morphology and absorptive surface areas of chickens. Two hundred day-old Ross 308 chickens (100 male and 100 female) were equally allocated into five groups. Throughout the whole study, the control group of chickens was fed with a basal diet, while the experimental groups of chickens were fed with the same diet supplemented with propolis and bee pollen: P1 = 0.25 g of propolis/kg + 20 g of bee pollen/kg; P2 = 0.5 g of propolis/kg; P3 = 1.0 g of propolis/kg; P4 = 20 g of bee pollen/kg. The duodenal villi of chickens from all experimental groups were significantly higher and wider ($p < 0.001$), while their duodenal villi crypts were significantly deeper ($p < 0.001$) in comparison with these parameters in chickens from the control group. The villus height to crypt depth ratio, as well as the absorptive surface areas of broiler chickens, were significantly increased ($p < 0.001$) in experimental groups of chickens in comparison with the control group. These findings suggest that dietary supplementation with propolis and bee pollen has a beneficial effect on broilers chickens' intestinal morphophysiology.

Keywords: intestinal morphology; duodenum; intestinal villi; intestinal absorption; broilers feeding; propolis; bee pollen

1. Introduction

Propolis and bee pollen belong to a group of natural substances of animal and vegetable origin with intense antioxidant and antimicrobial properties [1]. The bioactive components of propolis and bee pollen include flavonoids, phenolic acids and their derivatives, which are also responsible for the

bactericidal, antiviral, antifungal, analgesic, anti-inflammatory, antioxidant, immunostimulating and immunomodulating effects of these compounds in humans and animals [1–3].

A large number of previous studies have suggested an increase in the production performance of chickens fed with propolis and/or bee pollen [4–9]. These effects could be related to the effect of propolis extract on gastrointestinal microbiota, which increases levels of beneficial bacteria and decreases pathogenic types [10]. This modulation of microbiota could promote intestinal health, since the beneficial bacteria could provide improved feed digestibility and protection against pathogens via competitive exclusion through a variety of mechanisms [11,12].

With consideration of the above, and also the fact that the European Commission banned the use of antibiotics as growth agents in 2006 [7,13], the use of natural feeding additives such as propolis or bee pollen is very important in terms of improvement of performance, health status and immune systems in broiler chickens [1,14].

The small intestine is an important organ responsible for the digestion and absorption of nutrients from the diet. Any changes in its function affect the function of other organs and systems in the organism [15]. There are only a few studies that have previously evaluated the effect of propolis and bee pollen on the intestinal morphology of broiler chickens, and their results are contradictory [12,16–19]. However, some of them have proven that these natural additives improved intestinal morphophysiology [16–19].

The aim of this study was to determine the influence of dietary supplementation with propolis and bee pollen on the intestinal morphology and absorptive surface areas of broiler chickens.

2. Materials and Methods

2.1. Animals and Diets

A total of 200 (100 male and 100 female) day-old Ross 308 broiler chickens were evenly distributed by gender for use in the present study. The feeding trial of the broilers was carried out on a farm in Eastern Croatia under the supervision of the Division for Animal Production and Biotechnology, Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek. The experimental protocol was approved by the Committee for Animal Welfare of the Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek (Approval code: 602-04/18-01/01; 2158-94-02-18-01).

The experiment was a completely randomized design, and broilers were allocated into five dietary treatments with two replicate groups of 20 birds per pen (5 diets × 2 replicates). The groups of broilers were housed under the same conditions during the whole experimental period. Temperature, humidity, and lighting in the facility were maintained within the optimum limits according to the manufacturer's recommendations for the Ross 308 hybrid [20]. Breeding was conducted on wooden sawdust (10 cm depth) and lasted for six weeks (42 days). During the study, feed and water were offered to broilers ad libitum. For ensuring effective monitoring of all the investigated indicators, all the broilers were marked with a leg ring on the seventh day of the feeding trial.

During days 1–21 of the study, broilers were fed a mixture of broiler starter. During days 22–42 of the study, broilers were fed a mixture of broiler finisher. The composition and calculated analyses of feed mixtures used in the feeding of the broilers are shown in Table 1. Throughout the study, the control group (K) of the broilers was fed a standard diet without additives, while the experimental groups of broilers (P1, P2, P3 and P4) were fed the same diet supplemented with propolis and/or bee pollen: the P1 group was offered a diet supplemented with 0.25 g of propolis and 20 g of bee pollen per kg of diet; the P2 group was offered a diet supplemented with 0.5 g of propolis per kg of diet; the P3 group was offered a diet supplemented with 1.0 g of propolis per kg of diet; the P4 group was offered a diet supplemented with 20 g of bee pollen per kg of diet. The doses of bee pollen and propolis were selected on the basis of known broiler chicken gastrointestinal tract physiology and through series of pilot studies on a small number of animals. The inclusion of propolis and bee pollen into the feed mixture was performed using a vertical mixer (Briketstroj Ltd., Valpovo, Croatia).

Table 1. The composition and calculated analysis of feed mixtures used in the feeding of the broilers.

Ingredients, %	Starter	Finisher
	Day 1–21	Day 22–42
Corn grain	45.00	46.10
Flour middling	2.80	3.00
Dehydrated alfalfa	2.80	4.00
Soybean meal	20.20	10.00
Sunflower meal	4.00	4.00
Yeast	4.00	3.00
Full fat soybean	12.40	20.00
Vegetable oil	3.70	5.00
Monocalcium phosphate	1.20	1.20
Limestone	1.60	1.40
Salt	0.30	0.30
Premix *	1.00	1.00
Pigozen 801	1.00	1.00
Total	100.00	100.00
Calculated Analysis		
Crude protein, %	21.02	19.15
Crude fat, %	8.36	10.96
Crude fiber, %	4.96	5.05
Lysine, %	1.11	0.96
Methionine, %	0.66	0.61
Tryptophan, %	0.26	0.23
Calcium, %	1.04	0.98
Phosphorous, %	0.70	0.67
ME, MJ/kg	12.30	13.10

* Each 1 kg of premix contained: vitamin A 1200,000 IU; vitamin D3 200,000 IU; vitamin E 3000 mg; vitamin K3 250 mg; vitamin B1 150 mg; vitamin B2 600 mg; vitamin B6 200 mg; vitamin B12 1 mg; folic acid 50 mg; niacin 4400 mg; Ca pantothenate 1500 mg; biotin 10mg; choline chloride 50,000 mg; iron 5000 mg; copper 700 mg; manganese 8000 mg; zinc 5000 mg; iodine 75 mg; cobalt 20 mg; magnesium 750 mg; selenium 15 mg; antioxidant butylated hydroxytoluene (BHT) 10,000 mg; methionine 100,000 mg; herbal carrier 1000 g.

Samples of raw propolis and bee pollen used in this study were obtained from apiaries located in naturally preserved areas of continental Croatia (around the city of Osijek, Eastern Croatia). Propolis and bee pollen were crushed mixed, in powder form, with dry feed mixture using a vertical mixer. Bearing in mind that the biological activity of propolis and bee pollen depends on the components of polyphenolic fraction, mainly flavonoids, in the propolis and bee pollen samples used in this study, the amount of total flavonoids (expressed as equivalents of quercetin) was determined by a colorimetric method according to Chang et al. [21]. The results of this analysis are shown in Table 2. The analysis was performed at the Department of Health Ecology within the Croatian Institute of Public Health in Zagreb, Croatia accredited according to HRN EN ISO/IEC 17025:2000.

Table 2. The amount of total flavonoids (mg/g) in propolis and bee pollen, expressed as equivalents of quercetin.

The Amount of Total Flavonoids (mg/g), Expressed as Equivalents of Quercetin	Propolis	Bee Pollen
	248.24	31.80

2.2. Sample Collection, Measurements and Analysis

At the end of the feeding trial (i.e., day 42), 10 birds from each group were randomly selected and slaughtered for a necropsy examination. Fifty duodenal samples (10 from each group) were collected from the birds directly after slaughter and fixed in 10% neutralized formalin. The duodenal samples were 2 cm long and dissected at the midpoint of the duodenum. The fixed tissue samples

were transported to the Department of Pathology and Forensic Medicine, Faculty of Medicine Osijek, where they were further processed. The tissues were then dehydrated with increasing concentrations of ethyl alcohol (70%, 90%, 96% and 100%), cleared in xylene and embedded in paraffin. The paraffin blocks were then cut using microtome, into four 5-µm-thick discontinuous paraffin-embedded sections per broiler duodenal sample that were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus CX40), while representative fields were photographed and digital images were captured for morphometric analysis. A computer morphometric program, Quick Photo Micro 3.0, was used for morphometric measuring the duodenal villi height and base width of the villi. The same computer program was used for measuring the duodenal villi crypt depth. For the measurement of duodenal villi height, cross-sections of 10 villi were randomly selected. The criterion for villus selection was based on the presence of intact lamina propria. Villus height and width, as well as crypt depth, were measured at 40× the objective magnification. The villus height was measured as the distance from the apex of the villus to the junction of the villus and crypt [22]. The villus width was measured as the distance from the junction to the basement membrane of the epithelial cell at the bottom of the crypt at the bottom third of the length of the villus (base width of the duodenal villi) [23]. All the measurements taken from 10 villi per one sample were counted from four different preparations from each duodenal segment for each bird, and were expressed as the average duodenal villi height and average base width of the duodenal villi for each bird. Finally, 10 average heights of duodenal villi, as well as 10 average base widths of the duodenal villi from 10 birds were expressed as the average height of the villi for a group and the average base width of the villi for a group [22]. The duodenal villi crypt depth was measured from the base of the villus to the mucosa [23]. All the measurements from 10 crypts were counted from four different preparations from each duodenal segment for each bird. Averaged depth measurements of 10 crypts were expressed as the average duodenal villi crypt depth for each bird. Finally, 10 average depths of duodenal villi crypts of 10 birds were expressed as the average depth of duodenal villi crypts of the group [22]. The ratio of villus to crypt was estimated by dividing the villus height by the crypt depth [23]. The absorptive surface area of the duodenal villus was estimated by considering a villus as a cylindrical structure [23]. Villus absorptive surface area was calculated using the formula: Villus absorptive surface area = $2\pi \times$ (average villus width/2) $\times$ villus height [23,24].

2.3. Statistical Analysis

The normality of the data distribution was tested by a Kolmogorov–Smirnov test; all data were processed by methods of descriptive statistics. The numerical variables were described as the median and interquartile ranges. A Kruskal–Wallis test was used for the comparison of numerical variables among the groups. The level of statistical significance was set at $p < 0.05$. Statistical analysis was performed using the statistical package Statistica for Windows 2010 (version 10.0, StatSoft Inc., Tulsa, OK, USA).

3. Results

Morphometric analysis of the duodenal villi of broiler chickens revealed differences between the control and experimental groups of chickens at the tissue structure level on the 42nd day of the feeding trial, as shown in Table 3. The duodenal villi of chickens from all the experimental groups were significantly higher ($p < 0.001$), while their base was significantly wider ($p < 0.001$) in comparison to those in chickens from the control group. There was a statistically significant difference in duodenal villi crypt depth between the groups of chicken ($p < 0.001$).

Table 3. The values of evaluated parameters of duodenal villi of broiler chickens on the 42nd day of the feeding trial.

Parameter	Group of Chickens Median (Q1–Q3)					* *p*
	K	P1	P2	P3	P4	
Duodenal villi height (μm)	718.50 ^{a} (584.50–841.50)	834.00 ^{b} (695.00–990.00)	992.00 ^{c} (814.50–1111.50)	886.00 ^{bcd} (697.00–1134.50)	798.50 ^{bde} (658.75–1088.00)	<0.001
Base width of the duodenal villi (μm)	48.00 ^{a} (38.00–68.00)	59.00 ^{b} (52.00–69.50)	67.00 ^{bc} (51.00–77.25)	54.00 ^{bd} (45.50–67.00)	65.00 ^{ce} (58.75–75.25)	<0.001
Duodenal villi crypt depth (μm)	78.00 ^{a} (66.00–93.00)	85.00 ^{a} (75.50–93.50)	71.50 ^{b} (63.75–80.25)	64.00 ^{c} (54.00–74.50)	78.00 ^{ad} (70.00–85.25)	<0.001

* Kruskal–Wallis test. abcde: Medians within a row with different superscripts are different; K = control group; P1 = feed mixture + 0.25 g of propolis/kg of feed mixture + 20 g of bee pollen/kg of feed mixture; P2 = feed mixture + 0.5 g of propolis/kg of feed mixture; P3 = feed mixture + 1.0 g of propolis/kg of feed mixture; P4 = feed mixture + 20 g of bee pollen/kg of feed mixture.

The histological representations of the duodenal villi of broiler chickens from all the groups are shown in Figures 1–5.

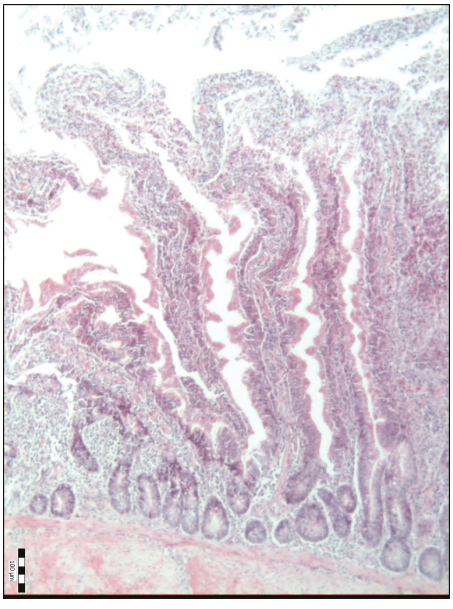

Figure 1. Histological representation of the duodenal villi of broiler chickens from the control group of chickens (K) (H&E; ×100).

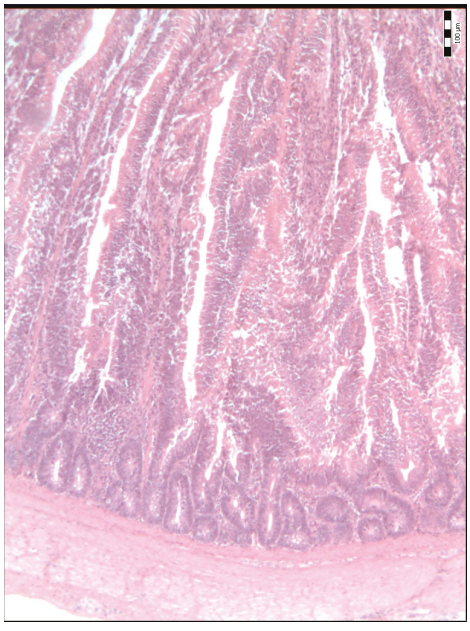

Figure 2. Histological representation of the duodenal villi of broiler chickens from the P1 experimental group of chickens (H&E; ×100).

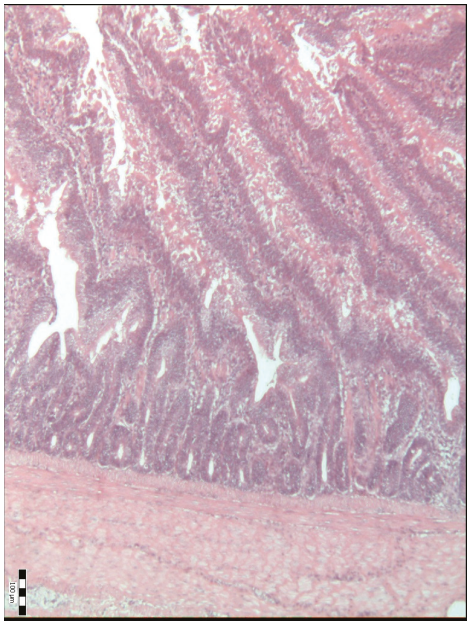

Figure 3. Histological representation of the duodenal villi of broiler chickens from the P2 experimental group of chickens (H&E; ×100).

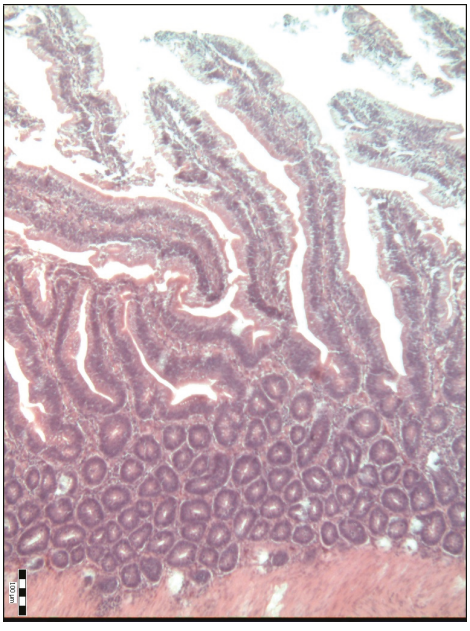

Figure 4. Histological representation of the duodenal villi of broiler chickens from the P3 experimental group of chickens (H&E; ×100).

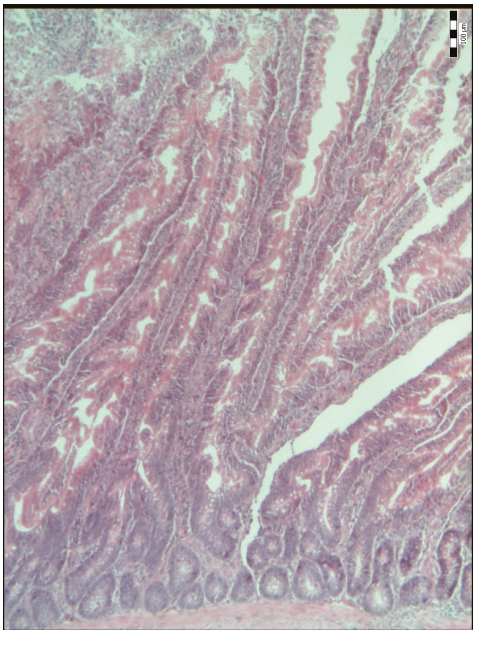

Figure 5. Histological representation of the duodenal villi of broiler chickens from the P4 experimental group of chickens (H&E; ×100).

The study also revealed that there was a statistically significant difference in the villus height-to-crypt depth ratio on the 42nd day of the feeding trial between the control and experimental group of chickens ($p < 0.001$), as shown in Table 4.

Table 4. The villus height-to-crypt depth ratio of broiler chickens on the 42nd day of the feeding trial.

Parameter	Group of Chickens Median (Q1–Q3)					* *p*
	K	P1	P2	P3	P4	
The Villus Height-to-Crypt Depth Ratio	8.86 [a] (7.16–10.60)	9.81 [b] (8.51–12.09)	14.24 [c] (11.64–16.36)	13.61 [cd] (10.54–16.70)	10.89 [be] (8.70–12.73)	<0.001

* Kruskal–Wallis test. (Q1–Q3) = interquartile range; K = control group; P1 = feed mixture + 0.25 g of propolis/kg of feed mixture + 20 g of bee pollen/kg of feed mixture; P2 = feed mixture + 0.5 g of propolis/kg of feed mixture; P3 = feed mixture + 1.0 g of propolis/kg of feed mixture; P4 = feed mixture + 20 g of bee pollen/kg of feed mixture.

The study further showed that there was a statistically significant difference between the average values of the absorptive surface areas of the duodenal villi of broiler chickens on the 42nd day of the feeding trial between the control and experimental group of chickens ($p < 0.001$) (see Figure 6).

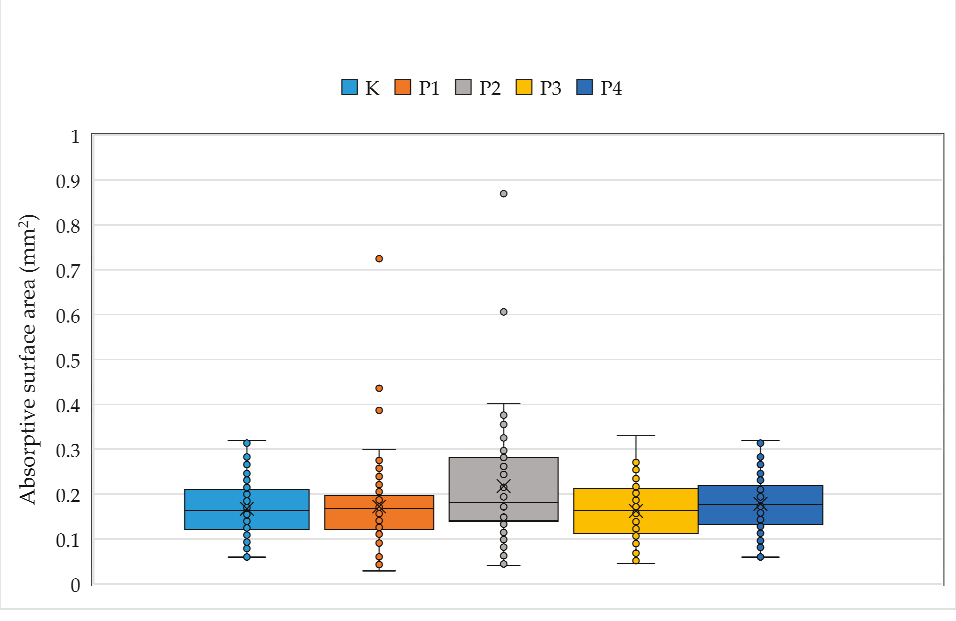

Figure 6. The average absorptive surface area of duodenal villi according to the group of broiler chickens on the 42nd day of the feeding trial (Kruskal–Wallis test, $p < 0.001$).

4. Discussion

Morphometric results of the duodenal villi in chickens on day 42nd of feeding trial revealed that it was significantly higher, while its base was significantly wider in the experimental groups compared to the controls. These results are consistent with the results of the study by Wang et al. [16], who demonstrated that chickens fed a diet supplemented with a mixture of bee pollen had significantly higher and wider intestinal villi of the duodenum, jejunum and ileum in comparison to the chickens fed a control diet. The same authors further determined that the observed differences were greater during the early stages of development of the gastrointestinal system [16]. The results of the present study are also consistent with the results of a study by Tekeli et al. [25], who showed that the addition

of ginger and propolis extract both separately and in combination in the diet resulted in a significant increase in the length of the intestinal villi of the jejunum in chickens from the experimental groups when compared to chickens of the control group. On the other hand, Eyng et al. [17] showed that the intestinal villi of the duodenum of chickens fed a diet supplemented with various amounts of propolis were shorter or lower when compared to the intestinal villi of the chickens in the control group.

Considering the morphometric results of the duodenal villi crypt depths in chickens on the 42nd day of fattening, this study showed that there were significant differences in the depths of duodenal villi crypts between the chickens from the experimental groups compared to the chickens from the control group. This result is consistent with that of Eyng et al. [17], who showed that the crypt of the intestinal villi of the duodenum of chickens fed a diet supplemented with various amounts of propolis were deeper compared to crypt of the intestinal villi of the duodenum of chickens from the control group.

All the previously mentioned results of this study can be attributed to the beneficial effect of the biologically active components of propolis and/or bee pollen. These components participate in controlling the proliferation of pathogenic bacteria and the consequent avoidance of possible damage to the intestinal mucosa, which also leads to the reduction of morphometric measures of the intestinal villi [17,26].

Within the explanation of the identified influence of propolis and/or bee pollen on the histological features of chickens' intestines, it is important to keep in mind that diet composition is in fact the main factor that can modify the histological appearance or morphology of the intestine and, consequently, its absorptive capacity, which ultimately defines the growth performance of fattening chickens [27]. It is further known that the intestinal villi are quickly and continuously adjusted as a response to conditions in the lumen of the intestine (that are strongly influenced by diet composition) reflecting the dynamic environment inside the intestines of animals. Accordingly, longer intestinal villi are associated with an increase in the absorptive surface of the intestines and also with an increase of the absorption capacity of the intestine [28]. This finding was also demonstrated in the present study, since the absorptive surface area of duodenal villi in all experimental groups were increased in comparison to that of the control broilers.

Previous studies have already confirmed that longer intestinal villi indicate an improved ability to absorb nutrients in the intestine [29,30]. In addition, it has been proven that longer villi are associated with active cell mitosis, which provides a greater absorptive potential of villi for various nutrients [31,32]. Deeper intestinal villi crypts indicate a rapid metabolism of tissue in order to allow the renewal of the intestinal villi, if there is a need for its regeneration [27]. Lowering the height of the villi or reducing crypt depths of intestinal villi may lead to a reduction in the absorption of nutrients [33].

This study further showed that there was a statistically significant difference in the villus height to crypt depth ratio on the 42nd day of the feeding trial between the control and experimental groups of chickens. This result is highly important, bearing in mind that a higher ratio of villous height and crypt depth refers to a greater capacity of nutrient digestibility and absorption in chickens [34]. Namely, it has been proven that shorter intestinal villi relative to crypt depth are related to a smaller number of absorptive cells and a larger number of secretory cells. Secretory cells are responsible for the secretion of mucins that form a mucinous lining of the intestinal epithelium, thus increasing the number of secretory cells and leading to an increased secretion of mucin. Changes in the quantity or composition of mucin of the intestinal mucosal surface can reduce the absorption of nutrients and/or increase the amount of energy required to maintain function of the intestines [27,35].

In present study, all the experimental groups of chickens had deeper crypts of the intestinal villi of the duodenum in relation to chickens from the control group, which is a clear indicator of higher proliferative activity in the mucosa of these intestinal villi. Higher proliferative activity in the mucosa of the intestinal villi indicates better digestibility and absorption of consumed feed mixtures in the experimental groups of chickens that were fed a mixture with the addition of propolis and/or bee

pollen. The latter has also been shown in studies of other substances of pronounced antimicrobial and antioxidant properties, such as, for example, garlic and some herbal extracts [36–38].

The clarification of antimicrobial and antioxidant effects of all the previously mentioned substances, including propolis and bee pollen, re-emphasizes the role of their phenolic components such as various flavonoids, phenolic acids and their derivatives that they have the ability to protect the intestinal villi and increase the absorption of nutrients [38]. It is believed that these biologically active components exert their antioxidant activity both at the cellular and at the tissue level [39]. Apart from their antioxidants, their antimicrobial activity should also be significant as these bioactive agents can modulate the gut ecosystem. Due to the synergism of antioxidant and antimicrobial activity of biologically active phenolic compounds from propolis and bee pollen, a further positive effect on the utilization of nutrients has been achieved [40,41].

The present study revealed some original solutions regarding the applied dosage of investigated natural supplements and their specific combinations in broilers feeding, but was not without limitations. Due to the commonly accepted '3Rs', the authors had the justifiable wish to minimize the number of animals used in this study that had already been used in similar studies [3]. However, considering the tested natural feeding additives and main objective of this study, the authors believe that the described design of the study did not affect the results.

In conclusion, the present study showed that the addition of propolis and/or bee pollen to feed mixtures has a significant protective effect on the gut tissue of chickens, which is reflected through better morphometric measures of the duodenal villi and duodenal villi crypts of chickens from all the experimental groups in relation to chickens from the control group. Following the results of this study, the addition of 0.5 g of propolis per kg of feed mixture showed the strongest positive effect on chicken guts. The promising and encouraging results of this study emphasize the importance of the further evaluation of the administration level of investigated supplements in order to maximize their positive effects on the gut tissue of chickens and, consequently, the overall health of broiler chickens.

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Whole-Life or Fattening Period Only Broiler Feeding Strategies Achieve Similar Levels of Omega-3 Fatty Acid Enrichment Using the DHA-Rich Protist, *Aurantiochytrium limacinum*

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Simple Summary: In many parts of the world, the human population does not consume sufficient quantities of omega-3 fatty acids. Humans can potentially reduce the risk or severity of a variety of illnesses by simply increasing their dietary intake of omega-3 fatty acids, with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) being particularly beneficial. Fish are a rich source of these important fatty acids, but many individuals do not consume fish and so the enrichment of more commonly consumed foods has been explored as a method to increase the consumption of omega-3 fatty acids. The fatty acid content of chicken meat reflects the fatty acid composition of their diet and so poultry meat can be easily enriched by introducing omega-3 rich ingredients into poultry diets. In this study we fed broilers diets supplemented with a DHA-rich protist, *Aurantiochytrium limacinum* for their whole life (42 days) or for the final 21-day fattening period to investigate which strategy represented a more efficient use of the DHA-rich ingredient. As similar levels of enrichment were found from both feeding durations tested, the study indicates that feeding for the 21-day fattening period is the more efficientperiod of dietary inclusion for *Aurantiochytrium limacinum*.

Abstract: The fatty acid composition of broiler chicken tissues can be increased by adding omega-3 rich ingredients to their diets. The purpose of this study was to compare the levels of tissue enrichment observed following the supplementation of broilers with the docosahexaenoic acid (DHA)-rich protist, *Aurantiochytrium limacinum* (AURA) for their whole life (42 days) or for the final 21-day fattening period. Day-old chicks (n = 350) were distributed among 35 pens (10 birds per pen) with each pen randomly assigned to one of five treatments: Control; 0.5% AURA from day 0–42; 1% AURA from day 0–42; 0.5% AURA from day 21–42; 1% AURA from day 21–42. Production parameters were recorded over the course of the study and the fatty acid profile of the breast, thigh, liver, kidney and skin with adhering fat was quantified at the end of the feeding period. The level of supplementation had a significant impact on the degree of omega-3 tissue enrichment, however, no differences were observed when the same dose was provided for 21 or 42 days. These results indicate that supplementation with AURA for a period of 21 days does not negatively affect broiler productivity and is the most efficient strategy to increase the nutritional value of broiler products.

Keywords: broilers; DHA; omega-3; fatty acids; enrichment

1. Introduction

Increasing consumer awareness of the health benefits associated with the consumption of omega-3 polyunsaturated fatty acids (n-3 PUFA) has resulted in a greater demand for n-3 PUFA rich produce [1]. As a result, the industry has been developing strategies to increase the n-3 PUFA content of commonly consumed foods [2]. By feeding chickens diets rich in n-3 PUFA, the fatty acid content of chicken meat can be altered to contain much higher quantities of n-3 PUFA, which can lead to health benefits for the consumer without making significant changes to their diet [2]. There are a variety of n-3 PUFA rich ingredients that have been used to supplement poultry diets in order to increase the n-3 PUFA content of their meet and eggs. Linseed, in various forms, can be a good source of alpha-linoleic acid (ALA) and can significantly increase tissue n-3 FA content [3]. However, some antinutritional features of linseed limit its inclusion in poultry diets [3]. In addition, most of the health benefits associated with omega-3 fatty acids have been attributed to increasing the intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [4]. While the human body can convert ALA to EPA and DHA, this process is inefficient and as such, the direct consumption of at least 250 mg of EPA+DHA per day is recommended [5]. Fish are a rich source of EPA + DHA but are only consumed regularly by small proportions of the population [6]. For those who do not consume fish, chicken has been found to contribute up to 28% of the dietary intake of EPA+DHA in the UK [7] and 24% of EPA + Docosapentaenoic acid (DPA)+DHA intake in Australia [8], despite the fact that chicken is not a naturally rich source of n-3 PUFA [9].

Fish meal and oil can also be used as a feed ingredient for poultry [10], but while this does increase the EPA + DHA content of the meat, it can also lead to a deterioration in consumer acceptability [11,12]. In addition, using fish meal/oil to supplement animal diets is not considered sustainable and could not supply the n-3 PUFA requirements of the world's growing population [13].

Schizochytrium spp. and *Aurantiochytrium limacinum* are both members of the Thraustochytrid family of heterotrophic protists, commonly classified as microalgae, but with fungus-like characteristics. The Thraustochytrids are primary producers of n-3 PUFA in the marine food chain and can be grown in a sustainable manner on a large scale using heterotrophic fermentation. Various authors have used *Schizochytrium* as a dietary supplement for broilers, and have detected increased tissue n-3 PUFA concentrations without any major impact on the organoleptic properties of the meat or on broiler productivity [14,15]. *A. limacinum* (AURA) is a closely related species that can be provided as a dietary supplement and has been shown to increase the DHA concentrations of cows milk, pork, chicken meat and eggs [16–21].

Previous work indicated that AURA is well tolerated by broilers with no negative impact on broiler health or productivity observed when broiler diets were supplemented at a level of 5% for their whole life [16]. Significant increases of tissue DHA concentrations were also observed when feeding broilers for the final 21-day fattening period only [17]. When comparing these similar studies, the results suggested that feeding for the entire life of the broiler may represent a more cost efficient use of dietary AURA, reaching higher levels of enrichment potentially due to the accumulation of DHA in the tissues over the whole life of the bird [17]. These comparisons were drawn from studies of similar design, carried out in the same facility, but at different times. As the studies did not occur concurrently, differences between the levels of enrichment could be attributed to factors other than the duration of supplementation. Therefore, in the current study we aimed to investigate the most cost-effective duration of supplementation by comparing the levels of enrichment achieved when birds were provided with supplementary AURA for their whole lives or for the final 21-day fattening period only.

2. Materials and Methods

The research protocol and animal care were conducted in accordance with European Union Directive 2010/63/EU covering the protection of animals used for experimental or other scientific purposes, and according to the recommendation of the Commission of 2007/526/CE covering the accommodation and care of animals used for experimental and other scientific purposes. The live

animal portion of the study was carried out at the CERZOO research center which is authorized by the Italian Ministry of Health to employ animals for experimental or other scientific purposes. The study was conducted to investigate the effect of supplementing broiler diets with *A. limacinum* (AURA; ALL-G-RICH®; CCAP 4087/2; Alltech Inc., Nicholasville, KY, USA) over their whole life (WL; Day 0–42) or for the 21-day fattening period only (FP; Day 21–42) on production parameters and the fatty acid content of breast, thigh, liver, kidney and skin samples. The analytical composition of AURA was determined prior to the start of the study using the following methods: crude protein (AOAC 990.03), crude fat (AOAC 954.02), fatty acid composition (AOAC 996.06, with matrix validation [22]), moisture (AOAC 930.15) and ash (AOAC 942.05).

The study was carried out using male Ross 308 broilers housed in 1.4 × 1.6 m pens. Food and water were provided ad libitum using one feeder per pen and using an internal water system network. The study site was equipped with a dynamic ventilation system. Heating was provided from positive water aerotherms and positive pressure ventilation was achieved by single, variable-speed fans linked to temperature sensors. The temperature program was recommended by the breeder [23] and was automatically controlled and programmed to gradually reduce the temperature as follows: 25–30 °C (D1); 22–27 °C (D1–D7); 19–25 °C (D7–D14); and 18–25 °C (D14–D42). The higher temperature at the beginning of the study was guaranteed by specific 150-watt lamps mounted in each pen. The relative humidity ranged from 60–80% (D1–D7) and 40–80% (D7–D42). The lighting program was 18 hours light, and 6 hours dark.

Day-old chicks (n = 350) were randomly distributed among 35 pens (10 birds per pen) with each pen randomly assigned to one of the five following treatments: 0% AURA day 0–42 (Control); 0.5% AURA from day 0–42 (0.5%WL); 1% AURA from day 0–42 (1%WL); 0.5% AURA from day 21–42 (0.5%FP); 1% AURA from day 21–42 (1%FP). The ingredients of the diets are shown in Table 1 and were designed to meet the nutrient levels recommended for Ross 308 broilers [24]. The experimental diets were produced at the CERZOO feed mill and provided as a crumble from day 0–10, or as pellets from day 10–42. The nutrient content of the experimental diets was determined at the Institute of Food Science and Nutrition (Faculty of Agricultural Sciences, Food and Environment, Catholic University of Sacred Heart, Piacenza, Italy) using the following methods: dry matter (ISO 6496); crude protein (Gazzetta Ufficiale Serie Generale n.92 of 21.04.96); crude fat (Directive EEC 84/4/EEC); crude fibre (Directive EEC n.92/89); crude ash (NEN 3329, ISO 5984-2002 (E)); starch by polarimetric method (Directive CEE n. 72/199 and ISO 10520:1997 (E)); sugars (Gazzetta Ufficiale CEE n. L155 of 12.07.71); energy content was calculated according to the equation proposed in G.U. CE n. L54 22.02.09. Individual fatty acids were quantified using AOAC Met. 996.06.2001, recently validated for chicken feed [25]. In brief, the fatty acids in each sample were trans-esterified in situ with 1.5 N HCl in methanol, in the presence of toluene. The toluene contained methyl tricosanoate which acted as the internal standard. The resultant fatty acid methyl esters (FAMEs) and toluene were then extracted. The FAMEs were then separated, identified, and quantified by gas chromatography equipped with a flame ionization detector (GC-FID).

The mg of FAME per 100 g of sample was then calculated using the following formula:

$$\text{FAME, mg FA/100 g wet sample} = \frac{A_X \times CF_X \times W_{IS} \times 100}{A_{IS} \times W_S \times 1.04 \times 1000}$$

where A_X = area counts for the FA; A_{IS} = area counts for internal standard (C23:0); CF_X = instrumental response factor for FA (EPA = 0.98, DHA = 0.99, GLA = 0.98); W_{IS} = weight of IS added to sample in mg; WS = weight of sample in mg; 1.04 = conversion factor from methyl ester to fatty acid.

The AURA used in the study contained 71.7 g of crude fat/100 g biomass and 16.0 g DHA/100 g biomass with a significant amount of palmitic acid (34.0 g/100 g biomass). Additionally, the AURA contained 13% crude protein, 2.63% ash and 1.97% moisture. The analytical composition of the experimental diets is shown in Table 2, while the fatty acid composition of the diets is shown in Table 3. Daily bird health, mortality and culling records were maintained. Pen live weight on days 0, 21 and

42 were recorded in addition to the feed intake and feed refusals. One bird per replicate was killed (according to annex I of the Reg. CE n. 1099/2009 of the council of the 24/09/2009 concerning the protection of animals during slaughter) on day 43 and both kidneys, the whole liver, breast, thigh and skin with adhering fat samples were taken post-mortem. The left breast and thigh were used for the determination of dry matter (UNI ISO 1442), crude fat (AOAC 991.36), protein (UNI ISO 937) and ash (AOAC 991.36). The fatty acid content of both kidneys, the liver and the right (skinless) breast, (skinless) thigh and skin with adhering fat were quantified using the method recently validated for chicken tissues [26]. FAME calculations were as described above for chicken feed.

Table 1. Ingredient composition of the five experimental diets supplemented with 0, 0.5 or 1% *Aurantiochytrium limacinum* (AURA) for their whole life (WL; day 0–42) and for the fattening period only (FP; day 21–42).

Diet Ingredient	Day 0–21			Day 21–42		
	0%WL 0.5%FP 1.0%FP	**0.5%WL**	**1.0%WL**	**0%WL**	**0.5%FP 0.5%WL**	**1.0%FP 1.0%WL**
Corn meal	46.20	46.50	46.25	52.96	52.90	52.90
Soybean meal 48%	36.70	36.58	36.60	30.12	30.00	29.88
Wheat meal	8.25	8.00	8.00	8.55	8.53	8.50
Soybean oil	4.50	4.13	3.90	4.58	4.28	3.93
Dicalcium phosphate	1.80	1.76	1.75	1.37	1.37	1.37
Limestone	1.00	0.98	0.97	0.98	0.98	0.98
Sodium bicarbonate	0.40	0.40	0.39	0.39	0.39	0.39
Lysine HCL	-	-	-	0.10	0.10	0.10
DL - Methionine	0.40	0.40	0.39	0.20	0.20	0.20
Vitamins and minerals [1]	0.50	0.50	0.50	0.50	0.50	0.50
Salt	0.15	0.15	0.15	0.15	0.15	0.15
Ronomix Hiphos	0.10	0.10	0.10	0.10	0.10	0.10
AURA	-	0.50	1.00	-	0.50	1.00

[1] The content of vitamins and trace minerals per kg of premix (Rovimix B extra /1), produced by DSM Nutritional Product—Istituto delle Vitamine, Segrate (MI), Italy, is as follows: vit. A: 2,000,000 IU/kg; vit. D3: 600,000 IU/kg; vit. E: 15,000 mg/kg; vit. K3: 612 mg/kg; vit. B1: 400 mg/kg; vit. B2: 1200 mg/kg; D-pantothenic acid: 2778 mg/kg; vit. B6: 1200 mg/kg; vit. B12: 6 mg/kg; vit. C: 20,000 mg/kg; Niacin: 8000 mg/kg; Folic acid: 250 mg/kg; Biotine: 30 mg/kg; choline chloride: 100,000 mg/kg; Mn: 26,000 mg/kg; Fe: 10,000 mg/kg; Cu: 1,000 mg/kg; Zn: 15,000 mg/kg; I: 200 mg/kg; Se: 40 mg/kg. Excipient: calcium carbonate: 42.00%; spent grapes: 25.06%.

Table 2. The analytical composition of the five experimental diets supplemented with 0, 0.5 or 1% *Aurantiochytrium limacinum* for their whole life (WL; day 0–42) and for the fattening period only (FP; day 21–42).

Nutrient Value	Day 0–21 [1]			Day 21–42		
	0%WL 0.5%FP 1.0%FP	**0.5%WL**	**1.0%WL**	**0%WL**	**0.5%FP 0.5%WL**	**1.0%FP 1.0%WL**
Dry matter (%)	89.4	89.0	89.2	88.9	88.8	88.9
Crude protein (%)	23.7	23.6	23.6	19.4	19.5	19.4
Crude fibre (%)	2.8	2.8	2.8	2.7	2.8	2.8
Crude fat (%)	6.5	6.5	6.9	6.8	6.5	6.9
Ash (%)	6.3	6.0	6.0	5.2	5.0	5.4
Starch (%)	40.2	40.5	39.9	43.5	43.9	43.5
Sugar (%)	4.6	4.6	4.6	4.5	4.6	4.6
M.E. [2] (Kcal/kg)	3180.5	3167.5	3169.0	3154.0	3158.0	3160.0

[1] The crumbled feed provided from day 0–10 and pelleted feed provided from day 10–21 were analysed separately and so the values presented here represent the mean of these two analyses [2] Metabolisable energy calculated according to Gazzetta Ufficiale CE n. L54, February 22, 2009.

Table 3. The fatty acid composition of the five experimental diets supplemented with 0, 0.5 or 1% *Aurantiochytrium limacinum* for their whole life (WL; day 0–42) and for the fattening period only (FP; day 21–42).

Fatty Acid (mg/g)	Day 0–21 [1]			Day 21–42		
	0%WL 0.5%FP 1.0%FP	**0.5%WL**	**1.0%WL**	**0%WL**	**0.5%FP 0.5%WL**	**1.0%FP 1.0%WL**
C12:0 Lauric	0.00	0.01	0.02	0.00	0.01	0.02
C14:0 Myristic	0.04	0.24	0.48	0.03	0.21	0.41
C15:0 Pentadecanoic	0.01	0.07	0.13	0.01	0.06	0.11
C16:0 Palmitic	6.24	7.91	10.47	6.44	7.65	9.89
C16:1 Palmitoleic	0.05	0.05	0.05	0.04	0.04	0.05
C17:0 Heptadecanoic	0.04	0.06	0.07	0.04	0.05	0.07
C18:0 Stearic	2.11	1.96	2.02	2.09	1.86	1.89
C18:1n9 cis Oleic	13.96	12.60	12.86	14.53	12.93	12.94
C18:1 cis11	0.61	0.55	0.56	0.59	0.52	0.52
C19:0 Nonadecanoic	0.11	0.10	0.10	0.11	0.09	0.10
C18:2n6c Linoleic	29.38	25.62	26.53	29.25	26.38	26.34
C18:3n3 α-Linolenic	2.27	1.95	1.98	2.20	1.94	1.96
C20:0 Arachidic	0.21	0.20	0.22	0.22	0.20	0.21
C20:1 cis-11-Eicosenoic	0.11	0.10	0.11	0.11	0.10	0.09
C20:5n3 EPA [2]	0.00	0.00	0.00	0.00	0.00	0.00
C22:0 Behenic	0.18	0.17	0.18	0.18	0.16	0.16
C24:0 Lignoceric	0.08	0.08	0.08	0.08	0.07	0.08
C22:6n3 DHA [3]	0.00	0.73	1.53	0.00	0.69	1.43

[1] The crumbled feed provided from day 0–10 and pelleted feed provided from day 10–21 were analysed separately; the values presented here represent the mean of these two analyses [2] Eicosapentaenoic acid [3] Docosahexaenoic acid.

Differences between the treatment groups were determined using the general linear model procedure of Minitab (Minitab, v18, State College, PA, USA) with Tukey's post hoc analysis used to determine the differences between the treatment groups. Regression analysis was used to determine whether the estimated DHA intake per bird could predict the DHA content of breast and thigh meat. DHA intake per bird was calculated by dividing the intake per pen by the number of birds present and then multiplying by the DHA content detected for each experimental diet.

3. Results

3.1. Bird Health and Performance

Mortality was below 5% for each treatment group with an overall mortality of 3.14% observed. The performance of the birds in terms of their weight, weight gain, feed intake and feed conversion ratio (FCR) are summarised in Table 4. There was no significant difference between the groups in terms of weight at the beginning of the study. The 1%WL and 0.5%WL groups differed significantly in terms of weight by day 21 and in terms of their average daily gain during the first period (D 0–21) with the latter gaining significantly more weight. By day 42, the 1%WL group differed significantly to the control in terms of weight and average daily gain, with lower values again observed for the 1%WL group. Average daily feed intake differed between the 1%FP group and the 0.5%WL groups between day 0 and 21 and between the control and 1%WL groups between day 21 and 42, and overall. Thigh weight differed significantly between the control and 1%WL group with the latter weighing significantly less than the control at the end of the trial.

Table 4. The effect of dietary supplementation with 0, 0.5 or 1% Aurantiochytrium limacinum for their whole life (WL; day 0–42) and for the fattening period only (FP; day 21–42).

Parameter	0 %WL D 0–42	0.5%WL D 0–42	1.0%WL D 0–42	0.5%FP D 21–42	1.0%FP D 21–42	Standard Error	*p* Value
Weight Day 0	37.3	37.1	36.9	37.0	37.2	0.27	0.920
Weight Day 21	883.7 ^{ab}	918.6 ^{a}	848.3 ^{b}	888.3 ^{ab}	883.7 ^{ab}	13.91	0.026
Weight Day 42	3072.1 ^{a}	2998.1 ^{ab}	2809.2 ^{b}	2861.0 ^{ab}	2947.8 ^{ab}	59.10	0.028
[1] ADG D 0–21	40.3 ^{ab}	42.0 ^{a}	38.5 ^{b}	40.5 ^{ab}	40.3 ^{ab}	0.65	0.016
ADG D 21–42	101.9 ^{a}	98.4 ^{ab}	91.2 ^{b}	93.9 ^{ab}	97.5 ^{ab}	2.52	0.051
ADG D 0–42	70.9 ^{a}	70.0 ^{ab}	64.8 ^{b}	67.2 ^{ab}	68.8 ^{ab}	1.43	0.036
[2] ADFI D 0–21	53.5 ^{ab}	58.9 ^{a}	53.4 ^{ab}	53.0 ^{ab}	52.6 ^{b}	1.48	0.031
ADFI D21-42	162.9 ^{a}	157.5 ^{ab}	148.2 ^{b}	151.2 ^{ab}	153.2 ^{ab}	2.95	0.014
ADFI D0-42	107.9	107.8	100.6	102.0	103.0	1.88	0.026
[3] FCR D0-21	1.3	1.4	1.4	1.3	1.3	0.03	0.144
FCR D21-42	1.6	1.6	1.6	1.6	1.6	0.03	0.723
FCR D0-42	1.5	1.5	1.6	1.5	1.5	0.02	0.441
[4] EPEF D21	317.5	315.0	287.6	323.7	322.8	9.01	0.049
EPEF D42	436.7	435.2	395.0	416.7	431.9	17.67	0.428
Breast weight (g)	604.6	590.3	556.6	552.1	575.0	20.39	0.388
Thigh weight (g)	581.4^{a}	552.1 ^{ab}	525.7 ^{b}	509.3 ^{b}	542.4 ^{ab}	13.46	0.009
Dressing (%)	86.0	86.2	86.2	86.3	86.3	0.44	0.990
Breast (%)	23.4	23.6	23.0	23.2	23.2	0.67	0.980
Thigh (%)	22.5	22.1	21.8	21.4	21.9	0.48	0.551

[1] Average Daily Gain; [2] Average Daily Feed Intake; [3] Feed Conversion Ratio; [4] European Production Efficiency Factor calculated as ((Average grams gained/day X % survival rate)/Feed Conversion X 10); ^{a,b} Means within a row that do not share a superscript differ significantly.

3.2. Meat Fatty Acid Content

The effects of supplementation with AURA on the concentration of selected fatty acids (i.e., those fatty acids that differed significantly between treatments or were of interest overall) in chicken breast, thigh, kidney, liver or skin with adhering fat are shown in Table 5. The full fatty acid profile determined for each tissue/organ is provided in Supplementary Table S1. The highest DHA concentrations were detected in the liver followed by the skin with adhering fat, thigh and kidney, with the breast meat having the lowest DHA content of all the sampled tissues. In all cases, the DHA content of the tissues/organs of supplemented birds were significantly higher than the unsupplemented control group. For breast and thigh samples both 1% groups had significantly more DHA than all other treatments with similar levels of enrichment observed for the 1%WL and 1%FP groups. The groups supplemented with 0.5% AURA were enriched to a similar degree with no difference observed between the 0.5%WL and 0.5%FP treatments. In the liver, both 1% treatments were again the most enriched with the duration of supplementation not effecting the level of enrichment. In the kidney, the 1%WL and both FP treatments were enriched to a similar degree, with the 0.5%WL group having significantly less DHA than the 1%WL group. In the skin the 1%WL treatment had significantly more DHA than each of the other treatments, with all remaining supplemented groups enriched to a similar degree. A quadratic relationship between DHA intake and tissue/organ DHA content was observed for the thigh, kidney and liver samples, while a linear relationship was observed for breast and skin samples (Figure 1). The efficiency of DHA transfer from the feed to the breast and thigh was estimated and indicated that feeding for the final 21 days resulted in a more efficient transfer of DHA from the feed to the tissues for both breast and thigh (Figure 2).

Table 5. The effect of dietary supplementation with 0, 0.5 or 1% Aurantiochytrium limacinum for the whole life (WL; day 0–42) or for the fattening period only (FP; day 21–42) on broiler tissue and organ fatty acid concentrations.

Fatty Acid (mg/100g)	0%WL D 0–42	0.5%WL D 0–42	1.0%WL D 0–42	0.5%FP D21–42	1.0%FP D 21–42	Standard Error	*p* Value
			Breast				
C15:0	1.5 ^{b}	2.2 ^{ab}	3.2 ^{a}	2.2 ^{ab}	2.7 ^{ab}	0.36	0.034
C20:5n3 EPA [1]	1.9 ^{b}	2.5 ^{b}	4.3 ^{a}	2.7 ^{b}	3.5 ^{ab}	0.39	0.001
C22:4n6	18.6 ^{a}	10.9 ^{b}	8.9 ^{b}	11.7 ^{b}	8.5 ^{b}	0.87	<0.001
C22:5n3 DPA [2]	12.1	11.3	12.4	11.5	11.7	0.76	0.841
C22:6n3 DHA [3]	6.9 ^{c}	32.6 ^{b}	55.2 ^{a}	30.3 ^{b}	50.1 ^{a}	2.26	<0.001
Total Omega 3	66.8 ^{b}	83 ^{ab}	111.9 ^{a}	82.8 ^{ab}	101.1 ^{ab}	8.82	0.010
Total Omega 6	818.7	689.8	711.5	710.3	650.9	102.6	0.829
Omega 6 / Omega 3	12.2 ^{a}	8.2 ^{b}	6.2 ^{c}	8.3 ^{b}	6.3 ^{c}	0.4	<0.001
			Thigh				
C12:0	0.7 ^{b}	1.1 ^{ab}	1.1 ^{ab}	0.8 ^{ab}	1.2 ^{a}	0.11	0.011
C14:0	14.6 ^{b}	25.3 ^{ab}	27.8^{a}	19.4 ^{ab}	30.0 ^{a}	2.66	0.002
C15:0	3.0 ^{c}	6.4 ^{ab}	6.9 ^{ab}	4.8 ^{bc}	7.4 ^{a}	0.59	<0.001
C17:0	5.8 ^{b}	8.2 ^{a}	7.0 ^{ab}	6.9 ^{ab}	8.1 ^{ab}	0.58	0.040
C20:4n6	115.5 ^{a}	97.8 ^{b}	89.5 ^{b}	100.1 ^{ab}	91.6 ^{b}	3.98	0.001
C20:5n3 EPA	2.9 ^{c}	5.0 ^{b}	8.2 ^{a}	4.5 ^{bc}	7.6 ^{a}	0.44	<0.001
C22:4n6	25.8 ^{a}	14.9 ^{b}	11.9^{b}	15.1 ^{b}	11.7 ^{b}	0.87	<0.001
C22:5n3 DPA	20.1	17.2	17.1	16.3	16.8	0.96	0.067
C22:6n3 DHA	11.7 ^{c}	53.6 ^{b}	86.0 ^{a}	46.6 ^{b}	79.6 ^{a}	2.05	<0.001
Total Omega 3	140.7 ^{c}	200.1 ^{ab}	207.8 ^{ab}	165 ^{bc}	223.3 ^{a}	13.3	0.001
Total Omega 6	1788	2087	1609	1695	1953	162.3	0.251
Omega 6 / Omega 3	12.7 ^{a}	10.4 ^{b}	7.7 ^{c}	10.3 ^{b}	8.6 ^{c}	0.252	<0.001
			Liver				
C15:0	1.5 ^{b}	2.0 ^{ab}	2.8 ^{a}	2.2 ^{ab}	2.8 ^{a}	0.22	0.001
C20:4n6	346.8 ^{a}	306.8 ^{ab}	270.5 ^{bc}	324.8 ^{a}	257.2 ^{c}	12.07	<0.001
C20:5n3 EPA	7.1 ^{b}	10.0 ^{b}	15.7 ^{a}	10.7 ^{ab}	15.3 ^{a}	1.25	<0.001
C22:4n6	37.4 ^{a}	24.7 ^{bc}	21.5 ^{bc}	26.5 ^{b}	18.2 ^{c}	1.8	<0.001
C22:5n3 DPA	27.7	23.6	26.9	29.2	24.1	1.79	0.153
C22:6n3 DHA	45.3 ^{d}	161.7 ^{c}	233.4 ^{a}	187.6 ^{bc}	214.4 ^{ab}	8.82	<0.001
Total Omega 3	104.4 ^{d}	215.9 ^{c}	300.2 ^{a}	251.4 ^{bc}	281.5 ^{ab}	11.16	<0.001
Total Omega 6	1143.9	1057.1	1074.1	1127.2	1085.2	59.31	0.820
Omega 6 / Omega 3	11.1 ^{a}	5.0 ^{b}	3.6 ^{c}	4.5 ^{bc}	3.9 ^{bc}	278	<0.001
			Kidney				
C20:2n6	35.0 ^{a}	34.8 ^{ab}	26.4 ^{c}	32.3 ^{abc}	27.9 ^{bc}	1.72	0.002
C20:3n3	5.0 ^{a}	5.2 ^{a}	3.6 ^{b}	4.8 ^{ab}	4.2 ^{ab}	0.33	0.010
C20:5n3 EPA	8.1 ^{c}	25.5 ^{b}	37.5 ^{a}	26.4 ^{b}	36.8 ^{a}	2.3	<0.001
C22:4n6	24.2 ^{a}	16 ^{b}	14.2 ^{b}	15.8 ^{b}	11.3 ^{b}	1.76	<0.001
C22:5n3 DPA	14.4	14.8	14.7	15.9	14.1	1.08	0.798
C22:6n3 DHA	21.2 ^{c}	53.3 ^{b}	70.8 ^{a}	60.1 ^{ab}	65.8 ^{ab}	3.95	<0.001
Total Omega 3	122.3 ^{b}	201.0 ^{a}	192.6 ^{ab}	180.7 ^{ab}	193.8 ^{a}	17.3	0.020
Total Omega 6	1798.1	2217	1600.5	1750.5	1710.6	256.43	0.506
Omega 6 / Omega 3	14.8 ^{a}	10.8 ^{b}	8.3 ^{c}	9.3 ^{bc}	8.7 ^{bc}	0.591	<0.001
			Skin with adhering fat				
C12:0	9.5 ^{b}	11.6 ^{ab}	15.0 ^{a}	11.0 ^{b}	13.1 ^{ab}	0.95	0.004
C14:0	181.3 ^{d}	248.5 ^{bc}	332.7^{a}	220.2^{cd}	305.4 ^{ab}	15.74	<0.001
C14:1	29.5	28.6	38.1	24.3	35.9	3.8	0.092
C15:0	34.6 ^{d}	59.7 ^{bc}	79.9 ^{a}	51.8 ^{c}	72.1 ^{ab}	3.93	<0.001
C18:3n6	101.8	81.5	79.3	80.6	90.6	5.78	0.049
C20:4n6	251.2 ^{a}	199.3 ^{ab}	228.1 ^{ab}	217.0 ^{ab}	180.4 ^{b}	16.35	0.047
C20:5n3 EPA	20.1 ^{c}	30.7 ^{bc}	71.6 ^{a}	30 ^{bc}	46.5 ^{b}	6.05	<0.001
C22:4n6	57.5 ^{a}	40.6 ^{b}	41.6 ^{ab}	40.9 ^{b}	31.8 ^{b}	4.02	0.002
C22:5n3 DPA	38.1	37.7	51.9	38.6	39.6	3.64	0.047
C22:6n3 DHA	18.5 ^{c}	90.8 ^{b}	210.1 ^{a}	87.3 ^{b}	147.3 ^{b}	14.69	<0.001
Total Omega 3[4]	1372.9	1307.8	1448.6	1231.9	1345.2	85.44	0.489
Total Omega 6[5]	19460.0	18141.0	16840.9	16994.7	17248.3	1029.4	0.368
Omega 6 / Omega 3	14.2 ^{a}	14.0 ^{ab}	11.7 ^{c}	13.8 ^{ab}	12.9 ^{bc}	0.317	<0.001

Means that do not share a superscript differ significantly. [1] Eicosapentaenoic acid; [2] Docosapentaenoic acid; [3] Docosahexaenoic acid; [4] Total Omega 3 = {C18:3n3+C20:3n3+C20:5n3+C22:5n3+C22:6n3}; [5] Total Omega 6 = {C18:2n6cis+C18:3n6+C20:2n6+C20:3n6+C20:4n6+C22:4n6}. ^{a,b,c} Means within a row that do not share a superscript differ significantly.

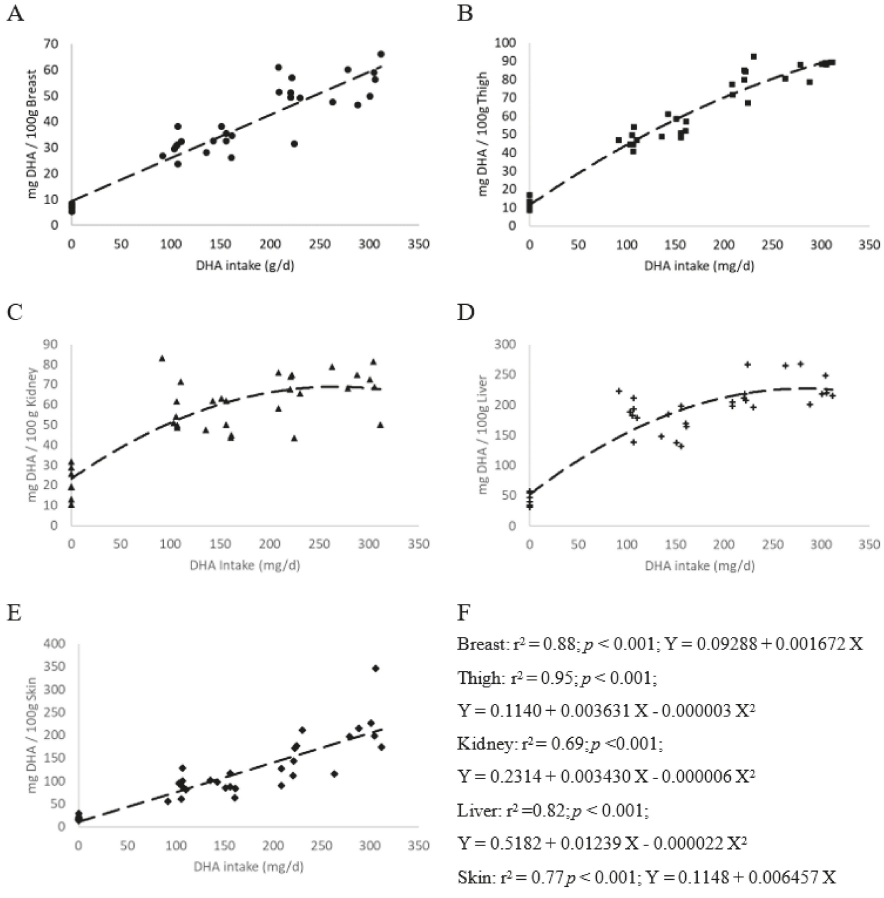

Figure 1. Scatterplots with regression lines for docosahexaenoic acid (DHA) intake (mg/day) against DHA content (mg/100g) detected in breast (**A**) thigh (**B**), kidney (**C**), liver (**D**) and skin with adhering fat (**E**). The r^2 value, significance of the relationship (p) and model equation are shown for each graph in panel **F**.

The concentration of DPA did not differ significantly between any of the treatment groups for any of the tissues/organs sampled. The highest levels of EPA enrichment were observed in the skin with adhering fat, followed by the kidney, liver, thigh and breast. For each tissue/organ sampled the highest EPA concentration was detected in the 1%WL group, with this group having more EPA than the control group in each case. The 1%FP group also had significantly more EPA than the control group in each case with the exception of the breast tissue samples. The 1%FP group was enriched to a similar degree as the 1%WL group in each tissue with the exception of the skin, in which the 1%WL group had significantly more EPA (Table 5). These differences contributed to significant differences between the groups in terms of their total n-3 PUFA concentration, with groups receiving the 1% AURA treatments generally enriched to a greater degree. For each tissue the C22:4n-6 concentration detected in the supplemented groups was generally significantly lower than the control, however, the same trend was not observed for the total n-6 concentrations, which did not differ between the treatment and control groups. The n-6/n-3 ratio for both 1% treatments were significantly lower than the control group in

every tissue/organ sample. Both 0.5% AURA treatments also had significantly lower n-6/n-3 ratios in every tissue/organ samples with the exception of the skin.

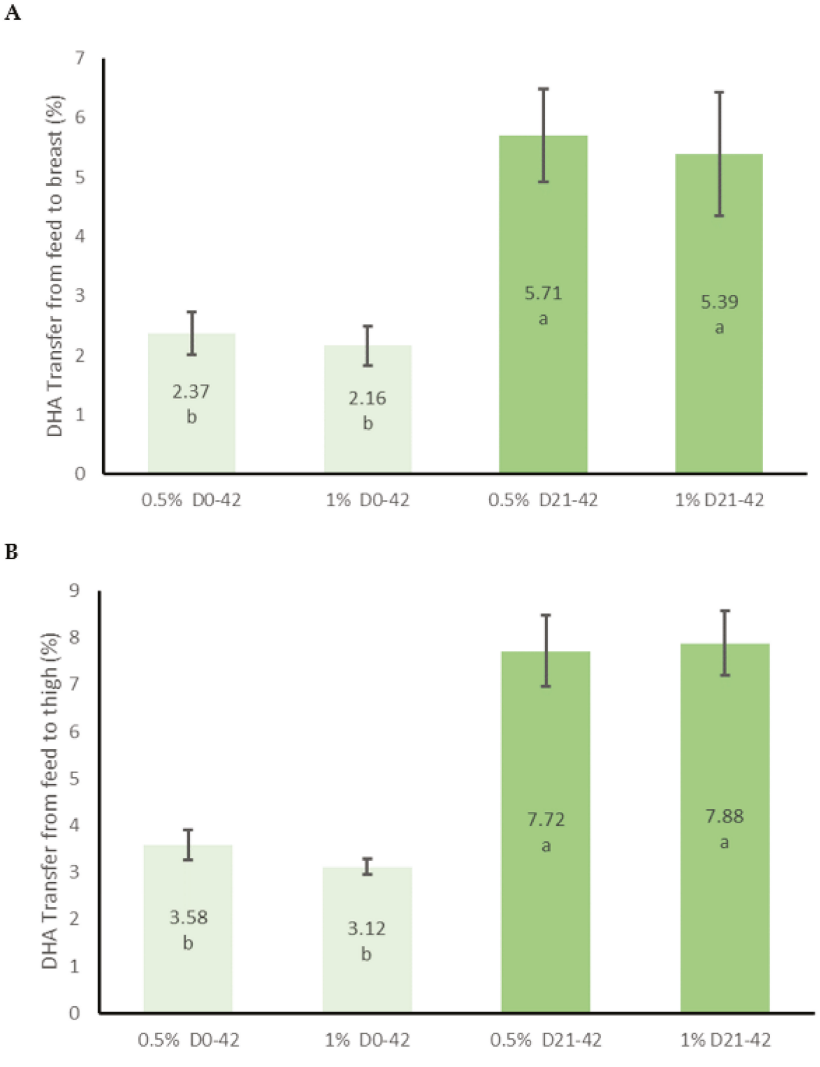

Figure 2. Mean efficiency of DHA transfer (± 95% C.I.) from feed to the breast (**A**) or thigh (**B**) tissues. Transfer efficiency was estimated for one bird per pen (n = 7 per treatment) as follows; (mg DHA in feed × average daily feed intake x 42 days) ÷ (mg DHA/g breast or thigh × breast or thigh weight) × 100. [a,b] Columns that do not share a letter differ significantly ($p < 0.001$).

4. Discussion

In our previous studies, the addition of AURA was found to have no effect on any of the broiler productivity parameters measured [16,17]. In contrast, the 1%WL group in the current study was found to have a lower average weight than the control group by day 42, likely due to a significantly lower average daily feed intake between day 21 and 42. For the whole 42 day period, feed intake did not

differ between the groups with no differences in feed conversion ratio, European Production Efficiency Factor, dressing % or breast weight observed. In our previous study, investigating supplementation at up to 5% of the diet, no differences in productivity were observed between the groups [16] which may suggest that the differences observed in the current study were not as a result of the inclusion of AURA. Some other n-3 PUFA rich ingredients have been shown to negatively impact broiler productivity, with the anti-nutritional properties of linseed limiting its inclusion in broiler diets [3]. In contrast, some authors have shown improvements in weight gain, feed intake and FCR when providing omega-3 rich *Schizochytrium* [10,27] or AURA [28]. The effects on the 1%WL group could be considered minor and considering there was no differences between the two groups supplemented for the fattening period only, supplementation with AURA is unlikely to reduce the productivity of broilers in practice.

It has previously been demonstrated that supplementing the diets of broilers with AURA is an effective method that can increase the concentration of DHA in broiler tissues [16,17]. When comparing the results of our previous studies, feeding 0.5% AURA for 42 days resulted in a similar level of tissue enrichment as feeding 1% AURA for 21 days [16,17]. Interestingly, the cost of supplementation (with an approximate AURA cost of €7 per kg) for 42 days at a level of 0.5% of the diet (€0.18 per bird) was lower than the cost of providing AURA at a level of 1% of the diet for 21 days (€0.26 per bird). These studies were carried out in the same research facility, over a two-year period using similar diets, however, differences in the level of enrichment observed between the studies could have been caused by factors other than the duration of feeding. As such, it was important to test the effect of AURA feeding duration on tissue DHA enrichment in a single experiment. Here we found that feeding a lower amount of AURA over a longer period did not lead to similar levels of enrichment as feeding higher levels over a shorter period. Furthermore, we found no effect of duration of feeding (i.e., 21 or 42 days) on the level of enrichment observed at each dose. The cost of supplementing the birds for 21 days at 1% of the diet in the current study would be an estimated €0.23 per bird. Feeding at a rate of 0.5% for the whole life of the bird would be less expensive (€0.16 per bird), but the degree of enrichment achieved remained significantly below that of birds supplemented at a rate of 1% for a shorter duration. Feeding for the final 21-day fattening period only is further supported by the significantly higher transfer efficiencies observed for the FP treatments when compared to the WL treatments. These results indicate that the most efficient feeding strategy is to feed for the final 21-day fattening period only.

The level of enrichment observed in the breast samples of birds supplemented with AURA at a rate of 0.5% fell within the range of values reported from our previous studies (23–35 mg DHA/100 g breast) when broilers were supplemented at the same rate. The DHA content of the thigh samples from the current study (47 and 54 mg DHA/100 g thigh following 21 and 42 days, respectively) exceeded the range of 24–43 mg DHA/100 g thigh reported from our previous studies. A similar trend was observed for the 1% AURA treatments, with the DHA concentration of breast samples from the current study (50 and 55 mg DHA/100 g breast following 21 or 42 days, respectively) and those from the thigh samples (80 and 86 mg/100 g thigh after 21 or 42 days respectively) exceeding the range reported from the breast (36–48 mg DHA/100 g breast) and thigh (42–50 mg DHA/100 g thigh) from our previous trials [16,17]. The fat content of the tissues in the current study was generally higher than those of the tissues from previous studies, increasing their capacity for DHA enrichment. However, the degree of fat deposition has been shown to be unaffected by increasing levels of n-3 PUFA with other nutritional and genetic factors likely responsible for the differences observed [29], between the overall levels of fat detected in the different studies.

Zuidhof et al. [30] investigated the effect of the duration of dietary supplementation of broilers using ground full-fat flaxseed as a source of n-3 PUFA and found that feeding for 24.1 days was the most cost effective duration that achieved an adequate level of tissue enrichment. Kanakari et al. [31] also used flaxseed to supplement broiler diets and showed that feeding for 2–4 weeks prior to slaughter would be sufficient for broilers to accumulate the same amount of n-3 LCPUFA as being fed for six weeks, which is similar to the findings of the current study. When supplementing the diets of

broilers with fish oil accompanied by either flaxseed, lard or rapeseed, Konieczka et al., [32] found that feeding for a duration of three weeks before slaughter resulted in significantly higher EPA + DHA concentrations than feeding for one or two weeks only. Our previous work in laying hens indicated that supplementation at a level of 1% of the diet increased the DHA content of eggs for the first 24 days, with no significant increase in the concentration of egg DHA after that point [18]. Overall, these results, using a variety of different n-3 PUFA sources, indicate that feeding for periods longer than 2–4 weeks is not a cost-effective use of the supplemental ingredient.

For a food to be labelled "a source of omega-3" in the European Union (EU) it must contain at least 40 mg EPA+DHA/100 g, while to be labelled "high in omega-3" it must contain 80 mg EPA + DHA/100 g [33]. For the breast meat, both the 0.5%WL and the 0.5%FP groups fell just short of this target with 35 and 33 mg EPA + DHA/100g detected respectively. The thigh samples however, did meet these criteria with 59 mg and 51 mg EPA + DHA/100 g detected in the 0.5%WL and 0.5%FP groups respectively. The breast samples of the 1%WL and 1%FP groups met the criteria to be considered a source of omega-3, with 60 and 54 mg EPA+DHA/100 g detected respectively, while the thigh samples could be considered high in omega-3 with 94 and 87 mg EPA+DHA/100 g detected respectively. Based on these results, the broilers would need to be supplemented at a rate of 1% of the diet so that both the breast and thigh meat could be labelled as "a source of omega-3" in the EU.

No EPA was detected in any of the diets provided, but despite this, an increased inclusion of AURA generally led to increased tissue concentrations of EPA. Similar results have been obtained in our previous trials [16,17], with the increase likely due to the retro-conversion of DHA to EPA [34]. In contrast, no differences were observed between the treatments in terms of the tissue n-3 DPA concentration. This is in keeping with the results of our previous study, when supplementing broilers with 0, 0.25, 0.5 and 1 % AURA, resulted in no differences in n-3 DPA concentrations between treatments in breast tissue samples, while in the thigh samples, the 0.5% treatment had significantly less n-3 DPA than the control group with no differences observed between the other groups [17]. Small but significant increases in breast n-3 DPA were observed when birds were supplemented with AURA at a rate of 2.5% and 5% of the diet with 10.19 and 10.52 mg n-3 DPA/100 g detected respectively, while at 0 and 0.5% of the diet 9.02 and 9.02 mg n-3 DPA/100 g were detected respectively. It does not appear that this increase in tissue n-3 DPA is due to some form of retro-conversion from DHA. It could more likely be a result of the elongation of EPA, which was present in higher concentrations in the 2.5 and 5% treatments. The elongation of EPA to n-3 DPA is more efficient than the elongation of EPA to DHA [35]. In humans, supplementary DHA from an algal source (containing no EPA) was found to significantly increase blood EPA concentrations while reducing the concentration of n-3 DPA indicating that DHA supplementation could negatively influence the concentration of n-3 DPA, possibly as result of competition at the level of fatty acid esterification [36]. These findings indicate that supplementary DHA-rich AURA, when provided at a level of 0.5–1% of the diet can also increase the tissue concentrations of the beneficial fatty acid EPA, despite it being absent from the experimental diets.

The significant increases in both EPA and DHA contributed to the higher total n-3 PUFA concentrations detected in many of the tissue/organ samples. The concentration of C20:4n6 was significantly lower in most of the tissue/organ samples from supplemented birds, however, this did not translate into lower total n-6 PUFA concentrations in any of the tissues/organs. Importantly, supplementation led to lower n-6/n-3 ratios, in every treatment and in every tissue/organ with the exception of the WL skin with adhering fat treatments. Reductions in a person's overall n-6/n-3 ratio have been shown to improve the health outcomes of patients suffering from a variety of illnesses, including cardio-vascular diseases and some forms of cancer, with the effective ratio based on the type of illness in question [37]. Consuming this AURA enriched broiler meat could help to reduce the overall n-6/n-3 ratio of a person's diet.

No attempt was made to assess the effect of supplementation on the consumer acceptability of the meat of supplemented birds. However, supplementing broiler diets with marine protists at a level

of 2.8% of the diet was previously found to have no impact on consumer acceptability [14]. As the inclusion rates observed in this trial were below this, it is unlikely that the consumer acceptability would be affected. However, it would be beneficial to determine the consumer acceptability of these enriched meats when fresh and following a period of storage. Recent work investigating the frozen storage stability of DHA found significant reductions in the tissue DHA content after a period of 12 or 24 weeks [26]. Chicken meat for human consumption is unlikely to be stored for this extended period of time and over this duration the DHA concentration of breast meat decreased by 35%, 35%, 29.4% and 27.8% in broilers supplemented with AURA at a rate of 0%, 0.5%, 1.5% and 2.5% of the diet. As such, it would be beneficial to establish the effects of storage on the stability of DHA and the consumer acceptability of the AURA enriched broiler meat over a shorter time period.

5. Conclusions

These results indicate that providing AURA as a dietary supplement for broilers for the final 21-day fattening period can improve the nutritional quality of broiler meat by significantly increasing the DHA and EPA concentrations of tissues and organs, as well as lowering the n-6/n-3 ratio without significantly impacting productivity.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/9/6/327/s1. Table S1: The full fatty acid profile of breast, thigh, liver, kidney and skin (with adhering fat), taken from birds fed diets supplemented with A. limacinum at a rate of 0, 0.5 or 1% for their whole life (WL; day 0–42) or for the fattening period only (FP; day 21–42).

Author Contributions: Conceptualization and methodology, C.A.M., M.M. and G.F.; supervision, M.M and G.F.; project administration, C.A.M. and M.M.; data curation, M.M. and J.D.K.; formal analysis, M.M., J.D.K. and G.F.; writing—original draft preparation, M.M. and J.D.K.; writing—review and editing, C.A.M., J.D.K., M.M., and G.F.

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Conflicts of Interest: The authors C.A.M. and J.D.K. are employees of Alltech which produces and markets ALL-G-RICH®, the commercial product used in this study

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Effect of Dietary Modulation of Selenium Form and Level on Performance, Tissue Retention, Quality of Frozen Stored Meat and Gene Expression of Antioxidant Status in Ross Broiler Chickens

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Simple Summary: Although the importance of usage of selenium as essential trace element in poultry production has been proven, the best source and level has not been fully addressed yet. Three different dietary selenium forms with three different levels were chosen to be added in broiler diet. Met-Se or nano-Se up to 0.6 mg/kg increased their performance and was more efficiently retained in the body than SeS. Frozen stored meat quality was improved in a dose-dependent manner especially with both Met-Se and nano-Se. Nano-Se was more potent than Met-Se, which in turn was more potent than inorganic Se against oxidative stress, which improved the quality of meat under frozen conditions.

Abstract: This study compares between different selenium forms (sodium selenite; SeS, selenomethionine; Met-Se or nano-Se) and levels on growth performance, Se retention, antioxidative potential of fresh and frozen meat, and genes related to oxidative stress in Ross broilers. Birds (n = 450) were randomly divided into nine experimental groups with five replicates in each and were fed diets supplemented with 0.3, 0.45, and 0.6 mg Se/kg as (SeS, Met-Se), or nano-Se. For overall growth performance, dietary inclusion of Met-Se or nano-Se significantly increased ($p < 0.05$) body weight gain and improved the feed conversion ratio of Ross broiler chicks at the level of 0.45 and 0.6 mg/kg when compared with the group fed the same level of SeS. Se sources and levels significantly affected ($p < 0.05$) its concentrations in breast muscle, liver, and serum. Moreover, Se retention in muscle was higher ($p < 0.05$) after feeding of broiler chicks on a diet supplemented with Met-Se or nano-Se compared to the SeS group, especially at 0.6 mg/kg. Additionally, higher dietary levels from Met-Se or nano-Se significantly reduced oxidative changes in breast and thigh meat in the fresh state and after a four-week storage period and increased muscular pH after 24 h of slaughter. Also, broiler's meat in the Met-Se and nano-Se groups showed cooking loss and lower drip compared to the SeS group ($p < 0.05$). In the liver, the mRNA expression levels of glutathione peroxidase, superoxide dismutase, and catalase were elevated by increasing dietary Se levels from Met-Se and nano-Se groups up to

0.6 mg/kg when compared with SeS. Therefore, dietary supplementation with 0.6 mg/kg Met-Se and nano-Se improved growth performance and were more efficiently retained than with SeS. Both sources of selenium (Met-Se and nano-Se) downregulated the oxidation processes of meat during the first four weeks of frozen storage, especially in thigh meat, compared with an inorganic source. Finally, dietary supplementation of Met-Se and nano-Se produced acceptable Se levels in chicken meat offered for consumers.

Keywords: broilers; selenium sources-levels; selenium retention; antioxidant capacity; frozen meat

1. Introduction

Selenium (Se) is an important trace nutrient for the maintenance, growth, and animals and humans health [1]. It improves the nutritive value and of meat quality [2]. As feed additives, Se can enhance growth productivity in broiler chickens [3]. Selenium is an important constituent of at least 25 selenoproteins involved in various physiological processes, including immune function, reproduction, and the maintenance of antioxidant defenses to avoid tissue damage. Selenium deficiency results in a number of disorders and injuries in poultry, such as skeletal myodegeneration, exudative diathesis (ED), muscular hemorrhages, atrophy of pancreas, decreased production of eggs, liver injury, reduced hatchability, and inhibited growth of bursal and thymic [4], and increase susceptibility of humans to certain degenerative diseases, such as cancer [5]. The fortification of poultry meat with Se represents a viable strategy for increasing human intake of Se. The national research council (NRC) [6] recommendations established a low selenium level (0.15 mg/kg) for the supplementation of broilers. This level is not adequate to avoid production losses resulted from selenium deficiency disorders [7]; consequently, there is a need to increase dietary selenium levels. Moreover, Se bioavailability not only depends on its physical form but also on dietary concentration and the levels of other trace elements. Excess levels of Se can be toxic when provided above the biological requirement. Thus, meeting Se requirements and optimizing performance is an important step in modern poultry production. Practically, selenium can be added for poultry's diet in the form of inorganic Se, organic Se, and most recently, nano-Se. The inorganic form of selenium (Se selenite) is primarily and commonly used for dietary supplementation, and exhibits a very narrow border between its dietary requirement and its toxicity [8]. Recently, it has been recognized that organic Se has a higher rate of tissue retention and bioavailability thus lower toxicity than inorganic Se, so it is preferable to inorganic Se in broilers [9]. In addition, organic Se is deposited more efficiently in breast muscle than inorganic forms [10]. With the development of nanotechnology, nano-Se has attracted widespread research interest due to its high catalytic efficiency and higher adsorbing capacity, and has exhibited strong absorption efficiencies and lower toxicity than inorganic Se [11]. Moreover, recent studies found that nano-Se has a higher effectiveness in controlling selenoenzymes and displays less toxicity than selenium-selenite [12]. Moreover, supplementation of dietary Se could also enhance oxide dismutase (SOD), glutathione peroxidase, (GPx) and catalase (CAT) activities, and reduce oxidative stress and lipid peroxidation biomarkers, consequently reducing oxidative stress in broilers [13]. Moreover, Se plays a key role in the signaling of redox via removal of hydrogen peroxide and lipid hydroperoxides using glutathione as an ultimate electron donor [14]. These antioxidant properties of Se have also been shown to continue in postmortem muscle tissue and prevent lipid oxidation [15]. For this reason, many dietary regimes in animal nutrition have been established to produce Se-enriched meat in order to increase human Se consumption [16]. The type and level of available Se is important to meet broilers' dietary requirements and optimize their production without producing any hazardous effects on broilers or human health. Definitive comparative studies to fully exploit the benefits of dietary supplementation with different available Se sources and levels in Ross broilers remains poorly investigated. Thus, the aim of the present study was to compare the bioavailability of different levels and sources of Se on performance,

Se retention, lipid oxidative stability of meat, meat quality, and mRNA expression of some selected genes related to antioxidant capacity in Ross broiler chickens.

2. Materials and Methods

2.1. Selenium Sources

Sodium selenite (SS) and selenomethionine (Met-Se) were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA and Sel-Plex; Alltech Inc., Nicholasville, KY, USA, respectively. nano-Se powder was prepared according to [17] where 4 mL of 25 mM GSH containing 15 mg of bovine serum albumin were mixed with one ml of 25 mM sodium selenite. The resulting red suspension was dialyzed against double-distilled water for 96 h. Every 24 h, the water was replaced to isolate the oxidized GSH from the nano-Se. Then, nano-Se and bovine serum albumin were lyophilized. The phase characterization and morphology of nanoparticles were analyzed by means of X-ray diffraction (XRD) using EMPYREAN diffractometer and JEM-200CX transmission electron microscopy (TEM) working at 30 kV as shown in (Figure 1). XRD patterns corresponding to the (100), (101), (110), (102), (111), (201), (003), (202), (210), and (211) planes of the formed nano-Se were observed at 20 angles of 23.6°, 29.9°, 41.4°, 43.8°, 45.4°, 51.8°, 55.9°, 61.8°, 65.3°, and 68.3°, respectively [18]. The nano-Se was spherical in shape with average size 42 ± 1.4 nm (total count of 100 NPs).

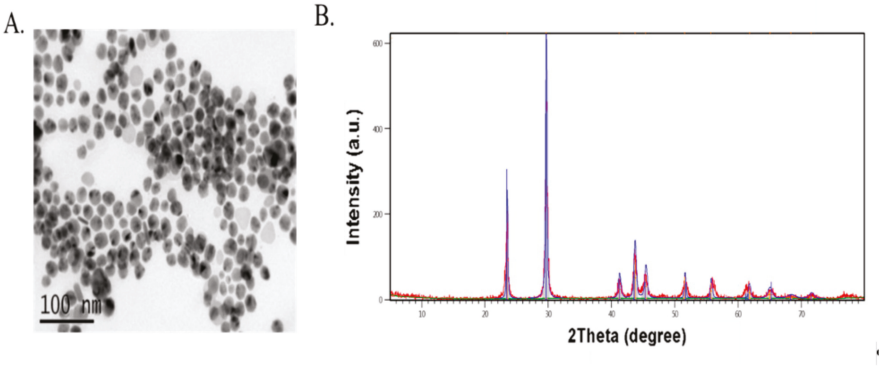

Figure 1. Characterization of nano-Se (**A**,**B**): (**A**) Morphology of the formed nano-Se pictured by transmission electron microscopy (TEM) and; (**B**) X-ray diffraction (XRD) pattern of the nano-Se.

2.2. Birds and Experimental Procedures

Four hundred and fifty, one-day-old, Ross broiler chicks (Ross 308) were individually weighed and divided to nine dietary groups, each group consisting of five replicates of ten chicks each per floor pen. Broiler chicks were fed diets containing inorganic Se (sodium selenite; SeS), organic Se, (selenomethionine, Met-Se), or nano-Se, each at three levels 0.3, 0.45, or 0.6 mg/kg Se (as fed). The basal diet was formulated to meet nutrient requirements of Ross broilers according to [19] except Se (Table 1). Diets were fed from 1 to 38 d including starter (1–11 d), grower (12–22 d), and finisher (23–38 d) diets. All chicks were given ad libitum access to feed and water. The environmental temperature was kept at 32 °C for the 1st week and then gradually decreased until reached 23 °C. All the experimental procedures were performed at the Institute of Nutrition and Clinical Nutrition and Poultry Farm following the Faculty of Veterinary Medicine guidelines and in accordance with the protocols approved by Institutional Animal Care and Use committee at Zagazig University (Approval no: ZU-IACUC/2/F/123/2018).

The proximate analysis of feed ingredients was done according to the standard method of [20]. For Se analysis in feed, one gram of feed was heated for 5 h in a furnace at 550 °C for ashing. Mixture from

3 N HCl (10 mL) and an ashed sample was heated until the solution became clear. After cooling, the sample was filtered and diluted to 50 mL with 0.1 N HCl. For analysis of selenium, lanthanum 185.4 L 50 gm/kg was added to 6 mL of the sample solution. Then, analysis was achieved by a spectrometer at a wavelength of 400 nm [20].

Table 1. The ingredients and nutrient levels of the basal diet (on dry matter basis).

Ingredients	Starter	Grower	Finisher
Corn, %	56	61.7	62.5
Soybean meal, %	34.86	28.1	25
Corn gluten, %	3.5	3.3	3.5
wheat bran, %	0	1	1.9
Soy oil, %	1.8	2.2	3.66
Calcium carbonate, %	1	1	1
Calcium diphasic phosphate, %	1.8	1.7	1.5
NaCl, %	0.3	0.3	0.3
Premix *, %	0.3	0.3	0.3
Methionine, %	0.18	0.14	0.11
Lysine, %	0.16	0.16	0.13
anti-mycotoxin, %	0.1	0.1	0.1
Total	100	100	100
	Nutrient Levels [b]		
Crude protein, %	23.2952	20.527	19.3087
ME (kcal/kg)	3042.271	3105.028	3200.17
Calcium, %	0.9656	0.92681	0.86886
Available P, %	0.467822	0.43785	0.3962
Methionine, %	0.569576	0.49246	0.456018
Lysine, %	1.380138	1.18469	1.092276
Ether extract, %	4.28232	4.8086	2.6345
Crude fiber, %	2.64082	2.6282	6.2493
Se mg/kg	0.06986	0.0696	0.07615

* Provided for each kilogram of diet: Vitamin A, 10,000 IU; vitamin E, 7200 IU; vitamin D3, 3000 IU; vitamin K, 2 mg; vitamin B1, 2640 mg; vitamin B6, 1200 mg; calcium pantothenate, 10 mg; nicotinamide, 50 mg; biotin, 40 mg; choline chloride, 500 mg; folic acid, 0.5 mg; cobalamin, 0.01 mg; calcium, 9000 mg; manganese, 120 mg phosphorus, 2100 mg; sodium, 3700 mg; iron, 110 mg; copper, 10 mg; zinc, 100 mg; iodine 1.1 mg. [b] Calculated values except selenium.

2.3. Growth Parameter Measurement

Live body weight (LBW) and feed intake of broiler chicks/pen were estimated individually at 21 and 38 d of age to calculate live body weight, body weight gain (BWG), total feed intake, feed conversion ratio (FCR) and relative growth rate (RGR).

2.4. Sampling and Analytical Procedures

At the end of the feeding trail, tissues samples (liver and breast meat) were collected from five birds/replicates that were slaughtered (slaughtering house under supervision of Institutional Animal Care and Use Committee at Zagazig University Faculty of Veterinary Medicine) and handled and kept at −20 °C until analysis of selenium content and meat quality tests. Blood samples were collected with

or without anticoagulant, then plasma and serum were kept at −20 °C until the analysis of selenium content and chemical analysis was performed.

2.4.1. Tissue Retention of Selenium

Briefly, liver and breast muscle were weighed (0.1 g) and mixed with of HNO_3 (8 mL) then digested by microwave. After that, deionized water was added to produce a 10 mL volume. The selenium content was determined following the procedure of [21] by atomic absorption spectrophotometer (Shimadzu Ltd., Shimane Shimadzu, Japan).

2.4.2. Selenium Content in Serum Constituents

Selenium content was measured in serum by atomic absorption spectrophotometer (AA6501, Shimadzu Ltd., Japan). Plasma samples were used for measuring of aspartate amino transferase (AST), alanine glutamyl transferase (ALT), and creatinine calorimetrically by diagnostic kits (MAK055, MAK052, and C4255, respectively) manufactured by Sigma-Aldrich.

2.4.3. Laboratory Analysis for Meat Quality

Meat pH and Drip and Cooking Loss in Meat Samples

Breast meat was used to determine postmortem pH (t = 0.5 and 24 h) by pH meter. Drip loss was estimated according to [22] (percent; proportional weight loss of a sample suspended for 72 h in a closed plastic bag under refrigerated conditions at 4 °C). After storage at −20 °C, cooking loss was determined (percent; weight loss proportionate of a sample after cooking for 40 min in a water bath at 70 °C followed by cooling).

Preparation of Samples for Total Antioxidant Capacity

Six hours after slaughter and handling, breast meat was cut into cubes of approximately 3 cm square); visible connective tissues and fat were removed. These muscle cubes mixed with distilled water then homogenized and centrifuged and used for measuring total antioxidative markers as free radical scavenging assay using 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) thiobarbituric acid reactive substances (TBARS) assay, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and ferric reducing/ antioxidant (FRAP) assay.

Thiobarbituric acid-reactive assay: Oxidation was evaluated on the first day and after one week from storage by the thiobarbituric acid assay described by [23]. Perchloric acid (27 mL, 3.83% v/v) was added to of meat sample (5 g) then homogenized for 1 min and filtered by filter paper, then 2 mL thiobarbituric acid was added to supernatants and incubated in a water bath (100 °C) for 20 min. Subsequently, immediate cooling to room temperature and centrifugation for 15 min was performed, then the absorbance was read by the spectrophotometer at 532 nm. The results were then calculated according to the standard curve and values were expressed as mg malondialdehyde (MDA)/kg meat.

ABTS assay: The total antioxidant capacity of chicken breast and thigh meat was analyzed by Trolox-equivalent antioxidant capacity (TAC) assay [24]. Briefly, the reaction between 14 mM ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] with an equal volume of 4.9 mM potassium persulfate was catalyzed to stimulate the formation ABTS+ radical cation formation, then incubated in the dark at room temperature for 12–16 h. After that, 10 µL of meat homogenate was added to the ABTS+ solution (1.0 Ml) and mixed thoroughly and after 60 s absorbance was read at 734 nm.

DPPH assay: The scavenging activity of the muscle samples was analyzed by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) [25]. Briefly, the meat samples were homogenized in distilled water and then centrifuged. The supernatant was mixed with ethanol and DPPH radical solution and incubated in a dark room for 10 min. Next, the absorbance measurement was read at 517 nm. The ability to scavenge the DPPH radical was expressed as µM per g of wet muscle tissue.

FRAP assay: Ferric reducing antioxidant power (FRAP) assay [26] was carried out on meat homogenates. The meat samples were homogenized in potassium phosphate buffer, centrifuged, and the supernatant was collected. Then, supernatant (1 mL) was collected and added to FRAP buffer (3 mL) containing 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine) in 40 mM HCl, and 20 mM Fe_2Cl_3 was added to 300 mM acetate buffer. Immediately after mixing, the absorbance was measured at 593 nm. A standard curve was prepared with $FeCl_2$. The antioxidant power of the samples was expressed as μM of Fe^{2+} per 1 g wet muscle tissue.

2.5. RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR.

At the end of the feeding trial (day 38), three birds per group were randomly selected, marked and injected with tert-butyl hydroperoxide, 0.2 mmol/kg body weight, intraperitoneally purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA, CAS Number 75- 91-2) to induce the oxidative stress. Birds were slaughtered, and liver samples were collected 48 h post-injection. From liver tissue, the total RNA was extracted by RNeasy Mini Kit; Qiagen, Cat. No. 74104. according to the manufacturer regulation. The extracted RNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA). The first strand cDNA was synthesized using kits of RevertAid™ H Minus (Fermentas Life Science, Pittsburgh, PA, USA). One μL of this cDNA was blended with 12.5 μL of 2× SYBR® Green PCR mix with ROX from BioRad, 5.5 μL of RNase free water, and 0.5 μL (10 pmol/μL) of each forward and reverse primer for the selected genes were added. The primers' sequences of catalase, glutathione peroxidase, and superoxide dismutase genes involved in antioxidant function were designed as previously described in [27]. The real-time PCR amplification was carried out with Rotor-Gene Q2 plex (Qiagen Inc., Valencia, CA, USA) with the following conditions; initial denaturation at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Relative fold changes in the expression of target genes measured in triplicate were estimated by the comparative 2−ΔΔCt method with the GAPDH gene as an internal control to normalize target gene expression levels [28].

2.6. Statistical Analysis

Data were submitted to a 2-way ANOVA, using PASW statistics 18 (SPSS Inc., Chicago, IL, USA) to clarify the effects of dietary Se sources, its levels, and their interaction. Gene expression data were statistically analyzed using one-way ANOVA and relevant figures were generated by Graphpad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). Tukey's test was used to separate the means when the treatment difference was significant ($p < 0.05$). All data were expressed as the mean ± SEM. Statistical significance was considered at $p \leq 0.05$.

3. Results and Discussion

3.1. Growth Performance

The effects of different dietary treatments on overall growth performance parameters (1–38 days) are presented in Table 2. The present study showed that the interaction between different dietary sources and levels of Se had significant ($p < 0.05$) effect on the body weight and gain of broilers at 38 days. The groups supplemented with selenomethionine (Se-Met) and nano-Se showed a significant increase ($p < 0.05$) in body weight and gain of Ross broiler chicks when compared with selenite selenium (SeS). Moreover, variety of levels and sources of Se played an important role in our study as when Se- Met or nano-Se were added to diets, body weight and gain increased as dietary Se levels increased, while higher levels in the SeS group at Se concentration of 0.3–0.45 mg/kg diet caused declines as dietary SeS levels increased. Different sources and levels of Se had no effect on feed intake ($p < 0.05$). Feed conversion ratio (FCR) of broilers was affected by the Se sources and levels as they play an important role in improving FCR. The FCR was improved by dietary supplementation of Se-Met or nano-Se, while the best FCR was established with a level of 0.6 mg Se/kg diet from Se-Met

or nano-Se, followed by groups supplemented with 0.3–0.45 mg/kg from Se-Met or nano-Se. Our data demonstrated that the application of dietary Se-Met or nano-Se up to 0.6 mg/kg resulted in the maximum growth rate of broiler chicks, while the same dose from SeS tended to reduce the growth performance of broilers chicks. These results proved that such selected dietary Se-Met or nano-Se levels had higher bioavailability than inorganic forms of Se. These results are in agreement with [29] who found a decline in body weight gain and feed utilization as supplemental inorganic Se increased, while for nano-Se, average daily gain, FCR, and survival ratio reached their highest levels at an Se concentration of 0.15–1.20 mg/kg. [13] Showed that feeding of broilers on 0.3 and 0.5 mg/kg from nano-Se significantly improved FCR and increased tissue selenium content. Our findings were also in agreement with those of [30], who described no differences in feed intake among broilers fed diets supplemented with either organic or inorganic forms of Se. Broilers chicks groups fed on 0.2 mg/kg diet from organic selenium or nano-Se had a similar growth rate as compared to the group supplemented with the same level of Se-selenite [31]. Our findings were also in agreement with those of [32] who reported that increased selenium levels had improved average daily gain in the same time there was no differences on average daily gain between nano-selenium and organic selenium in broiler chickens. The function of Se on growth rate may relate to its role in the selenoprotein P and selenoenzymes type I iodothyronine deiodinase expression, which have critical roles in the synthesis of thyroid hormones and Se transport [33]. Moreover, our results of increased growth performance with selenium methionine and nano-Se could possibly due to an increased thyroid hormone regulating the body's energy metabolism and increased digestibility of protein [34]. The results of this study suggest that different Se sources and levels may be necessary to optimize the performance of broilers, and that the form of organic Se may be of importance.

Table 2. Effects of dietary sources and levels of Se (mg/kg) on growth performance of broilers over 38 days.

	SeS			Met-Se			Nano-Se			SEM	*p*-value		
	0.3	0.45	0.6	0.3	0.45	0.6	0.3	0.45	0.6		Se Source	Se Level	Source × Level
BW	2184 ^{c}	2262 ^{b}	2155 ^{c}	2266 ^{b}	2304 ^{b}	2391 ^{a}	2253 ^{b}	2263 ^{b}	2372 ^{a}	4.82	<0.001	<0.001	<0.001
BWG	2139 ^{c}	2217 ^{b}	2110 ^{c}	2219 ^{b}	2260 ^{b}	2346 ^{a}	2211 ^{b}	2214 ^{b}	2327 ^{a}	4.18	<0.001	<0.001	<0.001
FI	3932	3877	3881	3823	3879	3859	3811	3826	3913	12.09	0.227	0.585	0.215
FCR	1.84 ^{c}	1.75 ^{b}	1.86 ^{c}	1.72 ^{b}	1.71 ^{b}	1.64 ^{a}	1.73 ^{b}	1.73 ^{b}	1.68 ^{a,b}	0.005	<0.001	0.007	<0.001
RGR	192.03	192.30	191.90	192.08	192.42	192.62	192.18	192.21	192.62	0.17	0.740	0.775	0.926

SeS = sodium selenite; Met-Se = selenomethionine; nano-Se = nano-selenium; BW = body weight; BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio, RGR = relative growth rate. ^{a,b,c} Means within a row carrying different superscript letters denote significant differences ($p < 0.05$).

3.2. Selenium Retention in Serum, Muscle, and Liver

In the present study, the different Se sources and levels had significantly affected ($p < 0.05$) Se concentrations in serum, liver, and breast muscle of broilers (Table 3). The groups fed on a diet supplemented with nano-Se and Met-Se showed higher ($p < 0.05$) serum, liver, and breast muscle Se concentrations when compared with those fed diets supplemented with SeS, indicating that nano-Se and Se-Met were better retained in the body than SeS, although the effect of Se-Met was more prominent for tissue Se retention than nano-Se at the same lowered level. Accumulation of minerals in tissues is considered an indicator for mineral utilization [35]. The concept of increasing Se content in human foods by altering dietary Se sources and level given to livestock is now of interest to nutritionists [36]. Wang et al. [37] stated that transport and uptake of selenium by broiler intestinal cells were higher in nano-Se than that of SeS. The difference in retention of Se between Se yeast and SeS or nano-Se may be clarified by the probable metabolic pathways and absorption process for Se from different Se sources [29]. The safe limit of Se in human food has been established at 2.0 mg/kg for the United States [38]. This level agreed with our results that up to 0.6 mg/kg of Se in broiler diets precipitates less than 1 mg/kg in meat with all sources of Se. Selenium uptake from Se-selenite occurs by passive diffusion contributing the poor availability of Se-selenite [39], and up to 50–75% of consumed Se-selenite

is lost through urine. Another limitation of adding selenite to feed is the short period storage of Se in the animal's body [40]. Our results of Se retention in tissue in accordance with those of [41], who demonstrated that broiler chicks fed on dietary organic Se had higher ($p < 0.05$) Se content in breast muscle and liver than those fed diets fortified by SeS. [36] also proved that the contents of Se in liver and muscles were affected by dietary Se supplementation, and retention of Se was increased when organic Se was supplemented as compared with inorganic Se. Cai et al. [13] stated that increasing dietary nano-Se increased the concentration of selenium in liver and muscle tissue ($p < 0.01$). An explanation for increased tissue content from nano-Se may be attributable to improved intestinal absorption of nano-Se due to smaller particle size and larger surface area [42]. SeS and nano-Se, on the other hand, are changed to the transitional selenide and then employed for synthesis of selenoprotein or methylated and after that excreted. However, Met-Se contains a large amount of selenomethionine. When recognized as a Se species, it can be altered to selenocysteine through the trans-selenation pathway and then lysed to selenide. So Met-Se might be simply utilized in the tissue than SeS or nano-Se [43]. Another property of Met-Se involves the chemical similarity between Met-Se and Met, which permits the body to use them interchangeably in protein synthesis as Met-tRNA cannot distinguish between Met and Met-Se, which makes it possible to build Se reserves in the body [2].

3.3. The Effect of Different Levels and Sources of Se on Selected Serum Parameters

The activity of liver enzymes including ALT and AST were not significantly affected by the interaction between different levels and sources of Se. The same trend was recorded for serum creatinine values (Table 3). Selim et al. [44] stated that activity of liver enzymes including ALT and AST were not significantly affected by addition of Zn-Se-Meth, P-Nano-Se, or L-nano-Se in broiler diets. Moreover, increasing the supplemental Se level from 0.3 to 0.45 ppm in broiler diets could not cause any significant difference in plasma creatinine level. In previous studies, [44] found that liver enzymes were not affected by adding different forms of Se (inorganic, organic, or nano) at levels up to 0.3 mg Se/kg diet.

Table 3. Effects of dietary Se sources and levels on Se concentration and some plasma biochemical parameters of broilers over 38 days [1].

	SeS			Met-Se			Nano-Se			SEM	*p*-Value		
	0.3	0.45	0.6	0.3	0.45	0.6	0.3	0.45	0.6		Se Source	Se Level	Se Source × Level
Se concentration													
Serum Se, mg/L	0.17 ^{f}	0.32 ^{e}	0.42 ^{d}	0.20 ^{f}	0.48 ^{c}	0.64 ^{a}	0.20 ^{f}	0.54 ^{b}	0.63 ^{a}	0.005	<0.001	<0.001	<0.001
Liver Se, mg/kg	0.32 ^{f}	0.94 ^{d}	1.16 ^{c}	0.52 ^{e}	1.27 ^{c}	1.52 ^{b}	0.59 ^{e}	1.55 ^{b}	1.83 ^{a}	0.011	<0.001	<0.001	<0.001
Muscle Se, mg/kg	0.16 ^{g}	0.27 ^{e}	0.32 ^{c,d}	0.28 ^{d,e}	0.41 ^{b}	0.75 ^{a}	0.22 ^{f}	0.35 ^{c}	0.77 ^{a}	0.005	<0.001	<0.001	<0.001
Plasma biochemistry													
AST U/L	83.50	84.28	84.78	83.59	83.63	83.71	83.43	84.12	84.17	0.12	0.173	0.049	0.559
ALT U/L	152.91	152.94	155.06	152.81	152.84	152.91	152.81	152.59	153.18	0.028	0.175	0.097	0.320
Creatinine mg/dL	5.68 ^{b}	5.84 ^{a,b}	6.50 ^{a}	5.70 ^{a,b}	5.58 ^{b}	6.04 ^{a,b}	5.94 ^{a,b}	5.92 ^{a,b}	6.20 ^{a,b}	0.009	0.162	0.002	0.521

SeS = sodium selenite; Met-Se = selenomethionine; nano-Se = nano-selenium. ^{a,b,c,d,e,f} Within a row, different superscript letters denote significant difference ($p < 0.05$). [1] Values are means ± standard error.

3.4. Antioxidant Potential of Different Sources and Levels of Se

These data showed that the expression pattern of selected antioxidant-related genes (glutathione peroxidase, GPx, super oxide dismutase, SOD and catalase, CAT) in relation to different Se levels and sources was addressed in (Figure 2). The expression of GPx mRNA significantly increased in groups fed nano-Se at 0.6 mg/kg diet followed by groups supplemented by 0.3 and 0.45 mg/kg diet from Met-Se and nano-Se when compared with SeS with the same levels. The highest expression of SOD was observed in groups supplemented with 0.45 and 0.6 mg/kg diet from nano-Se followed by the group supplemented by a 0.3 mg/kg diet from nano-Se and groups supplemented by 0.45 and 0.6 mg/kg diet from Met-Se, when compared with SeS supplemented group. The mRNA expression of catalase significantly increased with an increasing level of nano-Se and Met-Se when compared with SeS. The antioxidant enzymes such as SOD, CAT, and GPx [45], and non-enzymatic constituents such as glutathione (GSH) [46], play an important role for keeping the animal health, and physiological antioxidant systems. Selenium is a cofactor in several selenoproteins and the antioxidant selenoenzymes as glutathione peroxidase (GPx), thus its functional role is associated with the Se concentration in tissues [47]. Xiao et al. [48] demonstrated that the supplementation of Se in the maternal diet significantly ($p < 0.05$) enhanced the activity of GPx, T-SOD, and CAT in heat stress treated chick embryos when compared with the basal diet, as the levels of GPx1 mRNA were significantly ($p < 0.05$) elevated by adding Se. This may be clarified by higher Se retention in maternal Met-Se treatment [49], which aids in the production of more selenoproteins to preserve chick embryos with a higher antioxidant level. Under heat stress, [35] reported that the addition of organic Se significantly improved GPx activities as compared with broilers fed with inorganic Se. [50] established that the highest GPx activity and lowest MDA content in blood and testis were attained in the treatment of 0.5 mg/kg, as the GPx enzymes were involved in scavenging toxic H_2O_2 [51]. In animal research, the activity of GPx enzymes and their expression genes in tissues were correlated with the concentration of Se added to feed [47]. This finding is also in accordance with [52], who described that Se deficiency caused the reduction of GPx mRNA levels in four GPx genes found in chicken livers. The superoxide dismutase (SOD) and CAT are important antioxidant enzymes for poultry. The superoxide anion is transformed to H_2O_2 by SOD [53], and CAT changes H_2O_2 into water [54], although Se is not a component of SOD and CAT. Our results also indicated that nano-Se and Met-Se increased the mRNA expression of these genes. Yuan. [49] showed that in broiler breeding experiments, hepatic GPx1 and TrxR1 mRNA levels in Met-Se groups were higher ($p < 0.05$) than that in the SeS group.

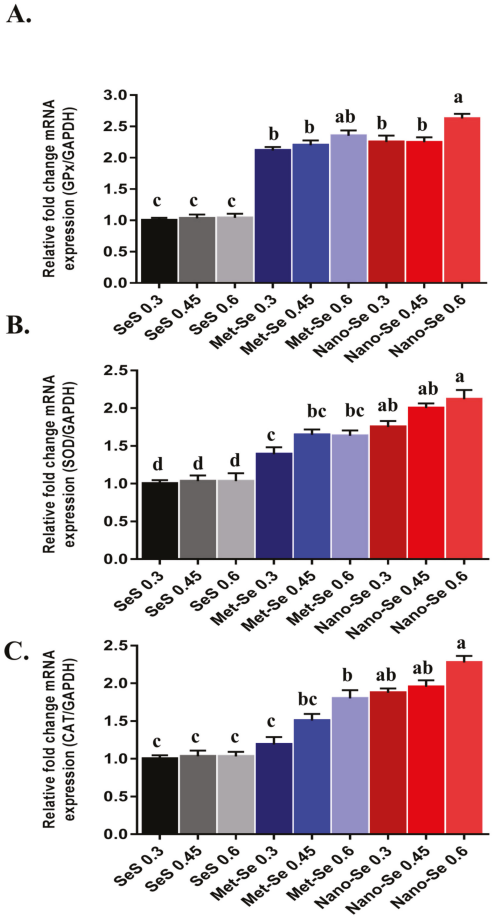

Figure 2. Effects of dietary Se source and level on the relative antioxidant enzymes expression (**A**–**C**). (**A**) Glutathione peroxidase (GPx); (**B**) super oxide dismutase (SOD); and (**C**) catalase (CAT) in the liver of broiler chickens at 40 days. SeS = sodium selenite, Met-Se = selenomethionine, nano-Se = nano-selenium. ^{a,b,c,d} Different superscript letters denote significant difference ($p < 0.05$). Values are means ± standard error.

3.5. Effect of Different Se Sources and Levels on Meat Quality

The role of diets supplemented with different Se sources and levels on breast meat quality in broiler chickens are shown in Table 4. Compared with SeS, dietary Met-Se and nano-Se inclusion in broiler diet improved meat quality, especially as Se levels increased from 0.45 to 0.6 mg/kg.

3.6. Post-Mortem pH of Meat, Cooking Loss and Drip Loss

Breast meat from groups that received an increased level of Met-Se and nano-Se exhibited increased ($p < 0.05$) pH 0.5 and 24 h later when compared with the SeS groups. In addition, birds in the Met-Se and nano-Se groups, specially at high levels, had lesser drip and cooking loss group ($p < 0.05$) compared to those in the SeS groups. The presence of Se in animal diets are a key influence on meat water retention, with the form and level regulating the variation in meat drip loss [55]. The results of our study agreed with [56], who found that the drip loss was lower and water-holding capacity was higher

in pigs fed with organic selenium. It has been reported by some authors that the mechanism by which antioxidants modify the water-holding capacity and drip loss of meat can be attributed to their ability to maintain muscle membranes' integrity post-mortem [57], while others have suggested that proteolysis and protein oxidation acts as an essential factor for determining the moisture retention of meat [58]. Lambert et al. [59] reported that the accumulation of a large amount lactic acid in the muscles, combined with a cessation of blood circulation which induces cellular hypoxia and results in a decreased pH after slaughter, changed the permeability of cell membrane and decreased the water-holding capacity. But our study demonstrated increased water-holding capacity of breast meat in broilers fed on Met-Se and nano-Se. This may be explained by the metabolic conversion of glucose to lactic acid in post-mortem muscle being delayed with organic Se or nano-Se supplementation, thus improving the water-holding capacity of meat and decreasing drip loss [60]. It has been reported elsewhere that 0.3 mg/kg Met-Se supplementation resulted in an increase in the pH of the breast meat of broilers [61] and in geese [62] as compared with 0.3 mg/kg SS supplementation. Other studies demonstrated that water-holding capacity is affected by organic Se supplementation [63] and nano-Se [64]. Cai et al. [13] proved that application of nano-Se increases the ability of broiler muscle proteins to attract water, thus reducing drip loss percentage. The present study further indicates that the role of Met-Se and nano-Se on the biochemistry of muscle tissue is more prominent than with SeS.

3.7. Thiobarbituric acid Reactive Substances (TBRAS) Content of Meat as a Marker for Lipid Oxidation

Frozen storage of all analyzed meat significantly increased ($p < 0.05$) the TBRAS values in breast and thigh meat, with the lowest values for TBRAS recorded in breast meat, which could be related to the total lipid content. With increasing dietary level of organic Se and nano-Se, the TBRAS values decreased in breast and thigh meat when compared with SeS supplementation (Table 4). Exposure to different physiochemical or pathological conditions has recently been shown to be one of the main predisposing agents controlling free radical formation in the body [65]. On the other hand, chicken meat enriched with polyunsaturated fatty acids (PUFA) augmenting the meat susceptibility to oxidation progressions [66]. Bakhshalinejad [32] reported that oxidation resistance of broiler meat was higher in case of supplementation of organic of Se and the higher concentration of Se the higher glutathione peroxidase activity, total antioxidant capacity and malondialdehyde formation. Oxidation of lipids produces free radicals, leading to mutagenesis, carcinogenesis, and aging of the cell [67]. The antioxidant role of Se has also been shown to continue post-mortem in muscle tissue, where it is reported Se reduced oxidization of lipids in meat and had an effect on its quality [15]. Providing Se-enriched meat for human consumption by manipulating animal feed therefore also protects the quality of meat [68]. In this respect, [69] showed that inclusion of Se in poultry diets provides Se-enriched meat and protects the meat from oxidation after slaughter, increasing the stability of the meat against various storage conditions which accelerate the oxidation processes that destroy membrane lipids, consequently reducing the meat's nutritional value [70]. Similarly, higher protection of muscle samples against lipid oxidation have been demonstrated by Se yeast with broilers [61] and turkey meat [71]. In addition, the breeders' diet supplemented with Se also provides antioxidant protection of lipid rich tissues, which was detected by lower TBARS values after slaughter [72]. Calvo et al. [73] found that birds supplemented with organic Se had lower malondialdehyde (MDA) concentrations in muscle samples than the SeS group with the same storage time. In agreement with our results on muscle pH, it has been reported that the pH reduction could accelerate lipid oxidation due to the enhanced autoxidation of hemoglobin at reduced pH [74]. With decreasing muscle pH, higher TBARS values have been reported [75].

3.8. Total Antioxidant Capacity of Meat

The presence of antioxidants in poultry meat is a powerful factor influencing its quality. Once antioxidant defense systems are debilitated, dysfunction of all body cells and tissues may occur. Thus to keep body functions optimal, antioxidant levels are important [76]. As Se plays major role in

protecting cells against oxidative stress, measuring the antioxidant biomarkers is a beneficial tool for evaluating the Se antioxidative role. In the present study ABTS, DPPH, and FRAP assays were used to estimate antioxidant capacities, as theses assay reflect the antioxidant properties of meat [77].

DPPH Assay: Thigh meat was characterized by significantly higher DPPH free radical scavenging ability than breast meat. The supplementation of nano-Se and organic Se at higher levels (0.6 mg/kg) into the Ross broiler diet increased the ability of meat to scavenge free radical DPPH and this capacity increased with the storage period (Table 4). Using specific sources from selenium in poultry diet increases the meat's ability to scavenge the free radical DPPH, due to Se antioxidative functions. During frozen storage, the removal ability of DPPH augmented in all examined samples of chicken meat, demonstrating that Se is stable in the meat [78].

ABTS Assay: The ability of breast and thigh meat to scavenge free radical ABTS were affected by dietary inclusion of Met-Se and nano-Se up to 0.6 mg/kg. During frozen storage, the ability of the meat parts to remove the free radical ABTS tended to increase, reaching the highest values after four weeks of storage (Table 4). These results agree with [78], who stated a higher antioxidative potential of chickens breast to remove free radicals tended to increase during frozen storage, reaching the highest values after storage period of 90 days. This can be accompanied by moisture loss as a result of evaporation, besides alterations in proteins structure and lipids due to oxidation progressions. Also, implementation of Met-Se and nano-Se to chicken diets significantly rises the breast's ability and thigh tissues to scavenge the synthetic free radical ABTS when compared with SeS.

FRAP Assay: In general, the capacity of the thigh myofibrillar protein to reduce Fe^{3+} to Fe^{2+} was higher than in breast myofibrillar protein. In the first three hours, dietary inclusion of 0.45 and 0.6 mg/kg diet of Met-Se and nano-Se had the same reducing capacity of Fe^{3+} to Fe^{2+}, while after four weeks the reducing capacity of Fe^{3+} to Fe^{2+} was more prominent in breast meat and thigh meat for groups supplemented with nano-Se. It is well understood that Se is vital for the intra- and extra-cellular antioxidant systems of the body [79]. Selenium is also effective in delaying post-mortem oxidation responses [15]. The association between meat quality and oxidation resistance of muscle is well recognized. Huff-Lonergan et al. [58] described that changes in the antioxidant defense system of animals and muscles would affect water-holding capacity, meat proteolysis and calpain activity, thus quality characteristics of meat. In former studies, the water-holding capacity and chicken muscles color were enhanced by dietary Se addition [80]. Se application to chicken diets causes a significant increase in the iron reduction ability for both sets of the leg and back muscles, which can be associated with the higher Se retention in the lipids-rich parts [78]. Li et al. [81] described how total protein solubility, pH at 45 min, and myofibrillar protein solubility decreased while cooking loss was improved after feeding broiler chickens 0.3 mg/kg of either Met-Se or nano-Se as compared with SeS. Muscle proteins comprise connective tissue, sarcoplasmic and myofibrillar, [82]. Protein solubility resulted from protein denaturation during muscle ageing. In addition, denaturation of muscle protein is associated with antioxidant capacity [83]. When muscle amino acids as cysteine, tryptophan are oxidized, disulfide bonds and carbonyl are produced. At that time, the protein structure is destroyed, which would decrease the solubility of protein [84]. Current study, revealed significant increases in the iron reduction capacity which can be related to higher deposition of Met-Se and nano-Se in breast and thigh of chickens specially when supplemented with higher dose (0.6 mg /kg diet) compared with SeS supplementation, which could be a consequence of improved antioxidant capacity.

Table 4. Effects of dietary Se source and level (mg/kg) on meat quality after slaughter and antioxidative potential of broiler meat (breast and thigh) during frozen storage.

	SeS			Met-Se			Nano-Se			SEM	*p*-Value		
	0.3	0.45	0.6	0.3	0.45	0.6	0.3	0.45	0.6		Se Source	Se Level	Se Source × Level
pH, 0.5 h	6.37 ^{d,e}	6.49 ^{c,d}	6.32 ^{e}	6.56 ^{c}	6.67 ^{b}	6. 80 ^{a}	6.78 ^{a}	6.82 ^{a}	6.83 ^{a}	0.007	<0.001	<0.001	<0.001
pH, 24 h	5.46 ^{d}	5.48 ^{c,d}	5.41 ^{d}	5.54 ^{b,c}	5.56 ^{b,c}	5.67 ^{a}	5.59 ^{b}	5.62 ^{a,b}	5.70 ^{a}	0.005	<0.001	<0.001	<0.001
Drip loss, %	2.74 ^{a}	2.62 ^{b}	2.83 ^{a}	2.38 ^{c}	2.17 ^{d}	2.13 ^{d,e}	2.35 ^{c}	2.16^{d}	2.04 ^{e}	0.007	<0.001	<0.001	<0.001
Cooking loss %	14.04 ^{b}	12.98 ^{c}	14.60 ^{a}	12.96 ^{c}	12.64 ^{c,d}	12.16 ^{e}	12.86^{c}	12.18 ^{d,e}	12.07 ^{e}	0.03	<0.001	<0.001	<0.001
Breast TBRAS, mg/kg 3 h	0.17 ^{a}	0.17 ^{a}	0.14 ^{a}	0.14 ^{a}	0.11 ^{a,b}	0.04 ^{c}	0.11 ^{a}	0.05 ^{b,c}	0.03^{c}	0.02	<0.001	<0.001	<0.038
Breast TBRAS, mg/kg 2 W	0.47 ^{a}	0.38 ^{b,c}	0.42 ^{a,b}	0.36 ^{b,c}	0.30 ^{c,d}	0.26 ^{d}	0.39 ^{a,b,c}	0.22 ^{d,e}	0.15 ^{e}	0.02	<0.001	<0.001	<0.001
Breast TBRAS, mg/kg 4 W	0.83 ^{a}	0.78 ^{a,b}	0.77 ^{a,b}	0.71 ^{b,c}	0.71 ^{b,c}	0.68 ^{c,d}	0.64 ^{d}	0.53 ^{e}	0.50 ^{e}	0.02	<0.001	<0.001	<0.007
Thigh TBRAS, mg/kg 3 h	0.23 ^{a}	0.26 ^{a}	0.24 ^{a}	0.25 ^{a}	0.21 ^{a,b}	0.13 ^{c}	0.23 ^{a}	0.16 ^{b,c}	0.11^{c}	0.02	<0.001	<0.001	<0.001
Thigh TBRAS, mg/kg 2 W	0.48 ^{a}	0.40 ^{b}	0.47 ^{a}	0.35 ^{b,c}	0.29 ^{c,d}	0.31 ^{c}	0.39 ^{b}	0.23 ^{d,e}	0.18 ^{e}	0.02	<0.001	<0.001	<0.001
Thigh TBRAS, mg/kg 4 W	0.86 ^{a}	0.80 ^{a}	0.80 ^{a}	0.72 ^{b}	0.72 ^{b}	0.69 ^{b,c}	0.65 ^{c}	0.56 ^{d}	0.54^{d}	0.02	<0.001	<0.001	<0.002
Breast ABTS, 3 h	2.30 ^{e}	2.47 ^{d}	2.09 ^{f}	3.47 ^{c}	3.60 ^{c}	3.75 ^{b}	3.87 ^{b}	4.20 ^{a}	4.21 ^{a}	0.03	<0.001	<0.001	<0.001
Breast ABTS, 4 week	5.37 ^{f}	5.59 ^{e}	5.27 ^{g}	6.31 ^{d}	6.52 ^{c}	6.93 ^{b}	6.92 ^{b}	8.14 ^{a}	8.21 ^{a}	0.02	<0.001	<0.001	<0.001
Thigh ABTS, 3 h	6.47 ^{f}	6.73 ^{d,e}	6.93 ^{c}	6.70 ^{e}	7.04 ^{b,c}	6.95 ^{c}	6.90 ^{c,d}	7.18 ^{b}	8.18 ^{a}	0.05	<0.001	<0.001	<0.001
Thigh ABTS, 4 week	7.47 ^{e}	8.06 ^{b,c}	7.94 ^{c}	7.70 ^{d}	7.73 ^{d}	7.95 ^{c}	7.90 ^{c}	8.18 ^{a,b}	9.18 ^{a}	0.04	<0.001	<0.001	<0.001
Breast DPPH, 3 h	5.91 ^{e}	6.19 ^{d}	5.81 ^{f}	5.98 ^{e}	6.29 ^{c,d}	6.30 ^{c,d}	6.34 ^{c}	6.74 ^{b}	7.13 ^{a}	0.04	<0.001	<0.001	<0.001
Breast DPPH, 4 week	6.39 ^{d}	6.71 ^{c}	6.35 ^{d}	6.48 ^{d}	6.79 ^{c}	6.80 ^{c}	6.85 ^{c}	7.34 ^{b}	6.77 ^{a}	0.05	<0.001	<0.001	<0.001
Thigh DPPH, 3 h	7.14 ^{d,e}	7.48 ^{a,b}	7.01 ^{e}	7.21 ^{c,d,e}	7.20 ^{c,d,e}	7.36 ^{b,c,d}	7.42 ^{a,b,c}	7.45 ^{a,b}	7.61 ^{a}	0.05	<0.001	<0.03	<0.001
Thigh DPPH, 4 week	8.54 ^{f}	8.82 ^{e}	8.83 ^{e}	9.23 ^{d}	9.39 ^{c}	9.58 ^{b}	9.41 ^{c}	9.58 ^{b}	9.72 ^{a}	0.02	<0.001	<0.001	<0.02
Breast FRAP, 3 h	0.15 ^{d}	0.22 ^{b,c}	0.16 ^{c,d}	0.22 ^{b,c}	0.32 ^{a}	0.33 ^{a}	0.25 ^{b}	0.33 ^{a}	0.37 ^{a}	0.01	<0.001	<0.001	<0.001
Breast FRAP, 4 week	0.28 ^{c}	0.38 ^{b}	0.30 ^{c}	0.37 ^{b}	0.43 ^{a,b}	0.42 ^{a,b}	0.40 ^{a,b}	0.43 ^{a,b}	0.45 ^{a}	0.01	<0.001	<0.001	<0.012
Thigh FRAP, 3 h	1.06 ^{f,g}	1.13 ^{e,f}	1.03 ^{g}	1.15 ^{e}	1.19 ^{d,e}	1.35 ^{a,b}	1.26 ^{c,d}	1.30 ^{b,c}	1.40 ^{a}	0.02	<0.001	<0.01	<0.001
Thigh FRAP, 4 week	1.24 ^{a}	1.28 ^{a}	1.26 ^{a}	1.31 ^{a}	1.40 ^{c}	1.52 ^{a,b}	1.39 ^{c}	1.50 ^{b}	1.59 ^{a}	0.02	<0.001	<0.001	<0.001

SeS = Sodium selenite; Met-Se = selenomethionine; nano-Se = nano-selenium; ^{a,b,c,d,e,f,g} means within a row carrying different superscript letters denote significant difference ($p < 0.05$).

4. Conclusions

Our results suggested that in Ross broiler chickens, dietary supplementation of either Met-Se or nano-Se up to 0.6 mg/kg increased their performance and was more efficiently retained in the body than SeS. In addition, under stress the antioxidant resistance of broilers fed selected higher levels of Met-Se or nano-Se was enhanced. Moreover, frozen stored meat quality was improved in a dose-dependent manner with both Met-Se and nano-Se. Nano-Se was more potent than Met-Se, which in turn was more potent than inorganic Se against oxidative stress, which improved the quality of meat under frozen conditions.

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Effect of In Ovo Injection of L-Arginine in Different Chicken Embryonic Development Stages on Post-Hatchability, Immune Response, and Myo-D and Myogenin Proteins

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Simple Summary: In the current study, we hypothesized that the in ovo injection of L-arginine (L-Arg) at different stages of embryonic development, which would have positive effects on the survival rate, hatching rate, immunoglobulin M (IgM) levels, heat shock proteins (HSPs) such as HSP-47, HSP-60, and HSP-70, and muscle development markers as well: Mainly, myoblast determination protein (myoD) and myogenin in pectoral muscles. As indicated, the in ovo injection of L-Arg resulted in an increased hatch rate and weight, survival rate, higher levels of IgM, and myogenin and MyoD expression in the muscles. At the same time, a decrease in the level of HSP-47, HSP-60, and HSP-70 expressions in the tissues was observed on the 14th day of injection compared to the eighth and 18th day of the injection period. In addition, the in ovo injection of L-Arg decreased the serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) concentration in serum as well micronuclei and nuclear abnormality in the blood on the 14th day of the incubation period. Hence, the 14th day would be a suitable day for the injection of L-Arg to promote the hatching rate and muscle growth of broiler chickens.

Abstract: The aim of this study was to evaluate the effect of in ovo injection with different ratios of L-arginine (L-Arg) into Ross broiler eggs at three different embryonic developmental stages (eighth day (d), 14th day, and 18th day) on the survival, hatchability, and body weight (BW) of one-day-old hatched chicks. Additionally, we have analyzed the levels of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT), the protein expression of heat shock proteins (HSPs), and we have also determined micronuclei (MN) and nuclear abnormality (NA). In addition, the genotoxic effect was observed in peripheral blood cells such as the presence of micronuclei and nuclear abnormalities in the experimental groups. The results showed that survival and hatching rates as well as body weight were increased on the 14th day of incubation compared to the eighth and 18th day of incubation at lower concentrations of L-Arg. Moreover, the levels of SGOT and SGPT were also significantly ($p < 0.05$) increased on the 14th day of incubation at the same concentration (100 μg/μL/egg) of injection. In addition, immunoglobulin (IgM) levels were increased on the 14th day of incubation compared to other days. The protein expressions of HSP-47, HSP-60, and HSP-70 in the liver were significantly down-regulated, whereas the expression of myogenin and myoblast determination protein (MyoD) were significantly up-regulated on the 14th day after

incubation when treated with all different doses such as 100 μg, 1000 μg, and 2500 μg/μL/egg, namely 3T1, 3T2, and 3T3, respectively. However, the treatment with low doses of L-Arg down-regulated the expression levels of those proteins on the 14th day of incubation. Histopathology of the liver by hematoxylin and eosin (H&E) staining showed that the majority of liver damage, specifically intracytoplasmic vacuoles, were observed in the 3T1, 3T2, and 3T3 groups. The minimum dose of 100 μg/mL/egg on the 14th day of incubation significantly prevented intracytoplasmic vacuole damages. These results demonstrate that in ovo administration of L-Arg at (100 μg/μL/egg) may be an effective method to increase chick BW, hatch rate, muscle growth-related proteins, and promote the immune response through increasing IgM on the 14th day of the incubation period.

Keywords: embryonic development; heat shock proteins; immunoglobulin; intracytoplasmic vacuoles; L-arginine

1. Introduction

The selection of chickens (*Gallus gallus*) for meat production has led to the generation of inbred strains that show accelerated growth performance, particularly enhanced muscle growth that mostly occurs during embryogenesis [1,2]. During embryogenesis, nutrients and energy are mainly acquired from yolk, which mainly contains lipids and low levels of carbohydrates [3]. Subsequently, the health of the embryo and post-hatch chicken depends on gluconeogenesis from essential amino acids [4,5]. In recent years, researchers have found that the administration of amino acids into fertilized broiler eggs, which is called in ovo feeding, may provide poultry companies with an alternative method to increase the hatchability and muscle growth weight of newly hatched chicks [6,7]. The supplementation of nutrients into fertilized broiler eggs influences embryo development and growth during incubation and the post-hatch growth performance of chicks [7]. The nourishment and supplementation with bioactive substances such as bioactive amino acids, polyphenols, and prebiotics can enhance the immune system, decrease osteoporosis, and decrease the risk of heart diseases [8]. Similarly, previous reports have indicated that the amino acids, carbohydrates, and vitamins that are applied to eggs through in ovo feeding can improve the hatching rate, body weight, survival rate, growth performance, and marketing size [9]. Moreover, an earlier study demonstrated that the in ovo feeding site and time can affect hatchability [10].

During embryonic development, the chorioallantoic membrane develops, which can vascularize on the 12th day of the incubation period. Moreover, the embryo is surrounded by the amniotic fluid, which remains in contact with the embryonic gastrointestinal tract and enables the transport of substances from the air chamber into the intestine [11]. Several genes are associated with cellular interactions and differentiation during the organogenesis of the eye, ear, brain, skin, and tissues such as bones and cartilages; the expression of those genes is either transient or initiated during later stages of embryogenesis [12]. Some authors have indicated that the injection of amino acids into the egg on the first day is sufficient to fully support embryonic development [13,14], leading to increased hatching and breast weight [15]. It has been demonstrated that the injection of sucrose and dextrin into chicken embryos can result in a greater percentage of pectoral muscle weight than the control [5,16]. Recently, it has been reported that chicken embryos injected with L-glutamine on the first day of incubation can increase the fiber area, pectoral muscle mass, and endothelial cell proliferation while stimulating vasculogenesis and angiogenesis [17]. The in ovo administration of amino acids or peptides increases the expression levels of MyoD1 and paired box protein 7 (Pax7), which are necessary for muscle growth during embryogenesis [18].

Standardization of the injection site, needle length, and embryonic age using amino acid (Lys + Met + Cys, Thr + Gly + Ser or Ile + leu + Val) with 11-mm and 24 mm-needles on the seventh and 14th day of incubation has resulted in poor hatchability and poor muscle growth markers [7]. An in ovo injection

of glutamine in conjugation with (silver nanoparticles) Ag NPs on the first day of chicken embryos increased the muscle mass [19], and L-arginine (L-Arg) in one-day-old quail embryos increased the hatchability and growth performance [20]. In our study, we checked different time intervals (eighth day, 14th day, and 18th day) and different doses of L-Ar (100, 1000, and 2500 μg/100 μL/egg) for responses related to the survival rate, hatchability, body weight, and muscle growth-related proteins such as myogenin and MyoD and immunoglobulin M (IgM) levels.

2. Materials and Methods

2.1. Ethics Statement

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Chonbuk National University, with the project number 2017R1D1A1B03032217. Animal care and handling are in compliance with the regulations of the IAEC Guidelines for the Euthanasia of Animals: 2015 Edition. The sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" (2015) No. CBNU 2015048 set by the Ministry of Science and Technology, Korea.

2.2. Chemicals

L-arginine, hematoxylin and eosin (H&E), and periodic acid-Schiff's (PAS) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Chemiluminescent for band detection was purchased from Thermo Scientific (Rockford, IL, USA). Antibodies were purchased from ENZO Life Science (Farmingdale, NY, USA). All the laboratory glassware was acquired from Falcon Lab ware (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.3. Experimental Design and Incubation

Ross 1040 broiler chicken eggs were obtained from Samhwa-Won Jong, South Korea. On the first day of incubation, eggs were weighed (60 ± 1.36 g) and separated into different groups. Eggs were randomly divided into 13 groups (4 × 20 × 3 = replication × eggs × injection) as described in Table 1. L-Arg was injected at three concentrations (100 μg, 1000 μg, and 2500 μg/100μL/egg) on the eighth, 14th, and 18th day of the incubation period, respectively. A 0.100-mL of L-Arg (1% PBS) was injected. Immediately after the injection, the hole was sealed with liquid paraffin. Then, eggs were placed in an incubator for 20 days under standard conditions (temperature, 37.8 °C; humidity, 60%). On the 18th day, eggs were transferred to hatching boxes and promptly placed in a hatcher incubator with humidity maintained at 60% and temperature set at 37 °C to hatch chicks.

Table 1. Experimental design for dose (L-Arg) and embronic stage (eighth day, 14th day, and 18th day) fixation.

Group	Dosage	No. of Eggs & No. of Replication	Total No. of Eggs
1C	Control	20 eggs × 4 replicates	80
1C1 (8th day)	PBS/100 μL/egg	20 eggs × 4 replicates	80
1T1 (8th day)	100 μg/100 μL/egg	20 eggs × 4 replicates	80
1T2 (8th day)	1000 μg/100 μL/egg	20 eggs × 4 replicates	80
1T3 (8th day)	2500 μg/100 μL/egg	20 eggs × 4 replicates	80
2C1 (14th day)	PBS/100 μL/egg	20 eggs × 4 replicates	80
2T1 (14th day)	100 μg/100 μL/egg	20 eggs × 4 replicates	80
2T2 (14th day)	1000 μg/100 μL/egg	20 eggs × 4 replicates	80
2T3 (14th day)	2500 μg/100 μL/egg	20 eggs × 4 replicates	80
3C1 (18th day)	PBS/100 μL/egg	20 eggs × 4 replicates	80
3T1 (18th day)	100 μg/100 μL/egg	20 eggs × 4 replicates	80
3T2 (18th day)	1000 μg/100 μL/egg	20 eggs × 4 replicates	80
3T2 (18th day)	2500 μg/100 μL/egg	20 eggs × 4 replicates	80

Note: In Ovo Injection and Treatment Groups.

2.4. Survival Rate Measurement

Embryos' survival rates during the incubation period were measured on the eighth day. Treated eggs were checked to determine the number of live and dead eggs as well as fertilized and non-fertilized ones among the total number of eggs. At 18th day of incubation, after injection, the eggs live eggs were moved to another hatching incubator with their respective experimental group. The survival rate was calculated with the following Equation (1):

$$\text{Survival rate \%} = \frac{\text{No. of live eggs}}{\text{No. of fertilized eggs}} \times 100 \quad (1)$$

2.5. Hatching Rate and Body Weight Measurements

On the 21st day, hatched chicks were moved from the hatcher incubator to hatching boxes to determine hatching rates. These hatched chicks were kept without food and water at 32 °C. Then, they were weighed to record their live body weights. The hatching rate was calculated with the following Equation (2):

$$\text{Hatching rate \%} = \frac{\text{No. of chicks hatched on 21st day}}{\text{No. of fertilized eggs that were } \textit{in ovo} \text{ fed}} \times 100 \quad (2)$$

2.6. Biochemical Indices

At the end of the experimental period, the hatched chicks were sacrificed under anesthesia (diethyl ether). Blood was collected from the jugular vein in tubes for serum separation. A small amount of collected blood was immediately smeared on clean grease-free microscope slides and air-dried for micronuclei (MN) and nuclear abnormality (NA). The breast muscle and liver were collected and washed in ice-cold saline for further study. The body was cut opened; muscle and liver samples were excised, washed with ice-cold saline, and then homogenized with 0.1 M of cold phosphate buffer, pH 7.4. Concentrations of serum glutamate pyruvate transaminase (SGPT) and erum glutamate oxaloacetate transaminase (SGOT) in serum were measured using commercial kits (Asan Pharamaceuticals Co., Ltd., Seoul, Korea).

2.7. Micronuclei (MN) and Nuclear Abnormality (NA) Tests Using Periodic Acid Schiff's (PAS) Staining

MN and NA were assayed in the liver by standard methods presented elsewhere [21,22]. Blood samples collected from the first day of the hatching period were immediately smeared on clean grease-free microscope slides and air dried. Afterwards, slides were fixed with methanol for 5 min at room temperature, gently rinsed with running tap water for 1 min, and immersed in a periodic acid solution for 5 min at room temperature. Then, these slides were rinsed using DH_2O, immersed in PAS Schiff's reagent for 15 min at room temperature, and gently washed with running tap water for 5 min. Finally, counter-staining was performed with a hematoxylin solution for 90 s. Then, slides were rinsed in running tap water for 30 s, air dried, and examined with a light microscope (100×) using immersion oil.

2.8. Measurement of IgM Concentration in Serum

Serum samples were collected from individual experimental animals to determine serum immunoglobulin (Ig) M levels using chicken IgM ELISA kit (Abcam, Suite B2304, Cambridge, MA 02139-1517, USA) following the manufacture's specification. IgM levels were analyzed based on absorbance values measured at 450 nm.

2.9. Analysis of Heat-Shock Proteins (HSPs) by Western Blot

Proteins were extracted from 100 mg of muscle samples using radioimmunoprecipitation assay (RIPA) buffer to determine the protein expression levels of HSP-47, HSP-60, HSP-70, myoD, and myogenin in experimental groups. Protein concentrations were determined using a BIO-RAD protein assay kit (BIO-RAD). Extract samples containing 50 µg of protein were solubilized in *Laemmli buffer*, separated by 12% acrylamide gel, and then transferred to Hybond-P PVDF membranes (GE Healthcare Inc., Amersham, UK) for 60 min at 200 mA. Then, these PVDF membranes were blocked with 5% skimmed milk powder in 0.5 M of Tris-buffered saline (pH 7.4) with 0.05% Tween 20 (TBST) at room temperature for 2 h. Western immunoblotting with HSP-47, HSP-60, HSP-70, Myo-D, and myogenin are primary antibodies (1:2500 dilution) took place overnight. After washing three times with TBST, these membranes were probed with HRP-conjugated secondary antibodies (1:5000 dilutions) for 60 min at room temperature, and then washed three times with TBST (10 min each wash). Protein bands were visualized using a Chemiluminescent assay kit from Thermo Scientific for 1–5 min. Bands were imaged with an iBright™ CL1000 Imaging System (Invitrogen in Thermo Fisher Scientific, Life Technologies Korea LLC, Jeonju-si, Jeollabuk-do, Korea) and quantified using Image J Software. The relative density of the band was normalized to that of β-actin as an internal control.

2.10. In Silico Molecular Docking Studies

To understand the mechanism of interaction of L-Arg with heat shock protein, crystal structures of GroEL mutant A109C (PDB ID: 5OPW) [23] and human HSP70 substrate binding domain L542Y mutant (PDB ID: 5XIR) [24] were downloaded from the Protein Data Bank. Molecular docking studies were performed using the GLIDE program [25] (Version 8.5, Schrodinger LLC, New York, NY, USA). To analyze docking results and execute the protocol, the Maestro user interface (Version 8.5, Schrodinger LLC, New York, NY, USA) was employed. Validation of the protocol was performed by redocking. The structure of L-Arg was sketched using ACD/chemsketch (Freeware version). The GLIDE grid generation wizard was used to define the docking space. Docking was performed using XP (Extra Precision mode) docking protocol.

2.11. Histopathological Study of the Liver

Livers were collected after chickens were sacrificed, immediately fixed with 10% neutral buffered formalin (NBF), and processed in an auto processor (Excelsior ES, Thermo Scientific, Waltham, MA, USA). After embedding in paraffin, 5-µm sections were made and subjected to H&E staining. Digital images were obtained using a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) at fixed 100× (200×) magnification.

2.12. Statistical Analysis

All the values are presented as mean ± SD from 12 determinations from each group and statistically analyzed using Duncan test following ANOVAs with SAS® software, version 9.4, (Institute of INC, North Carolina, USA).

3. Results and Discussion

3.1. Survival Rate and Hatchability

Survival rate was significantly ($p < 0.05$) increased in the 2T1 and 3T1 groups than that in other groups. The lowest survival rate was observed in 1T3, 3T2, and 3T3 groups (Figure 1). These results showed that the survival rates differed depending upon the injection period and the concentration of L-Arg. Embryos may utilize in ovo administered amino acids to improve energy status and save muscle protein to improve their enteric development, hatching, and survival rate [26]. In our study, the same mechanism might have occurred; the administration of L-Arg could improve the survival

rate at the minimal concentration (3T1) on the 14th day of the injection period (Figure 1). However, during incubation, an excess of amino acids such as glycine and proline failed to improve embryo development [27]. The same attributes could have been observed in our current study: that a maximum concentration of L-Arg affects the embryonic growth.

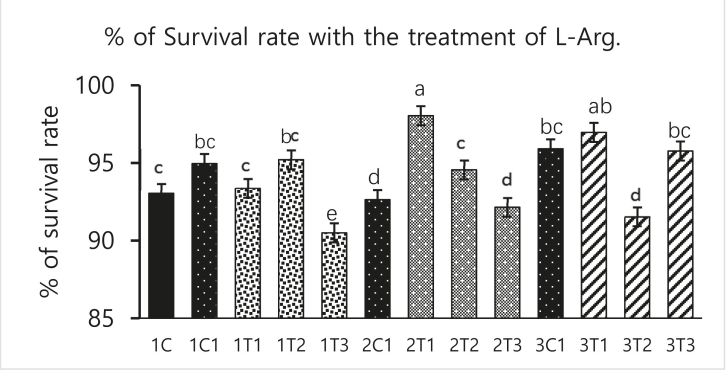


Figure 1. Effects of in ovo injections at different concentrations of L-arginine (L-Arg) with different developmental embryonic stages on survival rate. Small characters indicate significant differences among experimental groups at $p < 0.01$. Values are presented as mean ± SD from 12 determinations. Data were analyzed using Duncan test following ANOVAs with SAS® software, version 9.4, (Institute of INC, North Carolina, USA).

Different concentrations of L-Arg injected in embryos can influence biological molecules and toxicity during embryogenesis. However, several studies have reported that higher doses of L-Arg become toxic, which can cause significantly increased mortality rates and impaired weight gain, whereas chicks injected with lower concentrations of L-Arg (1.0%) showed better growth performance than those injected with a higher concentration (1.5%) of L-Arg [28]. In a parallel effect revealed in our current study to a lower concentration of L-Arg (2T1) on the 14th day of injection increasing the hatching rate (96.29%), body weight (64.25 g) also increased the survival rate (98.03%) compared to other groups (Figures 1–3). The in ovo injection of L-Arg to late-term embryos can increase the body weight (5% to 6%) compared to controls [5]. In addition, in ovo administration of all 20 different amino acids can increase chick weight by 3.6% and 2.1%, respectively [10]. The in ovo administration of amino acids might have stimulated the utilization of amino acids with a concomitant decrease in the degradation of amino acids by the embryo [29]. In ovo feeding of L-Arg resulted in higher embryo weight due to an increase in muscle mass [30]. The in ovo injection of L-Arg could be utilized by the embryo, resulting in increased muscle mass and a heavy embryo, which can increase the hatching rate [31]. L-Arg may attenuate adverse effects of rearing chickens under cold ambient temperatures or at high altitudes [32]. Furthermore, feeding broiler chickens with a diet that is deficient in L-Arg under cold stress at high altitudes can depress nitric oxide synthesis, decrease feed intake, reduce body weight gain, and increase the right ventricle to total ventricle weight ratio, mortality rate, and ascites mortality [31]. A previous study reported that a lower percentage (1.36%) of L-Arg supplemented to broiler eggs was more easily digestible than a higher percentage of arginine, and it could obtain the highest egg weight [33]. Albeit, a low dose of L-Arg stimulates the secretion of the growth hormone, which could increase the body weight [21]. The same mechanism might occur in our present study; L-Arg appeared to improve the body weight of chicks in group 2T1 (Figure 3). However, the body weights did not significantly vary among the other groups. Hatchability, gut microflora population, immune-related gene expression, and muscle fiber increased as a result of the 12–14-days in ovo injection of various substances such as Raffinose, *Lactococcus lactis*, and *Silybum marianum* extract [34–36]. Hence, a low dose (2T1) of L-Arg injected on the 14th day of the incubation period could improve body weight;

the reason behind this might be that the lower concentration of L-Arg may stimulate the growth hormones in the middle stages of embryonic development when compared to the early (eighth day) and late (18th day) embryonic stages.

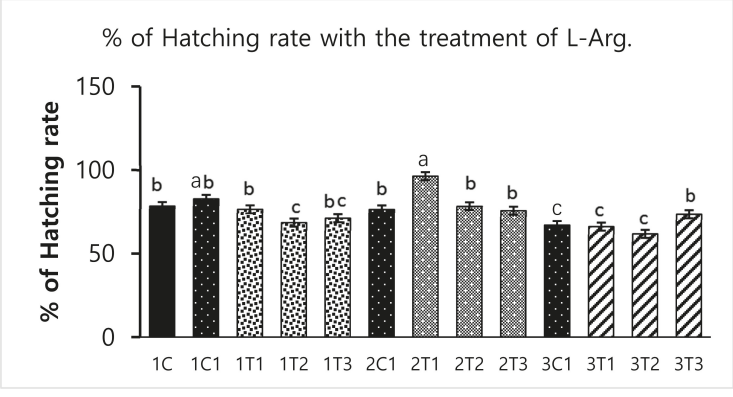


Figure 2. Effects of in ovo injections at different concentrations of L-Arg with different developmental embryonic stages on hatching rates. Small characters indicate significant differences among experimental groups at $p < 0.01$. Values are presented as mean ± SD from 12 determinations. Data were analyzed using Duncan test following ANOVAs with SAS® software, version 9.4, (Institute of INC, North Carolina, USA).

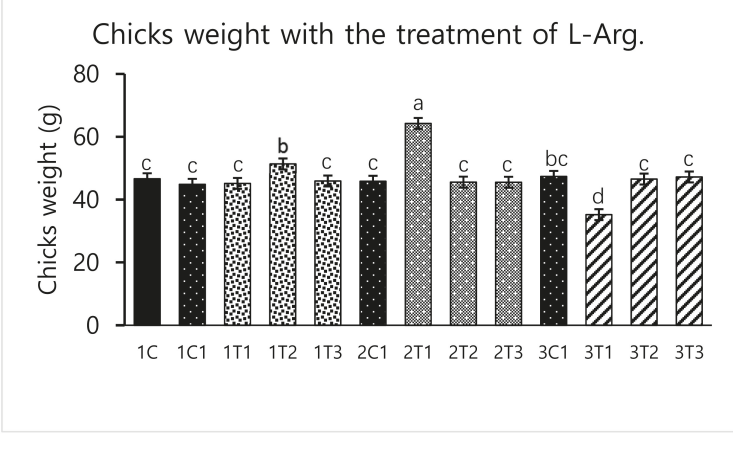


Figure 3. Effects of in ovo injections at different concentrations of L-Arg with different developmental embryonic stages on Chicks weight. Small characters indicate significant differences among experimental groups at $p < 0.01$. Values are presented as mean ± SD from 12 determinations. Data were analyzed using Duncan test following ANOVAs with SAS® software, version 9.4, (Institute of INC, North Carolina, USA).

3.2. Biochemical Indices (SGOT and SGPT)

Elevated SGOT and SGPT levels indicate improper liver function due to damages of the cell integrity and cell membrane in the liver. Our results revealed that the injection of L-Arg at all doses except the lower dose affected SGOT and SGPT levels on the eighth and 18th-day embryonic stages (Figure 6A). SGOT and SGPT levels were significantly decreased in the 2T1 and 2T2 groups of embryos compared to 2T3, 3C1, 3T1, 3T2 and 3T3. Increased levels of SGOT and SGPT in the blood are conducive

to liver function damage [37–39]. In fact, free radicals can attack hepatocytes and release stored SGPT to re-enter the blood serum [40]. A lower concentration of L-Arg supplementation caused a greater percentage reduction in SGOT and SGPT levels in sickle cell anemia subjects [39]. The supplementation of L-Arg to mice in higher concentrations showed that increased SGOT and SGPT levels had been linked to damage to hepatic cells and hemolysis [41]. The cause of liver damage is unclear. Hence, confirming that a higher concentration of L-Arg might have damaged the hepatic cell through the elevation of SGOT and SGPT in the 1T1, 1T2, 1T3, 3T1, 3T2 and 3T3 groups. On the other hand, the 2T1 group of injected chicken embryos could re-back the SGOT and SGPT levels compared to the other groups. Stimulating the action of nitric oxide (NO) production by L-Arg results showed that it improved the degree of the hepatocellular structure by blocking of B-cell lymphoma-2 (Bcl-2) and tumor necrosis factor-α (TNF-α) [42]. In addition, L-Arg at 1 g/day decreased the liver enzymes such as SGOT and SGPT through increasing the nitric oxide (NO) synthesis. NO synthase plays an important role in liver injury through inducible nitric oxide synthase (iNOS) pathways [43]. The same mechanism could be involved in our current study, too. This same mechanism that might have occurred in our study could be that the production of NO reduces necrosis and apoptosis by attenuation of the inflammatory pathway, which in turned prevented the hepatotoxicity. Moreover, it also improved the hepatobiliary function, and the ultrastructure of liver results reduced the SGOT and SGPT levels in L-Arg treatment in the lower dose (2T1) on the 14th day injection of embryos (Figure 4).

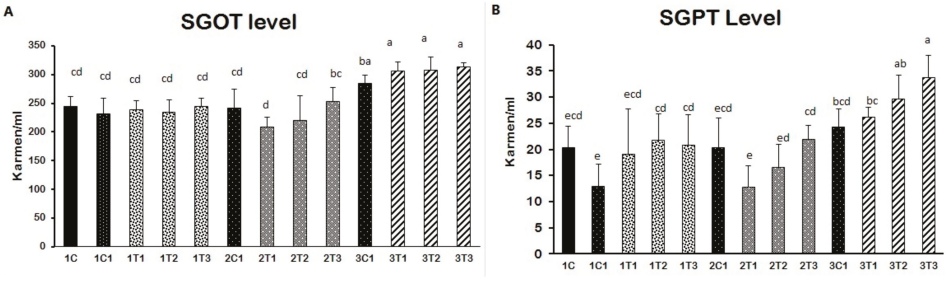


Figure 4. (**A**,**B**) Effect of in ovo feeding on broiler eggs with different concentrations of L-Arg at different developmental embryonic stages and effects on serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) concentrations in serum. Small characters indicate significant differences among the experimental groups at $p < 0.01$. Values are presented as mean ± SD from 12 determinations. Data were analyzed using Duncan test following ANOVAs with SAS® software, version 9.4, (Institute of INC, North Carolina, USA).

3.3. Micronuclei (MN) and Nuclear Abnormality (NA) Tests Using Periodic Acid-Schiff's (PAS) Staining

The wide use of different doses of L-Arg at three different incubation periods requires examining the genotoxic activity in peripheral blood by the method of [44]. MN and NA tests were conducted to examine peripheral blood cells in all the groups of experimental chicks (Figure 5). The MN test can measure subcellular processes of chromosomal breaks (clastogenesis) or cell spindle malfunctions (aneugenesis) as well as the formation of mitochondrial disruption and nuclear DNA, which can lead to mitochondria-dependent apoptosis in chicken embryos as an indicator of chromosomal damage [45]. Similar results were obtained in our current experiment: the MN and NA in peripheral blood erythrocytes were observed, which clearly demonstrates the higher genotoxicity of a high dose of L-Arg on the eighth, 14th, and 18th day of the incubation period. Moreover, the 2T1 and 2T2 groups showed a normal architecture of nuclei in peripheral blood cells, which was similar to the control group. Figure 5 shows marked inflammation around the periportal region with microvesicular and macrovesicular fatty infiltration (yellow arrows).

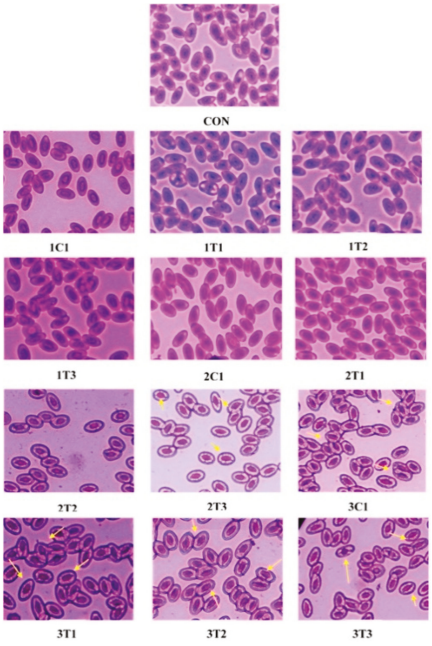
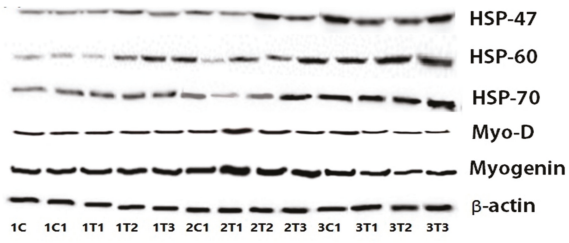


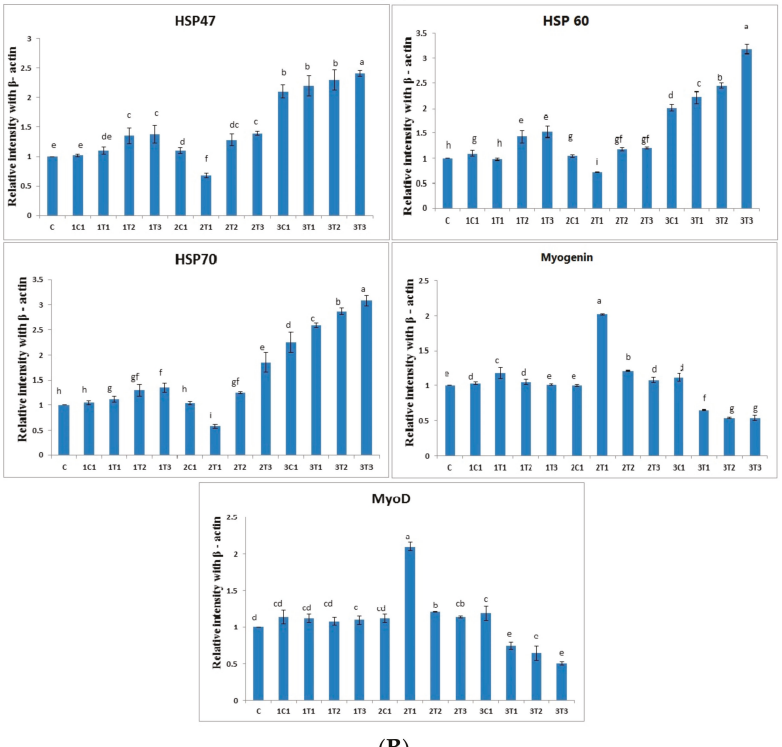
Figure 5. Photomicrographs of erythrocytes with normal nuclei in the peripheral blood cells of experimental groups. Micronuclei and nuclear abnormalities such as blebbed nuclei or lobed nuclei are indicated by an arrow ("→").

3.4. Protein Analysis by Western Blot

Western blot was performed in muscles to determine whether the different doses of L-Arg supplemented at various days of the incubation period may alter the protein levels of the HSP family such as HSP-47, HSP-60, and HSP-70. As shown in Figure 6, the protein expressions of HSP-47, HSP-60, and HSP-70 were significantly ($p < 0.01$) down-regulated in the 2T1 group compared to other groups. Moreover, their levels in the 3T2 and 3T3 groups were significantly (0.01) up-regulated compared to the 2T1 and 2T2 groups, although the protein expressions of HSP-60 and HSP-70 showed no significant difference ($p < 0.01$) among the 1T1, 1T2, 1T3, and 2T1 groups. Moreover, HSP-46, HSP-60, and HSP-70 were down-regulated in the 2T1 group compared to those in the other groups, whereas there was no significant difference in their levels between 1C and 2T1. HSP-70 is a reliable index of stress in chickens, while "3-hydroxyl-3-methyl-glutaryl coenzyme A reductase" has been used as an indicator of stress [46]. Pretreatment with L-Arg markedly reduced the dramatic down-regulation of HSP-60 and HSP-70 in hypoxic rat model. The increased expression of HSP-60 and HSP-70 might be related to their leakages from tissue, which can cause tissue injury due to free radical production [47,48]. Tissue injury might be caused by nitric oxide, a free radical, through the stimulation of endothelial cells and neutrophils that is generated from a higher dose of L-Arg [49]. Hence, the present results may suggest that the increased levels of HSP-47, HSP-60, and HSP70 in high doses of L-Arg may have a major role in tissue injury. The results of the study show that the increase of HSP-60 and 70 may be involved in tissue injury in the 3T1, 3T2, and 3T3 groups due to free radical production. The 2T1 group can prevent tissue injury via the down-regulation of HSP-46, HSP-60, and HSP-70. Moreover, the protein expressions of myogenin and MyoD were significantly up-regulated in the 2T1 group, whereas they were down-regulated in the 3T1, 3T2, and 3T3 groups compared to the other experimental groups.



(**A**)



(**B**)

Figure 6. (**A**) Effects of expression levels of L-Arg, heat shock protein (HSP)-47, HSP-60, and HSP-70 as well as myogenin and myoblast determination (MyoD) protein expressions in different stages of chicken embryos at different doses. Small characters indicate significant differences among experimental groups at $p < 0.01$. (**B**) The bar graph represents the quantitative expression of different proteins in all the groups. Data are expressed as the ratio of relative intensity with β-actin. Values are presented as mean ± SD from 12 determinations.

Oxidative stress can cause muscle atrophy by reducing myogenic differentiation markers such as myogenin and MyoD in skeletal muscles [50]. Some growth factors, namely cytokines and oncogenes, suppress the activity of myogenin and MyoD, thus resulting in decreased in the mass of muscle, which is defined as muscle atrophy [51]. A previous study reported that myogenic regulatory factors—mainly MyoD and MRF4—are only expressed later in different embryonic muscle groups as a result of increased muscle mass [52]. L-Arg increased the muscle cell as well as myogenin and MyoD under oxidative stress. Moreover, results from a previous experiment demonstrated that lower doses of

L-Arg could promote HSP70 expression in pig intestine [53]. Nevertheless, our present study has proved that increasing the concentration of L-Arg on the eighth and 18th day of the injection period could up-regulate the expression of HSP-60 and HSP-70; this effect might be through whey protein hydrolysate, which indicates the improper use of a functional food ingredient. Moreover, the L-Arg on the 14th day with (100 µg/100 µL/egg) promoted the muscle mass through the up-regulation of MyoD and myogenin due to their free radical scavenging activity.

3.5. Measurement of IgM Concentration in Serum

Concentrations of immune response markers such as IgM in all the experimental groups were analyzed. The duration and amount of L-Arg supplementation may influence immune status. Short-term supplementary L-Arg can influence the immunity power, because L-Arg has antioxidant and anti-inflammatory effects [54,55]. It can attenuate inflammatory reactions by suppressing the generation of inflammatory mediators such as inflammatory cytokines and C-reactive protein, which play major roles in the progression of tissue damage and organ dysfunction [56]. The treatment of L-Arg shows improved renal function through improved immune function [57]. Levels of IgM could provide an overall picture of immune function. It has been recently demonstrated that L-Arg can increase the specific immune response against infectious bursal disease (IBD) in chickens [58]. L-Arg provided by treatment has been reported to be the sole precursor of nitric oxide with lots of immune functions and growth performance [59]. These same biological attributes might be present after a low dose of L-Arg injection on the 14th day of the incubation period. It may improve immunity via the generation of IgM and the suppression of inflammatory cytokines and C-reactive protein (Figure 7).

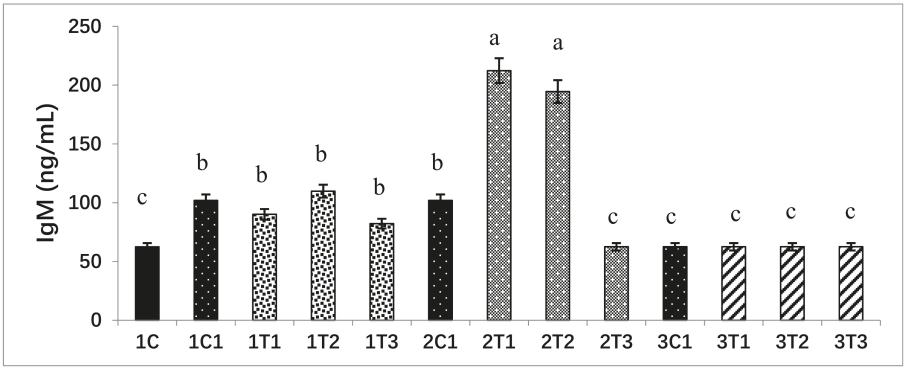


Figure 7. L-Arg induces immunoglobulin M (IgM) levels in different stages of chicken embryos at different doses. Small characters indicate significant differences among experimental groups at $p < 0.01$. Values are presented as mean ± SD from 12 determinations. Data were analyzed using Duncan test following ANOVAs with SAS® software, Version 9.4, (Institute of INC, North Carolina, USA).

3.6. Histopathology (H&E) Staining

Figure 8 shows the histology of liver of all the experimental groups. Sections from the control group exhibited a complete structure and regular shape of liver cells. Sections from the 1T1 and 2T1 groups showed normal hepatocyte gap compared to the 1C and 1C1 groups. Sections from the 1T3, 2T3, 3T1, 3T2, and 3T3 groups appeared with intracytoplasmic vacuoles in hepatocytes around the centrilobular regions. Moreover, hepatocyte tubes were surrounded by inflammatory cells and showed necrosis with nuclear fragmentation in the 3T2 and 3T3 groups. The hepatocyte gap was increased in the 2T3, 3T1, 3T2, and 3T3 groups. The hepatocyte gap appeared in normal architecture in the 1C, 1C1, 2C1, 3C1, 1T1, and 2T2 groups. The degeneration of livers was observed for birds when treated with 167 and 334 mg/L of L-Arg, which had an adverse effects on organs [60]. The liver after treatment with L-Arg (334 mg/L) had congested vascular spaces and periportal mononuclear inflammatory

infiltration [61]. The addition of L-Arg to poultry diets is required to avoid harmful influences of excessive free radicals produced during normal metabolism [62]. Dietary L-Arg supplementation plays a key role in enhancing meat quality. Increased L-Arg and betaine supplementation alleviates total body fat deposition and fatty liver [63,64]. Additionally, supplementation with high doses (50% and 100%) of L-Arg has negative effects on the structure of the liver of Sasso birds proved by H&E staining. However, our current results showed that in ovo injection with low doses (2T1) of L-Arg on the 14th day of egg embryo development did not have any negative effects compared to higher doses of L-Arg on the eighth or 18th day of the incubation period.

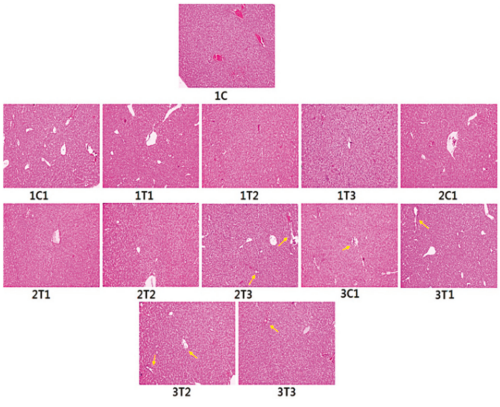


Figure 8. Histopathology of liver using hematoxylin and eosin straining. Sections from control chicks' hepatic lobule indicate complete structures. The liver cell has a regular shape that is within normal limits. Intracytoplasmic vacuoles are shown in hepatocytes around the centrilobular region in the 1T3, 2T3, 3T1, 3T2, and 3T3 groups. Hepatocyte marked inflammation around the periportal region, with microvesicular and macrovesicular fatty infiltration (yellow arrows) liver cells appearing near necrosis with nuclear fragmentation in the 3T2 and 3T3 groups. The hepatocyte gap was also increased in the 2T3 3T1, 3T2, and 3T3 groups. There was no difference between the control and the 2T1 group.

3.7. In Silico Molecular Docking Studies

The in silico molecular docking of L-Arg was studied. The entire glide, E model scores, and hydrogen bond interactions are presented in Figure 9. The main aim of the molecular modeling study is to understand the interactions of functional groups present in L-Arg with residues of targeted proteins theoretically. Heat shock protein 70 (HSP70) is one of the main nonglobin proteins, which has a similar structure in almost all living organisms. Between organisms as varied as yeast, chicken, Drosophila, and human, HSP70 is highly conserved. A previous study has described that HSPs are antigenically linked to the chicken HSPs by means of rabbit polyclonal antibodies [25]. In the same way, the similarity of HSPs extends to a DNA sequence. The chicken and human HSP70 genes are 64–72% similar (homologous) in the obtained amino acid sequences [65]. In addition, the HSP70 gene(s) of chicken, Drosophila, mouse, and human are very much conserved with the comparison of the DNA sequence [66,67]. Therefore, in the present study, solution structure for the human HSP70 substrate binding domain L542Y mutant (PDB id: 5XIR) was selected for the in silico molecular modeling study with L-arginine [68].

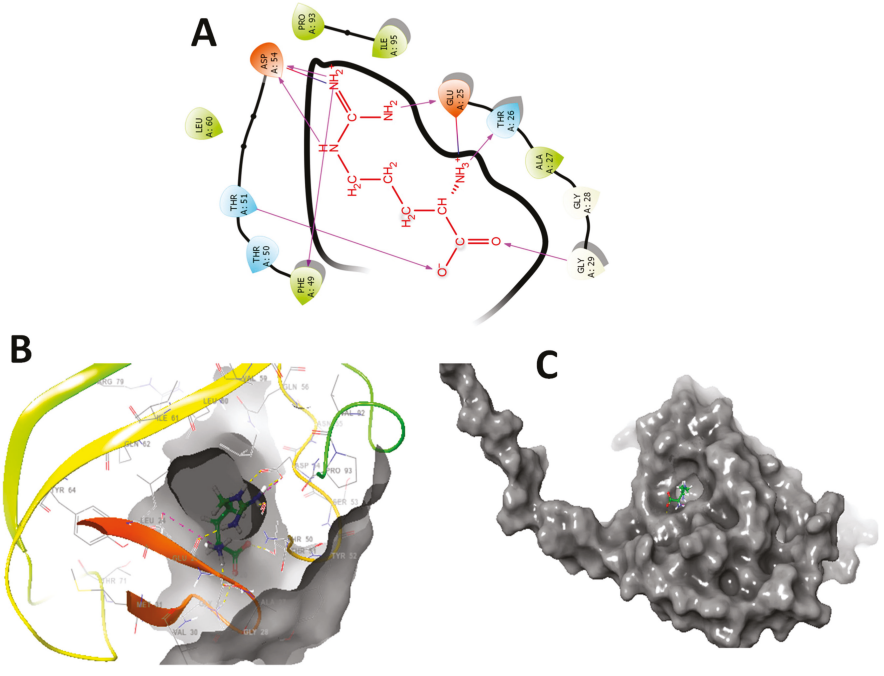


Figure 9. (**A**) 2D docking interaction of L-arginine with the active site of 5XIR. (**B**) 3D docking of L-arginine in the active site of 5XIR. (**C**) Docking packing representation of L-arginine with suitable binding pockets of 5XIR.

As presented in Figure 9, the main aim of the molecular modelling study is to understand interactions of functional groups present in L-Arg with residues of targeted proteins theoretically. In L-Arg, primary and secondary amines have strong hydrogen bonding interactions with protein residues. In brief, the molecular interaction of L-Arg with 5XIR protein and primary and secondary amines established a strong affinity with protein resides such as ASP A 328, ASP A 83, and HIS A 401. In addition, the carboxyl group present in the molecule also exhibits good hydrogen bonding interactions with LYS A 498. In a similar way, L-arginine with 5XIR protein showed good molecular interactions with ASP A 54, PHE A 49, GLU A 25, THR A 26 and GLY A 29 (Figure 9A).

The molecular modelling study revealed that L-Arg was bound onto similar active site cavity in the protein molecule. Superposition of interactions of an active site of L-Arg and amino acid residues of 5XIR protein is evidently portrayed in Figure 9B,C. The molecular docking of L-Arg with 5XIR protein is exposed to seven hydrogen bonding interactions, respectively with corresponding active site of the protein molecule. Besides, L-Arg showed strong hydrogen bonding interactions with good surface molecular interactions due to the presence of primary, secondary amines, and carboxylic acid groups. These relationships among 5XIR protein, and L-Arg might explain the experimental activity of them. Further research is going on in a due course to explore their possible modes of action. It is possible that L-Arg might block the activity of HSPs, and activation of Myogenin and Myo-D as well improved the immunoglobulin levels, thereby regulating the muscle growth (Figure 10).

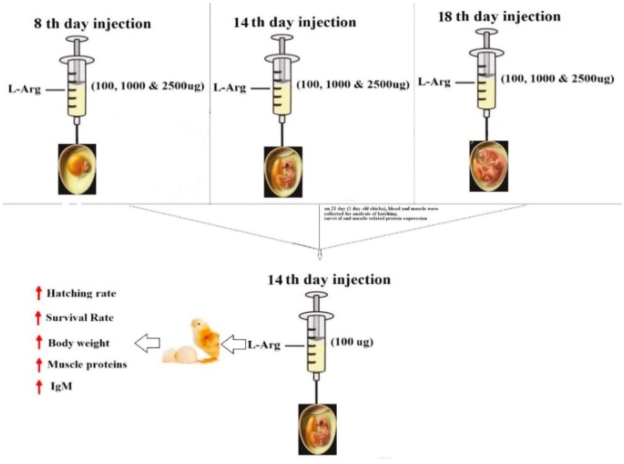


Figure 10. Possible mechanism of high doses and low doses of L-Arg on toxicity and muscle growth in chicken embryo.

4. Conclusions

In this study, we described a suitable embryonic developmental stage for the accessibility of in ovo injection using L-Arg at different concentrations for the first time. The injection of L-Arg on the 14th day at 100 μg/μL/egg enhanced both hatching and survival rates. It also increased body weight and immune response (IgM). In addition to oxidative stress, a sign of genotoxic effect was also observed in peripheral blood cells in which a presence of micronuclei and nuclear abnormalities such as blebbed nuclei, lobed nuclei, and notched nuclei were observed in the 2T3, 3T1, 3T2, and 3T3 groups. Histology from the control and 2T1 groups showed normal architecture, while injection on the 18th day of incubation and first day of chicks showed liver tissue damage. Overall results demonstrate that the optimum dose is 100 μg/μL/egg, and the optimum injection stage is on the 14th day to improve the immunity, hatching, and survival rate, which can be used for the poultry industry. In ovo injection in early and late embryonic stages could not offer good benefits for survival, hatching rate, or muscle development. If we choose the middle stage of embryonic development for in ovo injection, L-Arg might be able to promote muscle growth and improve the immune power without inducing adverse effects on the liver.

Author Contributions: Conceived and designed the experiments: S.A.S., K.S.S. Performed the experiments and measurements of serum biochemical parameters: S.A.S., D.R.K., J.R.P. and S.H.S. Protein analysis by western blot: S.A.S. Histopathology (H&E) staining: S.A.S. In silico molecular docking studies: P.R., D.J.Y and C.S.N.

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Effects of Dietary Supplementation of L-Carnitine and Excess Lysine-Methionine on Growth Performance, Carcass Characteristics, and Immunity Markers of Broiler Chicken

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Simple Summary: L-carnitine, lysine, and methionine are amino acids of important nutritional and nutraceutical interest and are used as dietary supplements to improve feed quality characteristics in broiler chicken. This study investigated the effect of different levels of L-carnitine and extra levels of lysine-methionine on growth performance, carcass characteristics, and some immune system markers. The findings of this study showed that the diet with around 30% of lysine-methionine content increased the back thoracic vertebrae and the proventriculus weights. A combination of lysine-methionine (level equal to NRC recommendations) with L-carnitine (15% and 75%) improved the immune response of broiler chickens against Newcastle and Gumboro diseases by stimulating the antibody production.

Abstract: L-carnitine as well as lysine and methionine are amino acids of important nutritional and nutraceutical interest and are used in nutritional strategies as dietary supplements to improve feed quality characteristics in animals and broiler chicken in particular. This study investigated the effect of different levels of L-carnitine and extra levels of lysine-methionine on growth performance, carcass characteristics, and some immune system markers. Two hundred seventy male Ross 308 broilers were a fed control diet (C) and eight different diets supplemented with an excess of amino acids. In the experimental diets, identified as D1, D2, D3, D4, D5, D6, D7, and D8, extra L-carnitine, lysine, and methionine were added in excess with respect to the American National Research Council (NRC) recommendations: L-carnitine equal to NRC (D1), control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine equal to NRC (D2), control diet supplemented with lysine equal to NRC, methionine equal to NRC, and L-carnitine at 15% in excess of NRC (D3), control diet supplemented control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 15% in excess of NRC (D4), control diet supplemented lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at

15% in excess of NRC (D5), control diet supplemented with lysine equal to NRC recommendations, methionine equal to NRC recommendations, and L-carnitine at 75% in excess of NRC (D6), control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 75% in excess of NRC (D7); and control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 75% in excess of NRC (D8). During the starter and growth phases, feed intake was not affected by dietary treatment ($p > 0.05$). By contrast, body weight and FCR were both affected ($p < 0.01$) during the starter period. During the finisher phase, feed consumption was affected ($p < 0.05$) by dietary treatment. Feed intake of broilers fed on C, D3, D6, and D7 were statistically similar ($p > 0.05$) (1851.90, 1862.00, 1945.10, and 1872.80 g/pen/day, respectively) and were higher ($p < 0.05$) than 1564.40 g/pen/day (D5). With the exception of drumsticks, neck, back thoracic vertebrae, and proventriculus weights, the economical carcass segments were not affected ($p > 0.05$) by the dietary supplementation of amino acids. Duodenum and ileum weights and lengths decreased with amino acid supplementation ($p < 0.05$). IgT and IgG titers against Sheep Red Blood Cells (SRBC) for both primary and secondary responses were not affected by dietary treatments ($p > 0.05$). Dietary amino acids supplementation did not affect IgM titer after the secondary challenge ($p > 0.05$) and had a significant effect ($p < 0.05$) on serum antibody titers in broilers vaccinated against Newcastle disease (NCD) and Gumboro 's disease at the 27th and 30th days, respectively.

Keywords: amino acids; dietary supplementation; broiler; growth performance; humoral immunity

1. Introduction

Nowadays, the growing demand for poultry meat has resulted in pressure on breeders to increase the growth rate of birds, the feed efficiency, the size of breast muscles, and the reduction in abdominal fatness [1]. Therefore, research is being oriented toward improving the techniques of poultry meat production. The improvement in carcass compositions with additives has become a focus on nutrition research. As an example, the addition of amino acids and metabolic intermediates to diets may lower the abdominal fat deposition in poultry. One example is L-carnitine, the biologically active form of carnitine, which is synthesized in the liver, kidney, and brain [2] from the essential amino acids' lysine and methionine, that can be considered as L-carnitine precursors [3,4]. L-carnitine (δ-trimethylamino-β-hydroxybutyrate) is a quaternary hydrosoluble amine with a small molecular weight that occurs naturally in microorganisms, plants, and animals [5]. Its concentration in animals varies according to species [6], tissue type [4,7], nutritional status of the animal [8,9], and the feed quality [10]. Dietary effects of L-carnitine, lysine, and methionine supplementations on the growth performance and body composition of broiler chickens are still poorly understood. Many studies have suggested that the dietary addition of lysine and methionine in excess with respect to the American National Research Council (NRC) [11] recommendations may result in enhanced performances, especially with regard to breast meat yield, body weight gain, and feed conversion ratio [12–21]. Corzo et al. [22] reported how a high dietary density of amino acids can lead to increased breast meat related to an increase in lean muscle tissue. Moreover, Mukhtar et al. [10] reported that a significant improvement in amino acids feed intake improves the average body weight gain and feed conversion ratio. On the other hand, Si et al. [15] concluded that the level of methionine should not be increased if lysine is in excess of its minimum needs.

It has been also reported that the dietary supplementation of lysine and methionine can improve the immunity of broiler chickens against different diseases [23–26]. Moreover, it has been reported that methionine constructively affects the immune system by improving both cellular and humoral responses [27–29]. The mechanisms proposed to explain methionine interference in the immune system is the T cells proliferation, which are sensitive to intracellular glutathione and cysteine levels, compounds that participate in the methionine metabolism [30].

L-carnitine supplementation is used to improve broiler productivity [31], and its bioavailability depends on the composition of the diet. Theoretically, supplementing broiler diets with adequate content of L-carnitine would facilitate the fatty acids β-oxidation and decrease the esterification reactions and triacylglycerols storage in the adipose tissue [3,32,33]. However, the impact of extra supplied L-carnitine may depend on the magnitude of its endogenic biosynthesis from lysine and methionine in the presence of Fe^{2+} and a number of vitamins (e.g., ascorbate, niacin, and pyridoxine), which are required as cofactors for the enzymes involved in the metabolic pathway of L-carnitine [4,34–36]. Some authors reported that abdominal fat deposition in broilers is reduced by L-carnitine supplementation without a significant effect on daily gain or feed conversion [37], while others observed no impact of dietary L-carnitine supplementation on abdominal fat composition [38]. Nonetheless, Bouyeh and Gevorgyan [39] and Celik et al. [40] showed that the growth performance of broilers was improved by L-carnitine supplementation. A study by Hosseintabar et al. [33] evaluated the effects of different levels of L-carnitine, lysine, and methionine on the blood concentrations of energy, protein, and lipid metabolites of male broiler chickens and concluded that, compared to a standard diet, the addition of 150 mg/kg of L-carnitine plus 15% lysine and methionine sustained a low plasmatic total cholesterol concentration compared to a standard diet. El-Wahab et al. [41] reported the broilers fed high levels of lysine and methionine with a surplus amount of L-carnitine (350 mg kg^{-1} to the diet) led to significantly lower cholesterol levels vs. a low L-carnitine intake. To the knowledge of the authors, the effect of simultaneous feed supplementation with L-carnitine and excess lysine-methionine on growth performance, carcass characteristics, and immunity markers of broiler chicken has not been studied before.

There is a need to standardize the dose of lysine-methionine and L-carnitine supplementation in the diet of broiler chickens not only to enhance their growth performance and carcass characteristics but also to improve their immune response. Hence, the main objective of this study has been to evaluate the effect of dietary supplementation of different levels of L-carnitine with or without an excess of lysine-methionine compared to dietary nutrient requirements on broiler chickens' growth performance, humoral immunity markers, and carcass characteristics during a 6-week rearing trial.

2. Materials and Methods

2.1. Animal Welfare and Ethics

All procedures related to animals' care and sampling were conducted under the approval of the Institution's Ethic Committee at the Department of Animal Science, Rasht Branch, Islamic Azad University, Rasht (Iran) (protocol N° 105/19) before the beginning of the experimental trial.

2.2. Experimental Diets Preparation

A control diet (C) for broiler chicken based on corn and soybean-meal was prepared according to the dietary nutrient requirements of broilers [1]. The ingredients and chemical composition of the control diet are given in Table 1. Thereafter, eight amino acids-supplemented diets (indicated as D1, D2, D3, D4, D5, D6, D7, and D8) were prepared by mixing the control diet thoroughly with the designated supplements at the required incorporation levels as shown in Table 2.

As stated above, the L-carnitine, lysine, and methionine levels in starter, grower, and finisher feeds for the control diet were determined according to the NRC [11] recommendations. In the experimental diets, D1, D2, D3, D4, D5, D6, D7, and D8, extra L-carnitine, lysine, and methionine (Carniking1, Lonza Ltd., Basel, Switzerland) were added in excess to the NRC [11] recommendations, as shown in Table 2, since NRC [11] recommended diets are suggested for feeding Ross 308 broiler chickens because of fewer phases of feeding periods and lower workloads [42].

Table 1. Ingredients and chemical compositions of the control diet.

Ingredient (%)	Starter Period (Day 1–22 of age)	Grower Period (Day 23–35)	Finisher Period (Day 36–42)
corn	59.0	62.0	65.0
soybean meal	35.0	30.0	27.0
soybean oil	2.0	3.5	3.5
Ca22% P18%	1.5	1.8	1.7
mineral oysters	1.2	1.5	1.5
NaCl	0.3	0.2	0.2
lysine-hydro-chloride	0.1	0.1	0.1
DL-methionine	0.2	0.2	0.2
mineral premix *	0.3	0.3	0.3
vitamin premix **	0.3	0.3	0.3
Sodium hydrogen carbonate ($NaHCO_3$)	0.1	0.1	0.1
Chemical Composition (%)			
metabolizable energy (kcal/kg)	2898	2955	3058
crude protein	21.7	20.8	18.2
crude fiber	2.3	2.2	2.1
calcium	0.80	0.80	0.79
available potassium	0.48	0.46	0.42
sodium	0.15	0.15	0.15
lysine	1.41	1.26	1.22
methionine	0.61	0.57	0.48
energy: protein ratio	133	142	167
Ca:P	1.7	1.7	1.9
linoleic acid	2.46	2.73	2.95
methionine + cysteine	0.97	0.91	0.77

* Calcium Pantothenate: 4 mg/g; Niacin: 15 mg/g; Vitamin B6: 13 mg/g; Cu: 3 mg/g; Zn: 15 mg/g; Mn: 20 mg/g; Fe: 10 mg/g; K: 0.3 mg/g; ** Vitamin A: 5000 IU/g; Vitamin D3: 500 IU/g; Vitamin E: 3 mg/g; Vitamin K3: 1.5 mg/g; Vitamin B2: 1 mg/g.

Table 2. Amount of L-carnitine, lysine and methionine in nutrient analysis of studied treatments.

Variation	Diets								
	C	D1	D2	D3	D4	D5	D6	D7	D8
L-carnitine *	NRC	NRC	NRC	NRC + 15%	NRC + 15%	NRC + 15%	NRC + 75%	NRC + 75%	NRC + 75%
Lysine **	NRC	NRC + 15%	NRC + 30%	NRC	NRC + 15%	NRC + 30%	NRC	NRC + 15%	NRC + 30%
Methionine ***	NRC	NRC + 15%	NRC + 30%	NRC	NRC + 15%	NRC + 30%	NRC	NRC + 15%	NRC + 30%

* L-carnitine (American National Research Council (NRC)): Starter period: 17.8 mg/kg; Grower period: 18.1 mg/kg; Finisher period: 22.9 mg/kg; ** lysine (NRC): Starter period: 1.41%; Grower period: 1.26%; Finisher period: 1.22%; *** methionine (NRC): Starter period: 0.61%; Grower period: 0.57%; Finisher period: 0.48%.

2.3. Animals and Experimental Design

Two hundred seventy 1-day-old, male Ross 308 broiler chicks obtained from a local commercial hatchery were used in this experiment. Chicks were randomly distributed into 27 pens (9 groups × 3 replications, each replication included 10 chicks). Each group was allocated to one of the nine dietary treatments indicated above. Birds were given starter feed from 1 to 21 days, a grower feed from 22 to 35 days, and a finisher feed from 36 to 42 days of age. Feed and water were provided ad libitum throughout the experimental assay. For the growth performance traits, the experimental unit was the pen. For the carcass and immunity traits, the experimental unit was the chicken.

2.4. Growth Performance Monitoring

Tens of birds per pen were weighed together on the 1st, 21st, and the 35th days of age to determine the live body weight and the weight gain. The feed consumption and feed conversion ratio (FCR) were also calculated for each growing phase as follows: from the 1st to the 21st day, from the 22nd day to

the 35th day, and from the 36th to the 42nd day of the 42 days experimental study as described by Bouyeh and Gevorgyan [39].

2.5. Carcass Characteristics Determination

As shown by Panda et al. [43–45], at the end of the experiment (day 42), three broilers per same treatment (e.g., one broiler per same diet per pens) (n = 3) were randomly selected, weighed without prior fasting, and scarified between 9:00 am and 10:00 am by cervical dislocation to evaluate the characteristics of the carcass. After skin removal and total evisceration, the feet were separated from the carcass in the tibio–tarsal joint. Economic carcass and gastrointestinal segments were removed, weighed, and the ratios of each segment to body weight were calculated.

2.6. Humoral Immune Response Measurements

Non-pathogenic antigens of Sheep Red Blood Cells (SRBC) were used to monitor the humoral immune response of broilers. The SRBC were purchased from a local Iranian supplier. A suspension was prepared by mixing 1 mL of phosphate-buffered saline (PBS) with 10 mL of SRBC. Six birds per same treatment (e.g., two broilers per same diet per pens) (n = 6) were subcutaneously injected in the breast with 0.5 mL of SRBC suspension on the 22nd and the 36th days of the experimental trial.

Then, seven days after each sensitization (28 and 42 days, respectively), antibody titers against SRBC were measured by a hemagglutination inhibition (HI) test according to Cunningham [46]. All antibody titers were recorded according to previous studies [47,48].

Birds were also vaccinated against infectious bronchitis (IB) on the 1st and 16th days of age, against Newcastle disease (NCD) on the 8th and 20th days of age, and against Gumboro's disease on the 14th and 23rd days of age. The humoral immune responses of chickens to the IB virus at the 23rd day of age, to the NCD virus at 27th day of age, and to the Gumboro virus at the 30th day of age were measured using the HI and ELISA methods as described by references [47,48]. Blood samples were collected from the brachial vein. Serum was separated by centrifugation (3000× g rpm for 15 min), and antibody titers against IB, NCD, and Gumboro virus were measured using commercially available ELISA kits (Bio-check BV, Gouda, Holland) according to the manufacturer's instructions. The absorbance of controls and samples were read at a wavelength of 405 nm using an ELISA reader (Bio-Tek Instruments Inc. ELX 800, Winooski, VT, USA).

2.7. Statistical Analysis

All data were subjected to an ANOVA statistical analysis with the General Linear Model (GLM) procedure of SAS [49]. The GLM was used according to the following model:

$$Y_{ijk} = \mu + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} = the j^{th} observation on the i^{th} treatment, μ = overall mean, α_j = the main effect of the L-carnitine level, β_K = the main effect of the methionine-lysine level, $\alpha\beta_{jk}$ = the effect of the interaction of L-carnitine and of methionine-lysine treatments, and ε_{ijk} = The random error.

3. Results

3.1. Growth Performance

Table 3 summarizes the results of the growth performance using the different diets. During the starter and growth phases, feed intake was not affected by dietary treatment ($p > 0.05$). By contrast, body weight and FCR were both affected ($p < 0.01$) during the starter period. In fact, D1, D2, D3, D6, D7, and D8 were associated with the highest live body weight ($p < 0.01$) with mean values of 706.31, 745.50, 671.10, 733.67, 741.00, and 723.27 g/pen/21day, and consequently, the feed conversion ratio was the lowest ($p < 0.01$) during this period compared to the Control Diet (C).

Table 3. Growth performance of Ross 308 broilers fed diets containing different levels of L-carnitine and lysine-methionine from day 1 to day 42 of age.

Parameters	Period	Diets									SEM	*p*
		C	D1	D2	D3	D4	D5	D6	D7	D8		
Feed intake, g/pen/period	1–21 d	997.30	1012.60	1009.90	988.67	977.43	983.57	1005.43	1012.30	1006.53	8.37	NS
	22–35 d	2815.30	2898.46	2892.30	2819.50	2810.20	2800.90	2814.60	2859.19	2793.13	50.49	NS
	35–42 d	1851.90 ^{a}	1702.40 ^{ab}	1781.90 ^{ab}	1862.00 ^{a}	1748.9 ^{ab}	1564.40 ^{b}	1945.10 ^{a}	1872.80 ^{a}	1757.70 ^{ab}	83.30	*
	1–42 d	5664.50 ^{ab}	5613.46 ^{ab}	5684.10 ^{ab}	5670.17 ^{ab}	5536.53 ^{ab}	5348.87 ^{b}	5765.13 ^{a}	5744.29 ^{a}	5557.36 ^{ab}	100.06	*
Body weight gain, g/pen/period	1–21 d	697.60 ^{ab}	706.31 ^{a}	745.50 ^{a}	671.10 ^{abc}	621.60 ^{bc}	610.17 ^{c}	733.67 ^{a}	741.00 ^{a}	723.27 ^{a}	18.57	**
	22–35 d	1207.10 ^{a}	1241.29 ^{a}	1165.50 ^{ab}	1066.67 ^{c}	1029.83 ^{c}	1042.00 ^{c}	1115.17 ^{bc}	1100.41 ^{bc}	1089.90 ^{bc}	29.31	**
	35–42 d	935.60 ^{b}	898.60 ^{b}	917.60 ^{b}	889.03 ^{b}	949.27 ^{b}	950.53 ^{b}	1037.36 ^{a}	1010.89 ^{a}	976.53 ^{b}	69.53	*
	1–42 d	2840.30 ^{a}	2846.20 ^{a}	2828.60 ^{a}	2626.80 ^{b}	2600.70 ^{b}	2602.70 ^{b}	2886.20 ^{a}	2852.30 ^{a}	2789.70 ^{a}	86.91	*
Feed Conversion ratio	1–21 d	1.43 ^{bc}	1.43 ^{bc}	1.35 ^{c}	1.47 ^{bc}	1.57 ^{ab}	1.61 ^{a}	1.37 ^{c}	1.36 ^{c}	1.39 ^{c}	0.03	**
	22–35 d	2.33 ^{c}	2.33 ^{c}	2.48 ^{bc}	2.64 ^{ab}	2.73 ^{a}	2.69 ^{a}	2.52 ^{ab}	2.60 ^{ab}	2.56 ^{ab}	0.06	**
	35–42 d	1.98	1.89	1.94	2.09	1.84	1.64	1.88	1.85	1.80	0.09	NS
	1–42 d	1.99 ^{ab}	1.97 ^{b}	2.01 ^{ab}	2.16 ^{a}	2.13 ^{a}	2.05 ^{a}	2.00 ^{ab}	2.01 ^{ab}	1.99 ^{ab}	0.04	*

C (Control) = diet with lysine, methionine, and L-carnitine equal to NRC recommendations; D1 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine equal to NRC; D2 = control diet supplemented with lysine at 30% in excess of NRC, at 30% in excess of NRC, and L-carnitine equal to NRC; D3 = control diet supplemented with lysine equal to NRC, methionine equal to NRC, and L-carnitine at 15% % in excess of NRC; D4 = control diet supplemented control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 15%in excess of NRC; D5 = control diet supplemented lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 15% in excess of NRC; D6 = control diet supplemented with lysine equal to NRC recommendations, methionine equal to NRC recommendations, and L-carnitine at 75% in excess of NRC; D7 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 75% in excess of NRC; D8 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 75% in excess of NRC; SEM = standard error of the mean; * $p < 0.05$, ** $p < 0.01$, NS = $p \geq 0.05$; $^{a, b}$: Means within the same row with common superscript letters are not significantly different ($p \geq 0.05$).

However, during the grower period, the live bodyweight of the broiler fed on D1 and D2 was not different from that of C (1241.29 g/pen/period and 1165.50 g/pen/period vs. 1207.10 g/pen/period) and was higher than those of D3, D4, D5, D6, D7, and D8 ($p < 0.01$). D1 and D2 were associated with the lowest feed conversion ratio ($p < 0.01$) with mean values of 2.33 and 2.48, respectively. During the finisher phase, feed consumption was affected ($p < 0.05$) by dietary treatment. Feed intake of broilers fed on C, D3, D6, and D7 were statistically similar ($p > 0.05$) (1851.90, 1862.00, 1945.10, and 1872.80 g/pen/d, respectively) and were higher ($p < 0.05$) than 1564.40 g/pen/day (D5).

However, live body weights were similar between dietary treatments ($p > 0.05$) with mean values of 976.53 g/pen/period and 949.27 g/pen/period, respectively. The FCR of this period was not affected ($p > 0.05$) by dietary treatment.

3.2. Carcass Characteristics

The results obtained for carcass parameters (economical carcass segments, body organ segments, and gut organs) are shown in Table 4.

Our results indicate that, with the exception of thigh weights, economical carcass segments were not affected by dietary treatment ($p > 0.05$). The neck weights of the bird given D1 and D8 were the highest ($p > 0.05$) with mean values of 64.50 g and 64.63 g, respectively, compared to 79.57 for the control diet. D2 was associated with the highest back thoracic vertebrae weight ($p > 0.05$) at 103.99 g versus 45.11 g for birds given the diet D8. However, all other body segments (heart, liver, gizzard, and abdominal fat) weights were not affected by dietary treatment ($p > 0.05$). With the exception of proventriculus weight, gut organ weights were not affected ($p < 0.05$) by dietary treatment. Our data showed that the dietary supplementation of lysine and methionine at 30% with a level of L-carnitine equal to NRC and of 15% of lysine, methionine, and carnitine increased ($p < 0.05$) proventriculus weight from 10.77 g to 11.26 and 11.43 g, respectively.

Table 4. Economical carcass segment means of Ross 308 broilers fed diets containing different levels of L-carnitine, lysine, and methionine from day 1 to day 42 of age.

Traits		Diets									SEM	*p*
		C	D1	D2	D3	D4	D5	D6	D7	D8		
Economical carcass segments	Live body weight, g	2873.70	2746.00	2826.00	2894.70	2883.30	2530.70	2600.40	2638.70	2523.30	172.46	NS
	Full abdomen carcass weight, g	2737.70	2619.00	2682.70	2738.30	2657.70	2356.00	2466.70	2487.30	2374.00	168.39	NS
	Breast weight, g	789.51	758.60	679.33	733.33	719.33	643.33	657.33	625.33	619.33	61.26	NS
	Drumsticks (thighs) weight, g	651.00 ^{a}	583.00 ^{ab}	520.67 ^{b}	560.67 ^{ab}	565.33 ^{ab}	496.67 ^{b}	514.670 ^{b}	502.00 ^{b}	508.00 ^{b}	37.72	*
Body organ segments	Neck weight, g	79.57 ^{a}	64.50 ^{ab}	61.46 ^{b}	58.66 ^{b}	59.37 ^{b}	51.88 ^{b}	59.97 ^{b}	60.20 ^{b}	64.63 ^{ab}	5.22	*
	Back thoracic vertebrae (notarium) weight, g	45.45 ^{b}	64.28 ^{ab}	103.99 ^{a}	82.34 ^{ab}	82.93 ^{ab}	85.18 ^{ab}	76.65 ^{ab}	78.41 ^{ab}	45.11 ^{b}	12.92	*
	Heart weight, g	17.19	16.52	17.24	16.39	18.23	15.17	16.21	15.66	15.40	1.51	NS
	Liver weight, g	70.13	64.20	72.31	72.40	69.66	58.38	61.27	65.60	59.97	4.82	NS
	Gizzard (ventriculus) weight, g	65.37	50.43	62.47	65.78	62.00	53.19	52.23	58.96	55.05	5.52	NS
	Abdominal fat weight, g	43.95	37.96	41.07	25.57	38.33	31.75	32.47	41.16	24.43	6.86	NS
Gut organs	Pancreas weight, g	6.07	6.77	6.62	6.84	7.01	7.37	6.65	6.58	5.71	0.61	NS
	Crop weight, g	8.63	7.38	7.47	10.10	7.58	7.35	7.23	7.90	6.98	0.99	NS
	Proventriculus weight, g	10.77 ^{ab}	8.59 ^{b}	11.26 ^{a}	9.15 ^{ab}	11.43 ^{a}	10.37 ^{ab}	10.91 ^{ab}	10.60 ^{ab}	9.13 ^{ab}	0.72	NS

C (Control) = diet with lysine, methionine, and L-carnitine equal to NRC recommendations; D1 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine equal to NRC; D2 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine equal to NRC; D3 = control diet supplemented with lysine equal to NRC, methionine equal to NRC, and L-carnitine at 15% in excess of NRC; D4 = control diet supplemented control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 15% in excess of NRC; D5 = control diet supplemented lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 15% in excess of NRC; D6 = control diet supplemented with lysine equal to NRC recommendations, methionine equal to NRC recommendations, and L-carnitine at 75% in excess of NRC; D7 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 75% in excess of NRC; D8 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 75% in excess of NRC; SEM = standard error of the mean; * $p < 0.05$; NS = $p \geq 0.05$; ^{a,b}: Means within the same row with common superscript letters are not significantly different ($p \geq 0.05$).

3.3. Intestine Segments

The effects of dietary treatment on the length, weight, width, and diameter of small intestine segments (duodenum, jejunum, and ileum) in broilers are shown in Table 5.

The dietary supplementation of amino acid decreased ($p < 0.05$) duodenum weight and length. Birds given a supplemented diet with 30% of lysine and methionine in excess of NRC and 75% of L-carnitine in excess of NRC had the lowest weight, with a mean value of 12.99 g. The lowest duodenum length was recorded when birds were given 15% of lysine and methionine in excess of NRC plus 75% of L-carnitine in excess of NRC. A combination of L-carnitine (75%) and lysine-methionine (30%) increased the duodenum width significantly ($p < 0.05$) from 7.50 mm to 9.38 mm. Concerning the duodenum diameter, the dietary addition of lysine-methionine at a level equal to NRC plus 15% of L-carnitine in excess of NRC (D3), of lysine-methionine and L-carnitine at a rate of 15% in excess of NRC (D4), and of lysine-methionine and L-carnitine at a rate of 30% in excess of NRC plus 15% of L-carnitine in excess of NRC (D5) increased the diameter from 0.94 mm (C) to, respectively, 1.61 mm, 1.67 mm, and 1.68 mm.

Dietary treatments did not affect ($p > 0.05$) the jejunum weight and length. The dietary addition of lysine-methionine at a level equal to NRC plus 15% of L-carnitine in excess of NRC (D3) and of lysine-methionine and L-carnitine at a rate of 15% in excess of NRC (D4) increased the jejunum width ($p < 0.05$) from 9.26 mm (C) to 9.50 mm (D3) and 9.80 mm (D4). The dietary supplementation of amino acids significantly decreased ($p < 0.05$) the ileum weight from 9.10 g (C) to 2.67 g (D4) and 2.97 g (D7) and the length from 18.16 mm to 9.26 mm (D8) and 8.00 mm (D7). The ileum width of the broiler group fed with lysine-methionine at a level of 15% plus L-carnitine at 75% in excess of NRC was the highest ($p < 0.05$) compared to the control diet 7.33 mm versus 7.97 mm (D7). Dietary treatment did not affect ($p > 0.05$) ileum diameter.

Caecum weights were not different between treatments and ranged from 12.62 g (D5) to 17.13 g (C) ($p > 0.05$). The colon weight of broiler bird fed with lysine-methionine at a level equal to NRC plus 15% of L-carnitine in excess of NRC (D4) was the highest ($p < 0.05$), with a mean value of 2.36 g. Rectum weights were similar between all the treatments groups and ranged from 1.78 g (D6) to 2.32 g (D7) ($p > 0.05$).

Table 5. Ross 308 broilers' intestine segment dimensions according to feed diets from 1–6 weeks of age.

Traits		Diets									SEM	*p*
		C	D1	D2	D3	D4	D5	D6	D7	D8		
Duodenum	Weight, g	20.27 ^{a}	15.53 ^{ab}	14.90 ^{ab}	16.37 ^{ab}	15.64 ^{ab}	14.61 ^{ab}	13.56 ^{b}	14.02 ^{b}	12.99 ^{b}	1.77	*
	Length, mm	34.33 ^{a}	33.00 ^{ab}	29.60 ^{abc}	30.30 ^{abc}	26.60 ^{bcd}	27.60 ^{abcd}	24.00 ^{cd}	22.00 ^{d}	25.00 ^{cd}	2.27	*
	Width, mm	7.50 ^{b}	7.97 ^{ab}	7.81 ^{b}	8.29 ^{ab}	7.64 ^{b}	6.84 ^{b}	8.22 ^{ab}	8.13 ^{ab}	9.38 ^{a}	0.57	*
	Diameter, mm	0.94 ^{b}	1.16 ^{b}	1.17 ^{b}	1.61 ^{a}	1.67 ^{a}	1.68 ^{a}	0.89 ^{b}	1.05 ^{b}	1.21 ^{b}	0.12	*
Jejunum	Weight, g	60.09	51.16	51.07	57.91	52.52	47.75	46.30	60.88	46.84	0.70	NS
	Length, mm	120.00	113.10	112.30	110.00	111.66	108.33	109.00	113.66	101.00	5.71	NS
	Width, mm	9.26 ^{abc}	8.44 ^{bc}	8.23 ^{a}	9.50 ^{ab}	9.80 ^{a}	9.34 ^{abc}	8.26 ^{bc}	8.02 ^{c}	9.30 ^{abc}	0.40	*
	Diameter, mm	1.26 ^{a}	1.40 ^{abc}	1.01 ^{c}	1.25 ^{bc}	1.34 ^{bc}	1.59 ^{ab}	1.09 ^{bc}	1.20 ^{bc}	1.80 ^{a}	0.15	*
Ileum	Weight, g	9.10 ^{a}	5.23 ^{bc}	5.06 ^{bc}	6.39 ^{b}	2.67 ^{d}	3.95 ^{dc}	3.39 ^{dc}	2.97 ^{dc}	3.09 ^{dc}	4.82	**
	Length, mm	18.16 ^{a}	16.80 ^{ab}	11.16 ^{cd}	15.23 ^{abc}	11.66 ^{cd}	12.66 ^{bc}	13.00 ^{bcd}	8.00 ^{d}	9.26 ^{d}	1.51	**
	Width, mm	7.33 ^{ab}	5.97 ^{ab}	7.26 ^{ab}	5.39 ^{b}	5.27 ^{b}	5.92 ^{ab}	6.45 ^{ab}	7.97 ^{a}	6.60 ^{ab}	0.61	*
	Diameter, mm	1.03	1.14	1.05	1.43	1.48	1.54	1.35	1.08	1.32	0.16	NS
Rectum weight, g		2.14	1.80	2.29	2.23	2.11	2.30	1.78	2.32	1.82	0.30	NS
Cecum weight, g		17.13	14.22	14.12	12.74	14.54	12.62	13.74	15.97	14.56	1.98	NS
Colon weight, g		2.10 ^{ab}	1.28 ^{b}	1.73 ^{ab}	1.52 ^{ab}	2.36 ^{a}	1.87 ^{ab}	1.98 ^{ab}	1.62 ^{ab}	2.06 ^{ab}	0.26	*

C (Control) = diet with lysine, methionine, and L-carnitine equal to NRC recommendations; D1 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine equal to NRC; D2 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine equal to NRC; D3 = control diet supplemented with lysine equal to NRC, methionine equal to NRC, and L-carnitine at 15% in excess of NRC; D4 = control diet supplemented control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 15% in excess of NRC; D5 = control diet supplemented lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 15% in excess of NRC; D6 = control diet supplemented with lysine equal to NRC recommendations, methionine equal to NRC recommendations, and L-carnitine at 75% in excess of NRC; D7 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 75% in excess of NRC; D8 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 75% in excess of NRC, SEM = standard error of the mean; ** $p < 0.01$, * $p < 0.05$; NS = $p \geq 0.05$; $^{a, b, c, d}$: Means within the same row with common superscript letters are not significantly different ($p \geq 0.05$).

3.4. Humoral Immune Response

3.4.1. Humoral Immune Response against Sheep Red Blood Cell (SRBC)

The dietary effect of amino acids supplementation on primary and secondary antibody responses are shown in Table 6. IgT and IgG titers against SRBC for both primary and secondary responses were not affected by dietary treatments ($p > 0.05$). Birds receiving diets supplemented with excesses of amino acids (D1, D2, D3, D4, D5, D6, D7, and D8) had significantly lower titers of IgM than that of those receiving C for primary response ($p < 0.05$), with mean values of 2.33 $\log_{10}$ versus 2.00 $\log_{10}$ (D1), 1.66 $\log_{10}$ (D2), 1.33 $\log_{10}$ (D3), 1.33 $\log_{10}$ (D4), 1.00 $\log_{10}$ (D5), 1.33 $\log_{10}$ (D6), 1.33 $\log_{10}$ (D7), and 2.00 $\log_{10}$ (D8). Dietary treatment did not affect ($p > 0.05$) IgM titer after a secondary challenge.

Table 6. Anti- Sheep Red Blood Cells (SRBC) antibody responses ($\log_{10}$) of broilers fed different levels of L-carnitine and lysine-methionine.

Dietary Treatment	Primary Response			Secondary Response		
	Total Antibody	IgM	IgG	Total Antibody	IgM	IgG
C	4.66	2.33 ^{a}	2.33	6.66	3.66	3.00
D1	4.00	2.00 ^{ab}	2.00	8.66	3.00	5.66
D2	3.33	1.66 ^{ab}	1.66	5.66	2.33	3.33
D3	2.66	1.33 ^{ab}	1.33	5.33	2.66	2.66
D4	3.33	1.33 ^{ab}	2.00	7.00	3.00	4.00
D5	2.33	1.00 ^{b}	1.33	6.33	2.66	3.66
D6	2.66	1.33 ^{ab}	1.33	6.33	3.00	3.33
D7	2.66	1.33 ^{ab}	1.33	7.00	3.00	4.00
D8	3.33	2.00 ^{ab}	1.33	8.00	3.00	5.00
SEM	0.80	0.38	0.58	1.03	0.52	0.95
p	NS	*	NS	NS	NS	NS

C (Control) = diet with lysine, methionine, and L-carnitine equal to NRC recommendations; D1 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine equal to NRC; D2 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine equal to NRC; D3 = control diet supplemented with lysine equal to NRC, methionine equal to NRC, and L-carnitine at 15% in excess of NRC; D4 = control diet supplemented control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 15% in excess of NRC; D5 = control diet supplemented lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 15% in excess of NRC; D6 = control diet supplemented with lysine equal to NRC recommendations, methionine equal to NRC recommendations, and L-carnitine at 75% in excess of NRC; D7 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 75% in excess of NRC; D8 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 75% in excess of NRC, SEM = standard error of the mean; * $p < 0.05$, NS = $p \geq 0.05$; $^{a, b}$: Means within the same column with common superscript letters are not significantly different ($p \geq 0.05$).

3.4.2. Humoral Immune Response against Bronchitis, Newcastle and Gumboro Diseases

The influence of different amino acids dietary supplementations on serum antibody titer in chickens vaccinated against IB, NCD, and Gumboro virus are shown in Table 7. Serum antibody titers in broilers vaccinated against bronchitis were not affected ($p > 0.05$) by dietary treatment on day 23. However, a significant increase ($p < 0.05$) in serum antibody titers in broilers vaccinated against NCD and Gumboro on the 27th and the 30th days was observed from, respectively, 3.66 $\log_{10}$ (C) to 6.00 $\log_{10}$ (D3) and from 3.38 $\log_{10}$ (C) to 3.59 $\log_{10}$ (D6). Chickens fed with 30% of lysine-methionine in excess of the NRC and of L-carnitine equal to the NRC supplemented diet had the heaviest thymus ($p < 0.01$) with a mean value of 24.60 g versus 15.59 for the control diet. However, chickens fed on L-carnitine (15%) and lysine-methionine (15%) in excess of the NRC had the lightest thymus with a mean value of 7.17 g. Bursa of fabricius and spleen weights were not affected ($p > 0.05$) by dietary L-carnitine and lysine-methionine supplementation.

Table 7. Bronchitis, Newcastle, and Gumboro hemagglutination-inhibition (log_{10}) titers of broilers fed on different levels of L-carnitine and lysine-methionine.

Traits	Diets									SEM	*p*
	C	D1	D2	D3	D4	D5	D6	D7	D8		
Antibody titer against injection of bronchitis at 23rd day of age	3.29	2.91	3.02	3.29	3.09	3.49	3.50	3.34	2.89	0.81	NS
Antibody titer against injection of Newcastle at 27th day of age (lg2)	3.66 ^{b}	5.00 ^{ab}	4.33 ^{ab}	6.00 ^{a}	4.66 ^{ab}	5.66 ^{ab}	4.66 ^{ab}	4.00 ^{ab}	4.66 ^{ab}	0.60	*
Antibody titer against injection of Gumboro at 30th day of age	3.38 ^{bc}	3.16 ^{c}	3.57 ^{ab}	3.51 ^{ab}	3.44 ^{abc}	3.54 ^{ab}	3.59 ^{a}	3.46 ^{ab}	3.44 ^{abc}	0.41	*
Bursa of fabricius weight, g	4.57	4.21	3.67	2.99	2.45	2.56	2.45	2.10	3.26	0.77	NS
Thymus weight, g	15.59 ^{bc}	19.00 ^{ab}	24.60 ^{a}	15.39 ^{bc}	7.17 ^{d}	11.50 ^{cd}	19.04 ^{ab}	15.50 ^{bc}	13.98 ^{bc}	1.89	**
Spleen weight, g	3.61	3.04	2.94	2.86	2.47	2.61	2.92	2.90	3.00	0.40	NS

C (Control diet) with lysine, methionine, and L-carnitine equal to NRC recommendations; D1 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine equal to NRC; D2 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine equal to NRC; D3 = control diet supplemented with lysine equal to NRC, methionine equal to NRC, and L-carnitine at 15% in excess of NRC; D4 = control diet supplemented control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 15% in excess of NRC; D5 = control diet supplemented lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 15% in excess of NRC; D6 = control diet supplemented with lysine equal to NRC recommendations, methionine equal to NRC recommendations, and L-carnitine at 75% in excess of NRC; D7 = control diet supplemented with lysine at 15% in excess of NRC, methionine at 15% in excess of NRC, and L-carnitine at 75% in excess of NRC; D8 = control diet supplemented with lysine at 30% in excess of NRC, methionine at 30% in excess of NRC, and L-carnitine at 75% in excess of NRC; SEM = standard error of the mean; ** $p < 0.01$, * $p < 0.05$, NS = $p \geq 0.05$; $^{a, b, c, d}$: Means within the same row with common superscript letters are not significantly different ($p \geq 0.05$).

4. Discussion

It has been observed that L-carnitine supplementation can enhance broiler productivity [26]. However, the effect of administrating an excess of L-carnitine may depend on the magnitude of its endogenic biosynthesis from lysine and methionine [20,29–31]. In the current study, we are reporting the effect of dietary supplementation of different levels of L-carnitine with or without an excess of lysine-methionine on growth performance, carcass traits, and some humoral immunity markers of broiler chicken.

4.1. Growth Performance

The results relative to the growth performance show that a combination of lysine and methionine at 15% and 30% in excess of the NRC recommendations was without impact on the feed intake, live body weight gain, and FCR of broiler chicken during the whole experiment period.

Live body weights at the end of the experimental trial varied from 2600.70 g to 2886.20 g. These values are consistent with those reported by Bouyeh and Gevorgyan [39], who showed that dietary supplementations of lysine-methionine at 0, 1.1, 1.2, 1.3, or 1.4% higher than NRC recommendations [11] led to body weight gains from mean values of 2960 g to 2920 g (1.1%), 2850 g (1.2%), 2970 g (1.3%), and 2730 g (1.4%), respectively.

On the other hand, Hickling et al. [12] showed that broilers fed with diets supplemented with methionine at a level as suggested by NRC and at a level 112% in excess with respect to the NRC recommendations weighed at six weeks 2221 g and 2248 g, respectively, and had feed conversion efficiencies of 1.81 (NRC) and 1.79 (112% of methionine in excess of the NRC), respectively. Birds fed with four levels of lysine—equal to the NRC recommendations and 106%, 112%, and 118% in excess of the NRC recommendations—weighed 2221 g, 2227 g, 2234 g, and 2238 g, respectively, at six weeks of age and had feed conversion efficiencies of 1.81 (NRC), 1.81 (106% of lysine in excess of the NRC), 1.80 (112% of lysine in excess of the NRC), and 1.79 (118% of lysine in excess of the NRC). Mukhtar et al. [50] found that dietary increasing of lysine from 53% to 78% and of methionine from 36% (control diet) to 61% had a significant effect on feed intake, body weight gain, and feed conversion. Mukhtar et al. [50] also reported a significant improvement of feed intake, average body weight gain, and feed conversion ratio when broiler chicken were fed with five diet: diet A (1.2% lysine + 0.49% methionine) without a broiler supper concentrate, used as control, diet B similar to diet A but with a broiler supper concentrate, diet C (1.3% lysine + 0.56% methionine), diet (D) (1.4% lysine + 0.6% methionine), and diet (E) (1.5% lysine + 0.63% methionine). Body weight gain increased from 1080.76 g (A) to 1806.75 g (B), 1828.31g (C), 1834.93 g (D), and 1940.0 g (E). Feed conversion ratio decreased from 2.32 (A) to 1.97 (B), 1.95 (C), 1.94 (D), and 1.93 (E). More recently, Bouyeh and Gevorgyan [51] reported that the dietary incorporation of lysine and methionine at 0, 10%, 20%, 30%, and 40% in excess of the NRC recommendations [11] did not affect the body weight gain at 42 days of age but that the feed conversion ratio was affected by dietary treatment at 21 days of age.

With regard to the L-carnitine dietary supplementation, our results show that dietary inclusion of L-carnitine at 75% or 15% in excess of NRC recommendations increased the live body weight, feed intake, and FCR of 308 Ross broilers. In this respect, our data are in line with those obtained by Celik et al. [40]. These authors reported that the growth performance of broilers was improved by L-carnitine supplementation at a level of 50 mg/L in drinking water.

As far as L-carnitine was concerned, the results reported here are consistent with those reported by Rodehutscord et al. [52] and by Farrokhyan et al. [32], although in both studies, carnitine supply was not supplied alone but in combination with other nutrients or additives. Indeed, Rodehutscord et al. [52] studied the effect of adding 80 mg of L-carnitine per kg of the diet with two dietary levels of fat (namely 4 and 8%) on growth performance of broiler chickens. At the end of the trial, on day 21, the live body weight averaged 853 g and feed conversion was improved by almost 5% in chicken groups receiving L-carnitine supplemented diets. Farrokhyan et al. [32] also examined the effect of dietary combinations of 0, 150, and 300 mg/kg of L-carnitine with or without 1 g/kg or 2 g/kg of gemfibrozil, on the growth

performance of broilers. It has been observed that, as dietary L-carnitine increased, weight gain and birds' feed intake increased and FCR decreased. However, our results are not in agreement with those of other studies. Barker and Shell [53] showed that the dietary addition of L-carnitine at 0, 50 or 100 mg/kg diet did not affect the weight gain or feed efficiency of broiler chicken. Lien and Horng [54] demonstrated that diets supplemented with 160 mg L-carnitine/kg for 6 weeks did not affect broilers' feed intake, body weight gain, and feed conversion ratio. Corduk et al. [55] also reported that the dietary addition of L-carnitine at 100 mg/kg did not influence body weight gain, feed intake, and feed conversion ratio of broiler chickens.

Our data show that the dietary combination of L-carnitine, lysine, and methionine had a significant effect on growth performance. In fact, L-carnitine is an amine compound biosynthesized primarily in the liver from the amino acids lysine and methionine. It is involved in energy metabolism, where it is required for the transport of long-chain fatty acids into the mitochondrial matrix for β-oxidation by the fatty acid oxidation complex [3]. One the other hand, Murray et al. [56] found that the addition of synthetic amino acids like lysine and methionine at high levels to the diet can stimulate insulin secretion from pancreas by aggregating in plasma which, in turn, releases amino acids and fatty acids [57] from the bodily saved sources and leads to protein synthesis.

Adding 75% of L-carnitine plus 15% or 30% of lysine-methionine or 15% of L-carnitine plus 15% of lysine-methionine had a significant effect on feed intake and live body weight at the end of the experimental trial. Furthermore, adding 15% of L-carnitine plus 30% of lysine-methionine had no benefits, and actually reduced feed intake and body weight and increased FCR significantly compared to the control.

To our knowledge, no information related to the effect of supplementing L-carnitine in combination with lysine-methionine on broiler growth performance is reported in the literature until now.

4.2. Carcass Characteristics

With the exception of drumsticks, neck, back thoracic vertebrae, and proventriculus weights, all other economical carcass segments weights were not affected by the dietary supplementation of L-carnitine and lysine-methionine. With respect to lysine-methionine supplementation, Mukhtar et al. [50] studied the effect of lysine and methionine on broilers' carcass characteristics and reported that the dietary inclusion of A (1.2% lysine + 0.49% methionine) without broiler supper concentrate, used as control, B similar to diet A but with a broiler supper concentrate, C (1.3% lysine + 0.56% methionine), D (1.4% lysine + 0.6% methionine), and E (1.5% lysine + 0.63% methionine) increased the eviscerated carcass weight from 1036.46 (A) to 1761.75 (B), 1783.21(C), 1790.93 (D), and 1894.6 (E) and the yield of commercial cuts (breast and drumstick). The percentage of meat in the drumsticks increased from 72.17% (A) to 76.54% (B), 81.76% (C), 78.67% (D), and 82.35% (E). Concerning the percentage of meat of the breast, it increased from 77.01% (A) to 80.33% (B), 85.84% (C), 86.55% (D), and 86.89% (E). However, Bouyeh and Gevorgyan [39] found that the dietary supplementation of lysine-methionine at levels of 0, 10, 20, 30, or 40% more than the NRC [11] recommendation did not affect the thigh and leg percentage to carcass weight. However, it had a significant effect on breast meat yield, carcass traits, and abdominal fat pad, and liver and heart weights. Concerning the effect of L-carnitine supplementation on carcass traits, previous studies have shown that the dietary inclusion of L-carnitine did not affect abdominal fat, heart, and liver weights [36,53,54,58]. On the other hand, Farrokhyan et al. [32] found that the dietary supplementation of L-carnitine (300 mg/kg) reduced abdominal empty carcass from 1826.6 g/chiks to 1793.3 g/chiks and breast weight from 1566.6 g/chiks to 1563.3 g/chiks. This dietary supplementation did not affect wing weight. The limited effect of L-carnitine observed in the present study could be attributed to a limited intestinal absorptive capacity of L-carnitine. Another possible explanation is that L-carnitine is easily degraded by intestinal microflora as suggested by Xu et al. [37].

4.3. Intestine Segments

In the present study, the dietary supplementation of lysine-methionine and L-carnitine had a significant effect on all intestine segments with the exception of jejunum weight and length, ileum diameter, and caecum and rectum weight. As far as we know, the effect of simultaneous dietary supplementation with lysine-methionine and L-carnitine on intestine segments is not documented. Some studies underline the relationship to villi surface area to better feed utilization, higher nutrient absorption, body weight gain, and growth performance [59,60]. However, Saki et al. [61] reported that the dietary addition of 0.36% of methionine in broilers feed did not affect intestinal villi characteristics on the 21st and 42nd days of age.

4.4. Humoral Immune Response

IgM primary response against the SRBC of birds fed with supplemented diets with L-carnitine and lysine-methionine was significantly lower than that of birds receiving the unsupplemented diet. No significant differences have been observed among dietary treatments for IgT and IgG titers against SRBC during both primary and secondary antibody responses. Latshaw [62] reported that antibodies are proteins. Therefore, any deficiency of essential amino acids, particularly during the growth of chickens, results in poor immune competence. Lysine is one of the amino acids that can influence the magnitude of antibody response [63,64]. This could be the reason that the lowest immune response was observed in the control diet where lysine was not supplemented. Therefore, a 15% of lysine broiler chicken diet was sufficient to stimulate optimum antibody production, and thus, a lower immune response was observed when lysine was added at 30% and 75% in excess of the NRC.

Reports on the effect of methionine supplementation on broiler chickens' humoral immune response are lacking. However, as far as L-carnitine was concerned, Deng et al. [65] found that the dietary addition of 0 (control), 100 mg/kg, or 1000 mg/kg of L-carnitine did not affect primary antibody responses to SRBC at the 4th week but that birds fed on a diet with 1000 mg of L-carnitine had a higher primary antibody response against SRBC than broilers in other groups at the 12th week. Moghaddam and Emadi [66] reported that there was a tendency for an increase in IgG, IgM, and IgA antibody titers as dietary threonine increased from 0.8% to 0.87%. However, IgG, IgM, and IgA antibody titers decreased when threonine was administrated at levels of 0.94% and 1.01%. The titers of IgG, IgM, and IgA antibodies for a secondary response were higher than those for a primary response.

5. Conclusions

The findings of this study suggest that the dietary supplementation of L-carnitine solely or in combination with excesses of lysine or methionine (with respect to NRC recommendations) did not affect body weight gain, feed conversion, and economical carcass segments of broiler chickens. By contrast, the diet with around 30% of lysine-methionine content increased the back thoracic vertebrae and the proventriculus weights. A combination of lysine-methionine (level equal to NRC recommendations) with L-carnitine (15% and 75%) improved the immune response of broiler chickens against Newcastle and Gumboro diseases by stimulating the antibody production.

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animals

MDPI

Review

Application of Moringa (*Moringa oleifera*) as Natural Feed Supplement in Poultry Diets

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Simple Summary: The wide application of in-feed antibiotics in poultry production has created public health hazards. A driving force for the interest of using natural herbs is to establish the antibiotics alternative in poultry production that has been reported in the literature. Therefore, the objective of the current review is to determine the effects of moringa (*Moringa oleifera*) tree leaves, seeds and their extracts on chickens' performance and health status. Based on previous findings, *M. oleifera* as natural feed supplement has sustained the production performance and improved the health status in chickens.

Abstract: Application of natural herbs with a view to enhancing production performance and health status has created an important demand in poultry production. With the increasing concerns on this issue, greater attention paid to alternatives to antibiotics for organic meat and egg production has led to a great demand. This study was conducted with view to assessing the possible role of *M. oleifera* as a natural feed supplement in poultry ration. Various scientific findings and published research articles were considered concerning issues including the study background, objectives, major findings, and conclusions of the review. *M oleifera* is known as a miracle tree because of its wealthy resource of various nutrients with high biological values. *M. oleifera* has been used as a growth promoter, immune enhancer, antioxidant, and has a hypo-cholesterol effect on chickens. It has both nutritional and therapeutic values. However, there is still much confusion in past published articles involving the major roles of *M. oleifera* in production performance and health status of chickens. Taking this into account, the present study highlights an outline of the experimental uses of *M. oleifera* on growth performance, egg production performance, egg quality, and health status in broilers and laying hens justified with the past findings to the present. The knowledge gaps from the past studies are considered, and the feasibility of *M. oleifera* in poultry ration is suggested. The findings have motivated further study on *M. oleifera* to find out the most active ingredients and their optimal doses in both broiler and laying hen rations. Finally, the present study highlights that supplementation of *M. oleifera* may play a role in the immunity, sound health, and production performance in poultry.

Keywords: *Moringa oleifera*; poultry; growth performance; laying performance; health status

1. Introduction

The human population is increasing globally day by day. Meeting the increasing demand of animal protein and providing safe food for human beings that is free from antibiotics by using herbal feed resources is a great challenge for the animal scientists in the future. The issue considering antibiotic resistance has created an augmented force to reduce antibiotic uses in livestock and poultry production [1,2]. Dietary inclusion of herbs and their extracts has growth-promoting roles in poultry [3]. Furthermore, different natural medicinal plants and their extracts as feed supplements have been used as a substitute for antibiotics in poultry production [4,5]. In addition, Mahfuz et al. [6] reported

that poultry scientists are now dedicated to applying unconventional natural feed supplement, which may play a role in possible therapies to improve the health as well as production performance of chickens.Thus, poultry researchers are searching for potential natural feed resources that will be both environmentally friendly and safe for human society [7,8].

Moringa oleifera is a well-known cultivated species in the genus *Moringa*, (family Moringaceae) under the order Brassicales. The common names of *Moringa oleifera* include moringa, drumstick tree, horseradish tree, and ben oil tree or benzoil tree or miracle tree [9–11]. The *M. oleifera* tree is native to South Asia, especially India, Sri Lanka, Pakistan, Bangladesh, Afghanistan; North Eastern and South Western Africa, Madagascar, and Arabia [12–15]. The moringa seed and leaves have a broad use in the food industry and therapeutic issues [12]. It is popular for its seeds, flowers and leaves inhuman food and as herbal medicine [16]. The different parts of the *M. oleifera* tree are used as a good source of human nutrition and in traditional diets in different countries of the world [17,18]. Furthermore, the seed powder of *M. oleifera* contains polyelectrolytes, which are the most important active ingredients for water purification [18,19].

Moringa oleifera is very useful as a feed supplement for animals, as its leaves are highly nutritious. The leaves of *M. oleifera* are the most nutritious part, being a significant source of vitaminB complex, vitamin C, pro-vitamin A as beta-carotene, vitamin K, manganese, and protein among other essential nutrients [20]. *Moringa oleifera* leaves have antimicrobial roles and are rich with fats, proteins, vitamins, and minerals [18,21]. The extracts from leaves of *Moringa oleifera* contain low amounts of polyphenols, which might have effects on blood lipid metabolism [20,22]. *Moringa oleifera* can be used as a source of micronutrient and as a dietary supplement in poultry [23,24]. In addition, *Moringa oleifera* leaf powder has anti-septic and detergent properties due to presence of different phytochemicalsin the leaves [25]. *Moringa oleifera* was reported to be an excellent source of vitamins and amino acids that reportedly boost immune systems [17]. The seed extracts of moringa are rich in polyunsaturated fatty acid [26,27]. *Moringa oleifera* exhibits anti-oxidant properties that can suppress formation of reactive oxygen species (ROS) and free radicals [27,28].

Until the present day, the application of *M. oleifera* in farm animals to improve the production performance and health status has been limited. Even though it was established that *M. oleifera* has medicinal importance for the health of chickens, unfortunately the inclusion levels of *M. oleifera* in poultry ration and their mode of actions are still under consideration. Taking this into consideration, the present study focuses on uses of *M. oleifera* as a natural feed supplement as well as an alternative to antibiotics that can improve the performance and health status of chickens.

2. Biological Role of *M. oleifera*

The *M. oleifera* tree is globally known for its economic and therapeutic roles (Figure 1). Ithas been honored as the "Botanical of the Year 2007" by the National Institute of Health (USA), [11]. The tree is also known as "never die" or "miracle tree"to the people of Africa [11]. Now the application of *M. oleifera* leaves in preparing foods is receiving great attention. Peoples from Ghana, Nigeria, Ethiopia, East Africa, and Malawi are consuming the moringa tree leaves directly in their diets [29]. Furthermore, *M. oleifera* leaves have been used for making soups, foods, breads, cakes, and yoghurts [30–33].

Figure 1. *Moringa oleifera* tree, tree leaves, and leaves powder.

2.1. Antioxidant Properties of M.oleifera

M. oleifera tree leaves possess various phytochemicals that have antioxidant properties and roles in controlling a wide range of diseases, like diarrhea, asthma, and various cancers [11]. The leaves of *M. oleifera* have also been reported to hold extensive amounts of total phenols, proteins, calcium, potassium, magnesium, iron, manganese, and copper [33]. They also contain rich sources of different phytonutrients, such as carotenoids, tocopherols, and ascorbic acid, which are good sources of dietary antioxidants [34,35]. A significant increase in activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and a decrease in lipid peroxide (LPS) content were found in moringa leaf extracts [11]. In addition, leaves extract from *M. oleifera* could improve the superoxide dismutase (SOD), catalase, glutathione, and peroxidase levels and reduce lipid peroxidation in albino mice [36]. Furthermore, total phenolic, flavonoid, and flavonol content in leaf extracts was found to be 120 mg/g of gallic acid equivalents (GAE), 40 mg/g of GAE, and 12.12 mg/g of GAE, respectively [37,38].

2.2. Therapeutic and Antimicrobial Properties of M.oleifera

M. oleifera leaf extracts have been distinguished as having anticancer, cytotoxic, anti-proliferative, anti-leukemia, anti-hepatocarcinoma, and chemo-protective properties [39–41]. The antitumor function of leaf extracts of *M. oleifera* is associated with the antioxidant and apoptosis inducing properties [42,43]. The antimicrobial properties of *M. oleifera* are well established. The extracts derived from *M. oleifera* tree leaves have been reported to be potential antibacterial and antifungal functions against various bacterial and fungal species [11,44,45]. Oluduro et al. [46] and Pandey et al. [47] have highlighted that *M. oleifera* exhibited 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate, methyl N-4-(α-L-rhamnopyranosyloxy) benzyl carbamate, and 4-(α-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy) benzyl thiocarboxamide that were able to play antimicrobial properties. The antimicrobial activities of the *Moringa oleifera* may be due to presence of lipophilic compounds and different metabolites (carboxylic acid, 2,4-diacetyl phloroglucinol, enzymes, and chitinases) in plant cell walls [48].

2.3. Immune Stimulating and Hypocholesterolemic Properties of M.oleifera

The immune functions of *M. oleifera* are also established by several in vitro studies [11]. Various biochemical ingredients, like quercetin, different glycosides, various isothiocyanate, kaempferol glucosides, that possess anti-inflammatory properties have been demonstrated from the extract of various parts of *M. oleifera* [49,50]. Different protein and various peptides' (isothiocyanates, glycoside cyanides etc.) presences in *M. oleifera* leaf extracts were able to modify the immune response positively [51,52]. An investigation was carried out to detect the immunomodulatory activity of *M. oleifera* on mice model. Chronic administration of *M. oleifera* significantly increased white blood cell (WBC) count and percent of neutrophils in experimental mice [51]. The exact mechanism of action of moringa leaves on stimulating the humoral and cellular immunity is not clear yet [51]. *M. oleifera* leaf extracts are reported to possess ahypo-cholesterolemic function [53]. β-sitosterol and 4-[α-(L-rhamnosyloxy) benzyl]-o-methyl thiocarbamate (trans) are two important active substances presence in the leaf extracts of *M. oleifera* that exhibit cholesterol lowering activities. These compounds could reduce the intestine uptake of dietary cholesterol in rats [49,54]. Furthermore, plasma cholesterol was decreased and fecal cholesterol was increased in rats fed with moringa leaf extracts [49,53]. In addition, another two components, moringine and moringinine, have been recently identified from *M. oleifera* leaves, which have roles in anti-hypoglycemic functions [49,55].

2.4. Nutritional Properties of M.oleifera

M. oleifera is also very popular for its nutritional values. It is reported as a good source of six major nutrients: Carbohydrate, especially dietary fibers; proteins; vitamins; minerals; lipids; and water. The unique features of *M. oleifera* are its richness in proteins, carbohydrates, and fibers with low fat. The leaves have been reported to enclose a range of essential amino acids and are a good source of

alpha linoleic acid [56]. *M. oleifera* leaves have been seen to exhibit high contents of vitamin A, C, and E [33]. The relative bioavailability of folate originated from *M. oleifera* leaves were about 82% in a rat model, which confirmed the fact that *M. oleifera* leaves exhibit rich source of dietary folate [57].

The nutritional composition of *M. oleifera* leaves (dry matter basic) showed dry matter (DM) about 93.63% to 95.0%, crude protein (CP) 17.01% to 22.23%, carbohydrate 63.11%to 69.40%, crude fiber (CF)6.77% to 21.09%, crude fat (EE) 2.11% to 6.41%, ash (total mineral) 7.96% to 8.40%, gross energy 14.790 (MJ/kg), and fatty acid 1.69% to 2.31% [58–60]. In addition, estimated calcium (Ca) was 1.91%; potassium (K) was 0.97%; sodium (Na) was 192.95, iron was (Fe) 107.48, manganese (Mn) was 81.65, Zinc (Zn) was 60.06, and phosphorus (P) was 30.15 parts per million (ppm) [59]. Magnesium (Mg) was 0.38%, and copper (Cu) was 6.1%, tannins 21.19%, phytates 2.57%, trypsin inhibitors 3.0%, saponins 1.60%, oxalates 0.45%, and cyanide 0.1% was also reported by Ogbe and John [59]. The leaves of the plant are enriched with methionine, phosphorus, calcium, and iron [11]. It is believed that the leaves of *M. oleifera* contain more calcium and twice as much protein than milk, higher vitamin C than oranges, higher potassium and iron than bananas, and higher vitamin A than carrots [10,61], and thus the plant is considered unique in nature [62]. Niaziridin, an active component that was identified from *M. oleifera*, can improve the absorption of different vitamins, minerals, and other micro nutrients in gastrointestinal tract of the host [50]. The nutritional composition of *M. oleifera* leaves are presented in Tables 1 and 2.

Table 1. Chemical compositions of *Moringa oleifera* leaves [†].

Nutrient Component	Fresh Leaves	Dry Leaves	Leaves Powder
Calories (cal)	92	329	205
Protein (g)	6.7–17.1	29.4–40.0	25.4–27.1
Fat (g)	1.7–2.11	5.2–6.5	2.3
Carbohydrate (g)	6.3–12.5	38.0–41.2	34.3–38.2
Fiber (g)	0.9–7.09	12.5–21.09	19.2
Vitamin A	0.9–11.05	16.3–18.90	-
Vitamin B1 (mg)	0.06	2.02–2.60	2.64
Vitamin B2 (mg)	0.05	19.82–21.3	20.5
Vitamin B3 (mg)	0.8	7.6–8.3	8.2
Vitamin C (mg)	220	15.8–17.3	17.3
Vitamin E (mg)	448	10.8–77.0	113
Calcium (mg)	440	2185–3050	2003
Magnesium (mg)	42–82	86–448	368
Phosphorus (mg)	30.15–70	204–252	204
Potassium (mg)	259	1236–1384	1324
Copper (mg)	0.07	0.08–0.49	0.57
Iron (mg)	0.85–10.7	25.6–490	28.2
Sulphur (mg)	-	363–630	870
Zinc (mg)	6.7	3.25–13.03	-
Manganese (mg)	81.6	86.8–91.2	-

[†] All values are in 100 g per plant material. References: [10,56,63].

Table 2. Amino acid contents in *Moringa oleifera* leaves [†].

Amino Acid	Fresh Leaves (mg g^{-1} DM)	Extracted Leaves (mg g^{-1} DM)
Lysine	13.25–26.77	14.06–18.09
Leucine	20.52–42.89	17.5–21.84
Isoleucine	11.91–22.53	8.08–11.30
Methionine	3.5–8.96	1.13–4.97
Cystine	3.8–5.18	1.0–3.39
Phenylalanine	16.31–27.14	8.9–15.51
Tyrosine	18.88	9.71
Valine	10.62–27.58	7.25–14.26
Histidine	5.17–13.57	7.16–7.50
Threonine	13.5–21.97	7.90–11.70
Serine	10.87–20.79	9.40–10.34
Glutamic acid	28.42–50.85	17.10–25.65
Aspartic acid	20.52–46.11	14.3–22.16
Proline	14.3–25.75	12.41–13.63
Glycine	15.33–26.62	10.3–13.73
Alanine	28.67–30.33	12.51–18.37
Arginine	18.9–30.28	13.25–15.64
Tryptophan	4.25–9.26	5.27–7.16

[†] References: [15,56,64,65].

It was thought that the moringa contains different anti-nutritional factors, such as tannins, phytates, oxalates and cyanide, which may affect normal digestion and metabolism of nutrients in animals [66]. In moringa, tannins and phytates are 12 and 21 g kg^{-1} of DM, respectively, which can be neutralized by different feed processing techniques, including chopping, socking, heat steaming, and fermentation with beneficial organisms [65]. Considering the health benefit effects of moringa, it is a unique plant due to its enriching minerals with lower anti-nutritional components [65].

3. Application of *M. oleifera* on Performance in Chickens

In most of the feeding experiments in poultry, the fresh, green, and undamaged mature *M. oleifera* leaves were properly air-dried, and then the dried leaves were ground to a fine powder in a hammer mill and considered as moringa leaf powder or leaf meal. Similarly, fresh mature moringa seeds were air-dried and ground and considered as moringa seed meal. In some experiments, the ground particles were then soaked into distilled water for 24 h, and the filtered aqueous solution was considered as moringa extract. Due to the rich nutrient content, especially the high amount of crude protein (CP), vitamins, and minerals, *M. oleifera* leaves can be used as a useful resource of dietary supplementation for livestock as well as poultry [65–67]. In addition, Briones et al. [68] stated that moringa leaves can be applied as a dietary supplement in layers and broilers due to high production performance and improved eggs quality. However, still there are many debates on the chicken's performance with different doses of *M. oleifera* in the previous studies. There are also many variables on doses and part of plant used, such as leaves, extract, sods, or seeds. Finally, many scientists agreed that *M. oleifera* plant might have a positive role in improving the production performance and health status in chickens. Further studies are still needed to detect the actual doses of application for optimum performance in chickens.

3.1. Effects of M. oleifera on Growth Performance in Broilers

The major findings on the role of *Moringa oleifera* on performance in broilers are summarized in Table 3. Alabi et al. [69] applied aqueous *M. oleifera* leaf extracts on the performance in broiler chickens. This study demonstrates that average daily body weight gain and final body weight were higher in 120 mL/L extract-supplemented groups than the control. Feed intake was highest in birds on positive control (having antibiotics) and lowest in birds that consumed 90 mL/liter of leaf extracts.

Feed conversion ratio (FCR) was lower in birds on 90 mL/L and 120 mL/L of leaf extracts fed groups. Collectively, the authors suggested that moringa leaf extracts can be added up to 90 mL/L in broiler chickens for optimum performance. The higher body weight and lower FCR in this study might be related to the presence of different bioactive components in moringa leaf extracts that may play a role in improved nutrient utilization in supplemented birds. Similarly, higher body weight was also recorded by Khan et al. [70] who used moringa leaf powder as dietary supplement with 1.2% levels in broilers. Abdulsalam et al. [71] conducted an experiment with moringa leaf meal in broilers and found that supplemented diets could enhance the growth performance at finisher period. The authors finally stated that moringa leaf meal can be applied as a natural source of protein in broiler diets. Similarly, inclusion of *Moringa oleifera* leaves at higher levels (15% and 20%) in broiler diets resulted in a higher growth rate and better health status in broilers [14]. In addition, dietary supplementation of *M. oleifera* leaves at 5% to 20% level showed higher growth performance in broilers [66]. Final live weight, average weight gain, and FCR were higher in 10% moringa leaf meal supplemented diets than the control through a 35-day trial period [72].Furthermore, feeding with *M. oleifera* leaf powder could improve live weight, body weight gain, dressing percentage, and FCR in broilers [73].

In contrast, no significant differences were observed on growth performance and economic parameters in broilers fed with *Moringa oleifera* leaf meal, according to Onunkwo and George [18]. Finally, the authors stated that *Moringa oleifera* leaf meal may be used at the level of 10% with view to reducing the production cost [18]. Similarly, feeding with moringa leaf meal in broilers led to a lower feed intake with higher FCR, as reported by Gakuyaet al. [74], which was due to presence of anti-nutritional factors in moringa leaves used in the experiment diets as row basis. No significant differences were observed on final live weight and dressing percentage by feeding moringa seed powder in broilers [75]. Gadzirayi et al. [76] applied *Moringa oleifera* leaf meal as supplementing part of conventional soybean meal in broiler diets at 0%, 25%, 50%, 75%, and 100% level. The author did not find any significant differences on feed intake and body weight gain between control and 25% level of moringa supplementation. However, significantly lower FCR was observed in moringa leaf meal fed groups. Finally, the study suggested using moringa leaf meal at a 25% level to promote growth in broilers. In addition, Ayssiwede et al. [77] noted that dietary application of moringa leaf meal up to a level of 24% had no adverse effects on body weight, average daily weight gain, FCR, mortality, and the weight of organs in broilers compared to the control diet. Olugbemi et al. [78] stated that average daily growth rate was lower with *Moringa oleifera* leaf meal at the inclusion level below 5% in diets, and the authors suggested to use maximum level of 5% without any harmful effects on growth performance and FCR in broilers. These findings confirmed the fact that feeding with moringa leaves had no deleterious effects on normal physiology and growth in the experimental broilers. However, collectively, some authors suggested that use of the *Moringa oleifera* leaf meal up to a 10% level would not have any adverse effects in broilers [78–80]

Table 3. Role of *Moringa oleifera* on performance in broilers. †

Types	Study Design	Main Findings	References
Moringa oleifera leaf powder	broilers (Hubbard) from 1–35 days, dose: 6,9,12, and 15 g/kg (supplementation type)	●higher pH of breast muscle ●higher weight and diameter of breast muscle fibers ●higher water holding capacity of breast muscle ●higher weight length index of tibia bone ●higher ash percentage of tibia bone ●no effects on alkaline phosphatase in tibia bone ●no effects on feed intake, FCR and bursa weight	[81]
Moringa oleifera leaf powder	broilers (Hubbard) from 1–35 days, dose: 0, 0.6%, 0.9%, 0.12%, 0.15% (supplementation type)	●higher final body weight ●higher length of small intestine ●higher empty weight of small intestine and ceca ●higher villus height (duodenum, jejunum, ileum) ●higher villus height/crypt depth (ileum) ●higher goblet cell number (total) in duodenum ●higher acidic mucin number in duodenum, jejunum and ileum	[70]
Moringa oleifera leaf extract	broilers (Hubbard)from 1–42 days, dose: 0, 60, 90, 120, 150 mL/L	●higher body weight gain ●lower FCR ●no effects on weight of inner organs ●no effects on dressing percentage	[69]
Moringa oleifera leaf meal	broilers from 0–42 days, dose: 0, 5%, 10% 15%, 20%, (inclusion type)	●higher body weight ●higher hemoglobin percent, and RBC number ●lower TC, LDL	[14]
Moringa oleifera seed powder	broilers from 1–42 days, dose: 0, 0.5%, 0.1%, and 2% (inclusion type)	●no effects on live weight and weight gain ●no effects on FCR ●no effects on dressing percentage, liver weight and heart weight	[75]
Moringa oleifera leaf meal	broilers (ANIK 2000 strain) from 0–49 days, dose: 0, 5%, 7.5%, 10 % (inclusion type)	●higher dressing weight in 7.5% and 10% level ●higher weight of liver, spleen, and gizzard ●no significant effects on body weight gain, feed intake and FCR	[18]
Moringa oleifera leaf meal	broilers (Cobb-500) from 1–35 days, dose: starter (1, 3 and 5 g/kg); grower (3, 9, and 15 g/kg); and finisher (5, 15, and 25 g/kg) (inclusion type)	●higher body weight and weight gain at grower period ●lower FCR ●no effects on feed intake ●higher Ca and P content in tibia bone ●no effects on tibia weight, tibia length, and weight-length index of tibia bone ●no effects on ash content in tibia, and bone breaking strength	[82]
Moringa oleifera leaf meal	broilers (Cobb-500) from 1–35 days, dose: starter (1, 3, and 5 g/kg), grower (3, 9, and 15 g/kg) and finisher (5, 15, and 25 g/kg) (inclusion type)	●higher body weight at starter and finisher period ●lower FCR ●no effects on feed intake ●higher dressing percentage, thigh muscle weight and bursa weight ●no effects on CP, CF, DM, EE, ash, NDF, ADF digestibility	[83]

Table 3. *Cont.*

Types	Study Design	Main Findings	References
Moringa oleifera leaf meal	broilers (Cobb-500) from 1–35 days, dose: 1%, 3%, and 5% (inclusion type)	●higher body weight and weight gain at starter period ●lower FCR ●no effects on feed intake ●higher thiobarbituric acid reactive values in breast muscle during storage ●higher fatty acid profile (C18:0, C15:0, C20:0, C20:3n6 and C22:6n3) levels ●no effects on thrombogenic index and atherogenic index in breast muscle	[84]
Moringa oleifera leaf meal	broilers (Ross) from 1–49 days, dose: 0, 3%, 5%, and 7% (inclusion type)	●higher final body weight and weight gain ●lower FCR ●higher feed intake ●higher dressing percentage ●higher meat tenderness and juiciness score	[85]
Moringa oleifera leaf meal	broilers from 1–42 days, dose: 0, 7.5%, 15%, and 30% (inclusion type)	●lower final body weight and weight gain ●higher FCR ●lower dry matter digestibility ●no effects on crude protein, crude fiber digestibility ●no effects on lipid metabolic profile (HDL, TC, LDL)	[74]
Moringa oleifera leaf meal	broilers from 1–35 days, dose: 0, 10%, 15% (inclusion type)	●higher meat color scores ●higher body weight gain ●lower FCR ●higher final body weight ●higher RBC number, PCV number, and HB percent	[72]
Moringa oleifera leaf meal	broilers (Habbard) from 0–42 days, dose: 0, 25%, 50%, 75 %, 100% (supplementation type)	●no effects on feed intake ●no effects on weight gain ●lower FCR ●higher body weight gain	[76]
Moringa oleifera leaf powder	broilers from 1–42 days, dose: 0, 0.05%, 0.10% (supplementation type)	●lower FCR ●higher final body weight ●higher dressing percentage ●higher live weight	[73]
Moringa oleifera leaf extract	broilers (Cobb)from 1–35 days, dose: 0, 30, 60, 90 mL/L	●lower FCR ●higher returns to investment ●lower feed intake ●lower weight gain and final body weight	[86]
Moringa oleifera leaf meal	broilers (Cobb) from 14–42 days, dose: 0, 5%, 10%, and 15% (inclusion type)	●higher FCR ●no effects on dressing percentage and carcass weight ●no effects on weight of inner organs ●no effects on CP and EE content in meat ●no effects on total cholesterol, HDL, LDL, total protein, glucose	[87]

† FCR, feed conversion ratio; HDL, high density lipoprotein cholesterol; TC, total cholesterol; LDL, low density lipoprotein cholesterol; RBC, red blood cell; PCV, packed cell volume; HB, hemoglobin; CP, crude protein; CF, crude fiber; DM, dry matter; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber.

3.2. Effects of M. oleifera on Meat and Bone Quality in Broilers

Dietary manipulation is an important way to improve the meat quality in poultry [2]. The meat derived from broiler chickens is an excellent source of protein, vitamins, minerals, and lower fat and has created a great demand among consumers [88]. Meat pH, tenderness, color (lightness, redness, and yellowness),and water holding capacity are very important meat quality characteristics to the consumers. An experiment on supplementation of *Moringa oleifera* leaf powder on the quality of meat and bone in broilers was conducted by Rehman et al. [81]. This study noticed that supplementation of leaf powder at 12 g/kg level could increase pH, water holding capacity, and muscle fiber diameter in the breast muscle of experimental broilers. In addition, higher weight, ash percentage, and the density of tibia bone in broilers fed with moringa leaf meal were also recorded in their studies [81]. In this study, authors hypothesized that higher muscle pH values in experimental groups were due to the stabilization of the myofibrils by activating antioxidant properties and preventing free radicals. Higher breast muscle weight could be the result of increased protein deposition in moringa-supplemented groups. The higher tibia bone weight and ash percent may be due to the presence of phytoestrogen flavonoids in moringa leaves powder. In contrast, Nkukwana et al. [82] found that *Moringa oleifera* leaf meal had no effects on tibia bone characteristics but could improve body weight gain and FCR. These differences might be related with inclusion levels and types of incorporation of moringa in broiler diets. However, it is a popular belief that dietary antioxidants can modify the meat color, minimize the rancidity, and retard lipid peroxidation, resulting in a well-maintained meat quality. The oxidative status of meat muscle is directly related to meat quality and has negative effects on cooking loss, drip loss, meat color, and pH [89]. Therefore, dietary supplementation of antioxidant-enriched moringa leaves would be a potential strategy to improve the meat quality in broilers. Moreover, it was reported that phytosterols could reduce malondialdehyde (MDA) content and increase glutathione (GSH) concentration in the breast muscle of experimental broiler chickens [88]. The inclusion of moringa leaf meal could improve fatty acid profile and could reduce lipid oxidation in breast muscle of broilers [84]. The authors assumed that improved fatty acid profile was due to the presences of saturated fatty acids in moringa leaves.

3.3. Effects of M. oleifera on Health Status in Broilers

Alnidawi et al. [14] has conducted an experiment with a view to examining the effects of *Moringa oleifera* leaf on health status in broilers.This study ensured that total cholesterol content was lower with higher level (at 15% and 20%)of *M. oleifera* fed in broiler diets. Similarly, high-density lipoprotein cholesterol (HDL) content in serum was increased and low-density lipoprotein cholesterol (LDL) was decreased with higher level of supplementation of *M. oleifera* in broilers. It was hypothesized that higher amounts of natural fiber in moringa leaves may have a role in lowering cholesterol level by increasing lipid metabolism in the host body. In addition, the blood parameters, like hemoglobin percent, total red blood cells number, and total packed cell volume, were found to be higher at 20% supplementation levels than the control diet [14]. *M. oleifera* leaf powder was considered as dietary supplement with 0.6%, 0.9%, 1.2% and 1.5% levels in broilers on growth performance and intestinal microarchitecture [70]. The intestine's morphological characteristics in chickens are vital for nutrient utilization and an indicator of sound physiology. The length and empty weight of small intestine were found higher in broilers fed with 1.2% leaves powder. In addition, higher villus height (duodenum, jejunum, ileum), villus surface area (duodenum), and villus height/crypt depth (ileum) were observed in 1.2% leaves powder fed group than the control. Higher villi suggest better absorption of nutrients due to enlarged surface area, which is a good indicator of gut system. Furthermore, the improvement of villus height and villus height/crypt depth ratio may be linked with high content of crude fiber in moringa-supplemented diets. This study further observed that total goblet cells of duodenum were higher in broilers fed with all levels of *M. oleifera* leaf powder in comparison with control group. The findings indicate enhanced mucosal protection with *M. oleifera* supplementation in broiler diets. Goblet cells are essential elements of innate gut immune system in poultry. Bursal follicle count was

also found to be higher in 1.2% *M. oleifera*-fed group than non-supplemented control diet. Finally, the authors concluded that dietary supplementation of *M. oleifera* at 1.2% level could modulate the intestinal structure and acidic mucin production without any adverse effects on growth performance in broilers [70].

The extract from the leaves of *Moringa oleifera* has apotential role as an anti-bacterial and antioxidant functions [22]. The roles of *Moringa oleifera* leaf meal at 10% and 15% level on the hematological parameters in broilers were examined by Ebenebe et al. [72]. Feeding *Moringa* leaf meal in broilers resulted in increased red blood cell (RBC), packed cell volume (PCV), and hemoglobin (HB) values in both levels of diets. Finally, the authors stated that *Moringa oleifera* leaf meal should be used within the 10% level in broiler diets. *Moringa oleifera* is known to a potential antioxidant with some antioxidant properties due to the presence of vitamins C and E, carotenoids, flavonoids, and selenium [15]. *Moringa oleifera* leaves contain various phytochemicals (carotenoids, flavonoids, chlorophyll, phenolics, xanthins, cytokines, alkaloids, etc.) that might have a role in improving health status [90].

3.4. Effects of M. oleifera on Egg Production, Performance, and Egg Quality in Laying Hens

The major findings on the role of *M. oleifera* in performance in laying hens are summarized in Table 4. The egg quality parameters, including egg size, shape, color, shell thickness, and egg yolk cholesterol, directly and indirectly influence egg consumers. In a recent study by Voemesse et al. [91], *M. oleifera* leaf meal was used in layer chickens' diet from 1 day old to 55 weeks of age to investigate the effects of moringa leaf meal on growth performance, egg production performance, and blood parameters. *M. oleifera* leaf meal was used at three different levels (0%, 1%, and 3%). In the growing period from 1 day to 20 weeks of age, this study did not find any significant differences on feed intake, but average daily body weight gain, final body weight, and FCR were improved in *M. oleifera*-supplemented groups. In the laying period, from 21 weeks to 55 weeks, feed intake was lower in moringafed groups, but the laying percent and FCR were higher in supplemented fed groups than the non-supplemented group. The higher body weight gain and egg production may be related to improved digestibility in supplemented groups due to different active components in moringa leaves. The author concluded that feeding moringa leaf meal at 1% level had positive effects on the growth and egg production in laying hens.In addition, *Moring oleifera* at 10% levels showed higher egg production in laying hens [66]. According to Abouz-Elezz et al. [92], *M. oleifera* supplementation could improve the egg production, egg mass, and egg yolk color scores compared with the non-supplemented groups. The improvement of yolk color scores could be due to high carotene content in moringa leaves. Higher feed intake, crude protein intake, weight gain, FCR, and protein efficiency ratios were recorded in laying chicks where *Moringa stenopetala* was the experimental supplement [93]. This is because of readily available proteins with their essential amino acids in the moringa leaf meal. The authors finally concluded that *Moringa stenopetala* leaf meal at up to 6% levels can be applied in growing chicks' ration.

In contrast, *Moringa oleifera* seed meal at 0%, 1%, 3% and 5% levels were used to examine the effects of egg production performance, egg quality, and egg fatty acid profile in Hy-Line laying hens [94]. Lower feed intake, egg production percent, egg mass, feed intake, and body weight were observed in moringa seed meal-fed groups than the control. Higher egg yolk color scores with higher linolelaidic acid in egg yolk were found in moringa seed meal supplemented groups than the non-supplemented diets [94]. The moringa seeds may contain different anti-nutritional factors, which may have deleterious effects on production performance in this study. In addition, Ahmad et al. [95] also reported that the decrease in production performance of layer chickens was due to high fiber and different anti-nutritional factors' presences in moringa pod meal. However, this study found a significant positive role in improving β-carotene, quercetin, and selenium levels in egg yolk with moringa pod supplementation. Moringa pods are naturally enriched with carotenoids and different flavonoids, which possess natural antioxidants that could modify the β-carotene and quercetin levels in egg yolk [74]. Egg yolk cholesterol was significantly lower in moringa pod meal fed groups than the control group, which may be due to presence of natural antioxidants in the experimental diets

containing moringa pod meal in this study. In addition, the nutrient profile of egg yolk was higher with the supplementation of moringa pod meal in Hy-Line layers [95]. In another study, Lu et al. [96] found that *M. oleifera* leaf meal had no effects on egg production, egg weight, and feed intake in Hy-Line Grey commercial layers, but birds fed with moringa leaf meal at 15% levels showed deeper egg yolk color than the non-supplemented fed group. Similarly, the albumen height and Haugh unit were higher in moringa-supplemented groups during storage of eggs at 4 °C and 28 °C for 4 weeks. Finally, the author stated that 5% moringa leaves meal can be included in laying hens' ration without adverse effects on egg production and egg quality. Similarly, Abou-Elezz et al. [80] found that *Moringa oleifera* leaf meal could improve egg yolk color scores and albumen percentage. This study further observed the lower egg laying percentage and egg mass in laying hens fed with moringa leaf meal. However, this study did not find any significant differences on final body weight and on other egg quality parameters (yolk percent, shell percent, and shell thickness). Finally, the author stated that 10% moringa leaf meal can be incorporated into the diets of Rhode Island Red laying hens. Feed intake, feed conversion ratio, and laying percentage were not influenced by adding moringa leaf meal at a 10% level, which was noticed by Olugbemi et al. [78]. However, inclusion of 10% moringa leaf meal could increase higher egg Roche color score [78]. A similar report on decreased egg mass and egg production percent with moringa leaf meal supplementation at higher levels (at 10% and 20%) in laying hens was observed by Kakengi et al. [79]. Interestingly, *Moringa oleifera* leaf meal at 5% level increased the egg weight, but the decreased egg weight was found when inclusion level was at 20%. The authors assumed that higher feed intake, FCR with lower egg production percent, egg mass, and egg weight at a higher-level supplementation was due to poor digestibility of nutrients because of different anti-nutritional phytochemical presences in moringa leaves [79].

Improving the egg quality by means of increasing its anti-oxidative properties by supplementing natural unconventional resources has gained a significant interest in poultry research [97]. The synthesis antioxidants, like butylated hydroxyanisole and butylated hydroxytoluene, are commonly used in food processing. However, they are found to be carcinogenic to human health, therefore, discovering natural antioxidant products as safe and effective alternatives is a very crucial need [98–100].

Table 4. Role of *Moringa oleifera* on performance in laying hens.[†,]

Types	Study Design	Main Finding	References
Moringa oleifera leaf meal	laying chickens and laying hens, from 1 day to 55 weeks, dose: 0, 1%, 2%, and 3% (inclusion type)	●no effects on feed intake in growing period ●higher body weight gain, and lower FCR ●lower feed intake in laying period ●higher egg production ●lower FCR ●higher serum albumen percent ●lower number of RBCs, WBCs, PVC, and lymphocytes ●lower egg production percent, egg mass, feed intake and body weight	[91]
Moringa oleifera seed meal	laying chickens (Hy-Line), from 20–28 weeks, dose: 0, 1%, 3%, and 5% (inclusion type)	●no effects on egg weight ●lower egg albumen height ●higher egg yolk color scores ●no effects on albumen weight, yolk weight, egg shell weight, and egg shape index ●lower palmitoleic acid in egg yolk ●higher linolelaidic acid in egg yolk	[94]
Moringa oleifera pod meal	laying hens (Hy-Line), from 50–66 weeks, dose: 0, 5, 10 and 15 g/kg (inclusion type)	●higher egg mass and lower FCR ●no effects on egg production, egg weight, and feed intake ●higher Haugh unit and egg shell thickness ●no effects on egg shape index and egg yolk index ●higher β-carotene, quercetin, and selenium in egg yolk ●lower cholesterol in egg yolk and serum ●higher nutrient composition in egg yolk	[95]
Moringa oleifera leaf meal	laying hens (Hy-line Gray) from 27–35 weeks, dose: 0, 5%, 10%, and 15% (inclusion type)	●no effects on feed intake and egg weight ●higher FCR ●lower egg production ●higher egg yolk color scores ●higher albumen height and Haugh unit during storage ●higher glutathione peroxidase in plasma ●lower malondialdehyde and uric acid in plasma	[96]
Moringa oleifera leaf powder	laying hens (Lohmann LSL) from 27–40 weeks, dose: 0, 0.2%, 0.4%, 0.6%, 0.8% (inclusion type)	●no effects on egg production, egg weight, and FCR ●no effects on sensory evaluation of eggs quality ●no effects on egg shell thickness ●higher egg production ●higher egg mass	[101]
Moringa oleifera leaf meal	laying hens (Rhode Island Red, RIR) from 27–38 weeks, dose: 0, 300 g/kg, (supplementation type)	●higher egg yolk color scores ●lower FCR ●higher albumen percent	[92]

Table 4. *Cont.*

Types	Study Design	Main Finding	References
Moringa oleifera leaf meal	laying hens (Rhode Island Red, RIR) from 36–41 weeks, dose: 0, 5%, 10%, and 15% (inclusion type)	●no effects on final body weight, egg weight, and FCR ●decreased egg production ●decreased egg mass ●higher egg yolk color scores ●higher albumen percent ●no effects on yolk percent, shell percent, and shell thickness	[80]
Moringa oleifera leaf meal	laying hens from 65–73 weeks, dose: 0, 5%, 10%, and 20% (inclusion type)	●no effects on feed intake, egg production percent, and FCR ●decreased feed cost ●no effects on albumen and yolk percent ●higher egg Roche color scores	[78]

† FCR, feed conversion ratio; RBC, red blood cell; WBC, white blood cell; PCV, packed cell volume.

3.5. Effects of M. oleifera on Health Status in Laying Hens

Analyzing blood parameters is very important in detecting the health status of birds. According to Voemesse et al. [91], serum albumin level was higher in laying hens fed with 3% level of moringa leaf meal than the control group, but the number of white blood cells (WBCs),red blood cells (RBCs), lymphocytes, and the packed cell volume were lower in moringa-fed groups than the control diets. The authors assumed that lower WBCs and lymphocytes in moringa-fed chickens may be due to the antimicrobial activity of phytochemicals in the moringa leaves. It is well known that a high WBC count is related to an infection caused by bacteria in the host. Lower level of cholesterol content in serum with dietary supplementation of moringa pod meal were observed, which might be influenced by antioxidants (flavonoids and carotenoids) and high fiber presences in the moringa pod meal in the experimental diets [95]. However, this study did not find any significant differences on antibody response against Newcastle disease virus. Lower values for malondialdehyde (MDA) and higher glutathione peroxidase in the plasma of laying hens fed with moringa leaf meal indicated the higher antioxidant activities [96]. Plasma total protein levels were higher by dietary 5% for moringa leaf meal supplementation, which is a good indicator of the liver's synthetic function. Furthermore, lower plasma uric acid in supplemented groups indicated higher protein retention in laying hens [96]. The improved antioxidant enzyme activities and the reduced MDA levels in the plasma and egg yolk indicated the fact that dietary moringa supplementation could improve the antioxidant activities. *Moringa oleifera* is an effective phytobiotic and is known to possess broad-spectrum antibacterial properties and immuno-modulatory functions [70,81,102].

4. Conclusions

This review study highlights that *M. oleifera* could be fruitfully used as an effective natural growth promoter as well as an immune-boosting agent in chickens' ration. Although *M. oleifera* was used in the experimental diets of poultry, further study was recommended by various researchers regarding the doses of *M. oleifera* on optimum performance and sound health in chickens. Thus, the future study should beconducted ina proper way so that it will examine the uses of *M. oleifera* in reaction to a pathogen challenge as well as dosages. In this study, we suggest future research with *M. oleifera* as an alternative for antibiotics in chickens so that it may be used as an effective strategy for organic meat and egg production. It could be concluded that *M. oleifera* can be used as an environmentally friendly feed supplement in chicken ration. The present study will help future researchers to discover the important effects of *M. oleifera* on immunity and health status that the past studies were not able to explore. Thus, the supplementation with *M. oleifera* may be a new concept of research in chicken production. The inclusion level of *M. oleifera* up to 10% in both broilers' and laying hens' diet could be recommended.

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Impacts of Graded Levels of Metabolizable Energy on Growth Performance and Carcass Characteristics of Slow-Growing Yellow-Feathered Male Chickens

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Simple Summary: Inadequate feed inhibits the potential performance of birds, and giving birds excess nutrients or levels higher than the requirement reduces production profits and may lead to negative effects on performance. Although recently there has been an expanding market worldwide for slower growing chickens to meet the consumer demand for a better tasting meat, little effort has gone into optimizing their dietary nutrient levels. Using fiv e different dietary energy levels, this study evaluated the optimal requirement of dietary energy for maximal growth rate, feed:gain ratio, meat quality indices, and blood metabolites of a Chinese yellow-feathered breed.

Abstract: A dose-response study was conducted to investigate the metabolizable energy (ME) requirement for Lingnan chickens from 9 to 15 weeks of age. One thousand two hundred 8-week-old slow-growing yellow-feathered male chickens were allotted to five dietary ME levels (2805, 2897, 2997, 3095 and 3236 kcal/kg). The results revealed that the daily metabolizable energy intake increased ($p < 0.01$), whereas the feed intake and feed:gain ratio decreased linearly ($p < 0.01$) with the increment in dietary ME level. The final body weight and daily gain of the highest ME treatment tended ($p > 0.05$) to be greater than those obtained with the lower ME levels. The fat content in breast muscle showed a quadratic response ($p < 0.05$) to the increase in dietary energy level. The shear force values of breast muscle in the 2897, 3095 and 3236 kcal/kg treatments were lower ($p < 0.05$) than those of the 2997 kcal/kg treatment. In conclusion, among the tested ME levels, 3095 kcal/kg was adequate for feed intake, shear force, and plasma uric acid, and 3236 kcal/kg tended to increase the body weight, body gain, and feed conversion ratio of Lingnan males between 9 and 15 weeks of age; further studies are still required for testing higher levels.

Keywords: energy requirement; meat quality; growth performance; slow-growing broilers; nutrient deposition

1. Introduction

In poultry production enterprises, feed cost accounts for around 70% of the total costs involved in production. Among the different feed-stuffs used in formulating poultry diets, the source of dietary energy resources is a major cost; 70% of the total poultry diet content are energy sources. Optimizing the dietary energy level, therefore, is important for lowering the feed cost per unit of poultry products [1].

Increasing dietary energy level provides fundamental benefits in the feed conversion ratio (FCR) of broilers, mostly by decreasing feed consumption [2–4]. On the other hand, using excessive energy or a level higher than the requirement can increase the deposition of undesirable abdominal fat in broiler carcass, considered to be an economic loss as it is often counted as a waste product [2]. The dietary energy can be optimized for both growth performance and for enhanced meat quality. Dietary nutrient levels alter meat color, energy content, and histological makeup as well as the metabolic characteristics of broiler muscles [5–7].

The optimal dietary energy for broilers has been estimated in several previous studies [8,9], but existing data require verification for modern genotypes [10]. In contrast, little effort has gone into optimizing the dietary energy level for slow-growing meat-type chickens. Recently, there has been an expanding market worldwide for slower growing meat-type chickens, giving them a place in contemporary production. This is mainly to meet the consumer demand for better tasting meat and for fulfilling organic production conditions [11], as well as avoiding some problems with the fast-growing broilers, such as sensitivity to environmental conditions, leg problems, metabolic failure, ascites, sudden death, and an increased mortality rate occurring during the finishing phase [12–14]. This relatively new interest in slow-growing meat chicken breeds is increasing worldwide, though it is associated with higher costs of production [11].

China is the second-largest global producer of chicken meat, almost half of which is from Chinese yellow-feathered breeds [15]; Chinese annual production of such breeds exceeds four billion birds. The distinct flavor and favorable color of the meat are highly desired by local consumers in China and in neighboring countries [16]. There are three types of such chickens [17], broadly classified as fast- (marketable around 8–10 weeks, 1.47–2.30 kg weight), medium- (marketable 9–14 weeks, 1.00–2.27 kg weight), and slow-growing (marketable 12–25 weeks, 1.06–1.88 kg weight). The increasing commercial importance of these indigenous birds means that comprehensive work is needed to improve their feeding standards. As the dietary energy requirement for slow-growing yellow broilers has not been estimated or optimized, the present study has evaluated the effects of different dietary ME levels on growth performance, blood biochemical variables, carcass quality, body composition, rate of energy deposition, and fat content in breast and thigh muscles.

2. Materials and Methods

2.1. Chickens, Diets and Management

The experimental conditions were approved by the Animal Care and Use Committee of the Institute of Animal Science, Guangdong Academy of Agricultural Sciences, China, with the approval number GAASISA-2015-011. The yellow-feathered male chickens (Lingnan breed, a meat-type breed that originated in South China) were obtained from a commercial hatchery (Guangdong Wiz Agricultural Science and Technology Co., Guangzhou, China) and were raised from day 1 to 8 weeks of age on a common, typical diet, provided ad libitum. One thousand two hundred birds were weighed at 8 weeks of age and randomly allocated to 30 equally-sized (4.55 m^2) floor pens of 40 birds, having a similar average body weight (BW) (771.25 ± 10.23 g). Five dietary treatments, each with six replicates, consisting of graded metabolizable energy (ME) levels (2900, 3000, 3100, 3200 and 3300 kcal ME/kg, calculated), were pelleted and provided ad libitum, as was water. These experimental diets (Table 1) were formulated to provide the nutrient requirements of Chinese yellow-feathered broilers [18], except for the ME level. The gross energy of the diets was analyzed according to the guidelines of Association of Official Analytical Chemists [19], and the ME was determined and calculated according to the methods and the equation of Jiang et al., [20], which showed 2805, 2897, 2997, 3095 and 3236 kcal/kg, respectively. The 2997 kcal/kg was considered to be the control dietary energy level diet according to the previously determined value [18]. The birds were raised under artificial lighting providing 18 h light:6 h dark. Relative humidity and average room temperature were approximately 70.0% and 18 °C throughout the 7-week experimental period (9–15 weeks of age).

Table 1. Composition and nutrient levels of the experimental diets (%, as fed basis).

Item	Metabolizable Energy Levels (Kcal/kg, Calculated [2])				
	2900	**3000**	**3100**	**3200**	**3300**
Ingredients					
Corn (yellow)	70.26	70.26	70.26	70.26	70.26
Soybean meal	16.23	16.23	16.23	16.23	16.23
Corn gluten meal	3.8	3.8	3.8	3.8	3.8
Soybean oil	1.05	2.25	3.44	4.64	5.83
Limestone	1.12	1.12	1.12	1.12	1.12
Di-calcium phosphate	1.10	1.10	1.10	1.10	1.10
Salt	0.30	0.30	0.30	0.30	0.30
Vitamin-mineral premix [1]	1.00	1.00	1.00	1.00	1.00
L-Lysine HCL (78%)	0.27	0.27	0.27	0.27	0.27
DL-Methionine (99%)	0.09	0.09	0.09	0.09	0.09
Corn cob meal	4.78	3.58	2.39	1.19	0.00
Total	100.00	100.00	100.00	100.00	100.00
Calculated composition [2]					
Metabolizable energy (Kcal/kg) [3]	2805	2897	2997	3095	3236
Crude protein (%)	16.00	16.00	16.00	16.00	16.00
Calcium (%)	0.80	0.80	0.80	0.80	0.80
Crude fiber	3.07	2.59	2.12	1.64	1.16
Total phosphorus (%)	0.56	0.56	0.56	0.56	0.56
Non-phytate phosphorus (%)	0.37	0.37	0.37	0.37	0.37
Lys (%)	0.85	0.85	0.85	0.85	0.85
Met (%)	0.37	0.37	0.37	0.37	0.37
Met+Cys (%)	0.65	0.65	0.65	0.65	0.65

[1] Supplied per kilogram of diet: VA 5000 IU, VD3 500 IU, VE 20 IU, VK 0.5 mg, VB1 2.4 mg, VB2 4.0 mg, VB6 3.5 mg, VB12 0.01mg, niacin 30 mg, D-calcium pantothenate 10 mg, folic acid 0.55 mg, biotin 0.15 mg, choline chloride 1200 mg, Fe 80 mg, Zn 65 mg, Cu 7 mg, Mn 60 mg, I 0.35 mg, Se 0.3 mg. The vitamins and minerals in the diet were supplied exactly as stated by the Ministry of Agriculture of the People's Republic of China [18]. [2] Values were calculated from data provided by the Feed Database in China [21]. [3] Analyzed values.

2.2. Growth Variables

The amounts of provided and refused feed were measured weekly on a replicate basis to calculate the average daily feed intake (ADFI), including adjustments for any dead birds. Mortality of birds was recorded daily. The initial BW, final BW (FBW), average daily body weight gain (ADG), and feed:gain ratio (g/g) (FCR) were measured on a per replicate basis. Metabolic BW was calculated according to the following equation: $[(\text{Initial weight} + \text{final weight})/2]^{0.75}$.

2.3. Sampling

At 15 weeks of age, after 12 h of feed-withdrawal, blood samples were collected in 5 mL heparinized tubes from the jugular vein of 12 birds per treatment (2/replicate) who had BW values within ± 10 g of the average; plasma was obtained by centrifugation at 1000× g for 15 min at 4 °C. The birds were slaughtered by approved methods for subsequent analyses. The right and left breast muscles were separately sampled, clear of observable connective tissues, and stored at −20 °C until analyses; the right breast muscle (*Pectoralis major* and *minor*) was sampled for meat quality determinations, and the left muscle was used in measuring the chemical composition.

2.4. Carcass Trait Determinations

Dressing percentage (bled and defeathered carcass weight (CW), including head and feet, expressed as a percentage of BW), semi-eviscerated (CW minus weights of trachea, crop, esophagus, intestine, pancreas, spleen, gallbladder, gonads, contents of the proventriculus, and gizzard lining, expressed as a percentage of BW), and eviscerated proportions (semi-eviscerated weight minus neck, head, liver, heart, gizzard, shank, abdominal fat, and proventriculus, expressed as a percentage of BW) were

calculated. In addition, the relative weights of de-boned thigh muscle, breast muscle, and abdominal fat, expressed relatively to BW, were calculated following the methods of the Chinese National Poultry Breeding Committee [22]. The breast and thigh muscles were placed in polyethylene bags and stored at −22 °C until chemical analysis.

2.5. Meat Quality Determinations

Meat pH, color (a* redness, b* yellowness and L* lightness), and drip loss were measured following the methods of Jiang et al. [23]. Meat pH was measured in the major right *Pectoralis* using a portable pH meter (version HI8424; Beijing Hanna Instruments Sci. & Tech. Co., Ltd., Beijing, China). Three readings of breast meat color were scored with a Chroma Meter (CR-410; Minolta Co., Ltd., Suita, Osaka, Japan) at different, but consistent, locations on the medial side of each muscle then averaged. Meat color scores, using L* a* b* color scales, were measured; L* is lightness (0 = black to 100 = white), a* is green (a*) to red (+a*), and b* is blue (b*) to yellow (+b*). Drip loss was estimated following a method modified from Shang et al. [24]. Briefly, about 11 g (fresh weight) of regular-shaped muscle section (4 cm (length) × 2 cm (width) × 1.5 cm (thickness)) cut from the same location in the breast muscle was weighed and suspended on a steel wire hook, without any contact, in a plastic bag inflated with air and stored at 4 °C for 24 h. The muscle samples were re-weighed to evaluate the drip loss percentage, according to the following equation: [(initial weight − final weight)/initial weight] × 100%. Finally, the shear force of cooked breast muscles was measured according to the methods described by Jiang et al. [23], using an Instron Universal Mechanical Machine (Instron model 4411, Instron Corp, Canton, MA, USA).

2.6. Composition of Body, Breast and Thigh Muscles, and Deposition Rate of Energy and Protein

The frozen samples of left breast and thigh muscles were dissected into small pieces and finely homogenized in a blender at −10 °C. To measure the fat and protein content, deposition rate of energy and protein in the whole body, ten birds at the age of 8 weeks (at the beginning of this experiment) and two additional birds per replicate at the age of 15 weeks were selected and prepared according to the methods of Zhou et al. [25] and Xi et al. [26]. Contents of crude protein (CP), crude fat, and gross energy were analyzed according to the guidelines of AOAC [19]. The deposition rate of protein and energy was estimated following the methods of Xi et al. [26].

2.7. Blood Biochemical Variables

The plasma contents of uric acid (UA), triglycerides (TG), and cholesterol (CHOL) were measured colorimetrically using a spectrophotometer (Biomate 5, Thermo Electron Corporation, Rochester, NY, USA) and commercial kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China).

2.8. Statistical Analysis

Each pen (replicate) served as the experimental unit. The effects of dietary ME levels were examined for each variable by ANOVA (JMP Ver. 8.0.2, 2009; SAS Institute Inc., Cary, NC, USA). Whenever significant effects of treatment were detected, Duncan's multiple range tests were used to compare the means. Where appropriate, orthogonal polynomial contrasts were used to estimate the linear and quadratic effects of the increasing levels of ME, and a probability level of 0.05 was applied to test significance (SPSS software version 17.0.1., IBM, Armonk, NY, USA). Based on the key indices (ADFI, feed:gain ratio, daily ME intake, uric acid, fat content of breast muscle, and fat content of thigh muscle), quadratic regression equations were used to determine the optimal dietary ME requirement of Chinese yellow-feathered chickens [27]. Data are expressed as means for each diet.

3. Results

3.1. Growth Performance

Daily ME intake increased, but ADFI and FCR decreased as linear responses to the increment in dietary energy level. The FBW, ADG, metabolic BW, and mortality rate were not affected ($p > 0.05$) by the dietary ME level, but the 3236 kcal/kg diet tended to have greater FBW and ADG than those of the lower ME levels (Table 2).

3.2. Carcass Quality

The tested dietary ME levels did not exhibit any significant effect on the carcass quality traits in terms of dressing percentage, eviscerated and semi-eviscerated proportions, relative weights of breast muscle, thigh muscle, and abdominal fat (Table 3).

3.3. Composition of Body, Breast and Thigh Muscles

As shown in Table 4, the fat content in thigh muscle increased linearly ($p < 0.05$) with the increase in dietary energy level, whereas the fat content in breast muscle showed a quadratic response ($p < 0.05$), and the highest value was obtained with the level 2997 kcal/kg. The protein, fat and energy content in the whole body as well as the energy and protein deposition were not affected by the dietary ME level. According to the regression model, the highest fat contents (%) in the breast and thigh muscles were obtained with diets containing 3047 and 3135 kcal/kg (Table 5).

3.4. Breast Meat Quality

The results of breast meat quality as affected by the dietary ME level are shown in Table 6. The 2897, 3095 and 3236 kcal/kg diets resulted in lower shear force values ($p < 0.05$) than those of the control diet, and those of the 2805 kcal/kg diet had an intermediate value ($p > 0.05$). The pH value, drip loss percentage, and meat color grades L*, a* and b* did not differ ($p > 0.05$) among the tested diets.

3.5. Blood Biochemical Variables

The results shown in Table 7 indicated that plasma UA decreased linearly ($p < 0.01$) with the increase in dietary ME level. The CHOL and TG concentrations were not affected by the diets. The regression model indicated that the optimal plasma UA was obtained with a diet containing 3200 kcal/kg (Table 3).

Table 2. Effect of dietary metabolizable energy level on average daily metabolizable energy intake and the performance of Chinese yellow-feathered chickens from 9–15 weeks of age.

Variables	Dietary Metabolizable Energy Levels (Kcal/kg), Analyzed Content					SEM	*p*	Linear	Quadratic
	2805	2897	2997	3095	3236				
Final body weight (g)	1386.6	1375.09	1408.08	1385.39	1430.12	16.85	0.0629		
Average daily gain (g)	12.82	12.58	13.27	12.79	13.73	0.35	0.0629		
Daily feed intake (g)	78.15 ^{a}	78.32 ^{a}	77.59 ^{a}	75.28 ^{b}	76.06 ^{b}	0.59	0.0005	0.000	0.002
Feed: Gain ratio (g/g)	6.10 ^{ab}	6.24 ^{a}	5.86 ^{bc}	5.90 ^{b}	5.56 ^{c}	0.13	0.0025	0.001	0.002
Daily metabolizable energy Metabolizable energy intake (kJ/d)	219.21 ^{d}	228.45 ^{c}	232.53 ^{bc}	233.52 ^{b}	246.06 ^{a}	5.69	<0.0001	0.000	0.000
Metabolic body weight (g)	188.25	189.04	189.79	188.60	190.71	1.98	0.5248		
Mortality (%)	3.33	4.16	5.12	6.67	4.17	2.06	0.6668		

Means within a row with different superscripts differ significantly ($p < 0.05$). SEM = pooled standard error mean. Metabolic body weight = [(Initial weight + final weight)/2] $^{0.75}$.

Table 3. Effects of dietary metabolizable energy level on the carcass quality of Chinese yellow-feathered chickens at 15 weeks of age.

Variables	Dietary Metabolizable Energy Levels (Kcal/kg), Analyzed Content					SEM	*p*
	2805	2897	2997	3095	3236		
Dressing percentage (%)	89.68	88.93	88.98	87.89	88.84	1.01	0.1116
Semi-eviscerated proportion (%)	83.10	82.80	82.31	82.01	82.16	1.27	0.5329
Eviscerated proportion (%)	68.99	68.51	68.74	67.63	68.05	1.01	0.2321
Breast muscle (%)	15.60	14.62	14.95	15.19	14.23	0.63	0.0906
Thigh muscle (%)	18.87	19.05	18.42	19.12	18.89	1.71	0.9304
Abdominal fat (%)	1.10	0.87	1.29	1.71	1.63	0.29	0.0889

SEM = pooled standard error mean.

Table 4. Effect of dietary metabolizable energy levels on the compositions of body, breast and thigh muscles, and deposition rates of energy and protein in slow-growing Chinese yellow-feathered chickens at 15 weeks of age.

Variables	Dietary Metabolizable Energy Levels (kcal/kg), Analyzed Content					SEM	*p*	Linear	Quadratic
	2805	2897	2997	3095	3236				
Composition of body									
Crude protein (%)	65.31	62.56	62.43	60.58	60.77	8.90	0.4481		
Fat (%)	22.73	25.06	24.19	25.85	27.14	7.95	0.5656		
Energy (kJ/g)	23.20	24.18	24.02	24.05	24.05	0.44	0.4839		
Intramuscular fat content (%)									
In breast muscle	0.94 ^{b}	1.12 ^{ab}	1.64 ^{a}	1.37 ^{ab}	1.31 ^{ab}	0.056	0.0441	0.386	0.016
In thigh muscle	5.12 ^{b}	5.56 ^{ab}	6.86 ^{a}	6.18 ^{ab}	6.64 ^{a}	0.36	0.0288	0.025	0.033
Nutrient deposition rate (%)									
Energy	12.71	14.82	14.80	15.48	15.18	0.85	0.0755		
Protein	25.03	25.58	26.21	25.01	26.88	1.41	0.2732		

Means within a row with different superscripts differ significantly ($p < 0.05$). SEM = pooled standard error mean.

Table 5. Dose-response regressions for Chinese yellow-feathered chickens fed diets with different metabolizable energy levels from 9–15 weeks of age.

Variable	Model [1]	Regression Equation [2]	Response [3]	*p*	R^2
Uric acid (mmol/L)	QP [1]	$y = 21.494x^2 - 575.47x + 3984$	3200	0.012	0.144
Fat content of breast muscle (%)	QP [1]	$y = -0.572x^2 + 14.587x - 91.435$	3047	0.016	0.142
Fat content of thigh muscle (%)	QP [1]	$y = -0.715x^2 + 18.765x - 116.62$	3135	0.033	0.116

[1] QP = quadratic polynomial; QP model = $Y = \alpha + \beta \times X + \gamma \times X^2$, where Y is the response variable, X is the dietary metabolizable energy (ME), α is the intercept; β and γ are the linear and quadratic coefficients, respectively. [2] Regression equations obtained using the analyzed metabolizable energy in the diets (2805, 2897, 2997, 3095 and 3236 Kcal/kg). [3] The response was obtained by $-\beta/(2 \times \gamma)$.

Table 6. Effects of dietary metabolizable energy levels on the breast meat quality of slow-growing Chinese yellow-feathered chickens at 15 weeks of age.

Variables	Dietary Metabolizable Energy Levels (Kcal/kg), Analyzed Content					SEM	*p*	Linear	Quadratic
	2805	2897	2997	3095	3236				
pH	6.11	6.00	6.08	6.06	6.02	0.009	0.736		
Drip loss (%)	2.06	2.22	2.19	2.06	2.08	0.076	0.948		
Shear force (kgf)	3.06 ^{ab}	2.49 ^{b}	3.52 ^{a}	2.31 ^{b}	2.62 ^{b}	0.001	0.023	0.241	0.487
Meat color									
L* value	55.54	55.99	55.91	54.01	55.96	1.649	0.399		
a* value	15.02	15.24	16.46	15.76	14.77	0.572	0.136		
b* value	20.16	20.54	21.98	19.19	21.58	2.278	0.279		

Means within a row with different superscripts differ significantly ($p < 0.05$). SEM = pooled standard error mean.

Table 7. Effects of dietary metabolizable energy levels on plasma variables of slow-growing Chinese yellow-feathered chickens at 15 weeks of age.

Variables	Dietary Metabolizable Energy Levels (Kcal/kg), Analyzed Content					SEM	*p*	Linear	Quadratic
	2805	2897	2997	3095	3236				
Cholesterol (mmol/L)	3.12	3.19	3.22	3.09	3.13	0.07	0.9687		
Triglycerides (mmol/L)	0.32	0.33	0.377	0.37	0.33	0.002	0.3375		
Uric acid (mmol/L)	197.00 ^{a}	159.83 ^{ab}	156.13 ^{ab}	117.21 ^{b}	134.79 ^{b}	3.81	0.0109	0.005	0.012

Means within a row with different superscripts differ significantly ($p < 0.05$). SEM = pooled standard error mean.

4. Discussion

4.1. Growth Performance

The present study tested five dietary ME levels (kcal/kg), consisting of a control level (2997), two lower levels (2805 and 2897), and two higher levels (3095 and 3236), respectively. The increase in dietary energy level did not affect the FBW or ADG of the slow-growing male yellow-feathered chickens, but the highest ME treatment (3236 kcal/kg) tended to result in greater FBW and ADG than lower ME levels. The daily ME intake increased, whereas ADFI and FCR decreased as linear responses to the increment in dietary energy level. Birds typically eat to fulfil their energy requirement [11,28], which can explain the reduced ADFI for the highest two dietary energy levels. The improved FCR for the highest ME level is attributable to the reduced ADFI and the relatively increased ADG. Supporting results were reported by Infante-Rodríguez et al. [4], indicating that BW and ADG were not affected by the dietary energy; however, ADFI was reduced by a high caloric level, and FCR was improved with a moderate increase in dietary energy. The present results were consistent with the findings of Kim et al. [29], who observed a reduced ADFI with higher energy levels than the standard diet. Other studies differed [30], where final BW and FCR in broilers increased with higher energy levels (2994 to 3013 and 3081 to 3111 kcal/kg ME, starter and finisher phases). Contrary to the present results, Houshmand et al. [31] found that broilers fed low-energy diets were heavier than those fed a standard diet. The results of Ferreira et al. [3] showed that a dietary energy close to 3000 kcal/kg did not affect BW in broilers, but a lower caloric level reduced BW, and a higher caloric level reduced ADFI. These varied responses to dietary energy levels in previous studies result from using different genotypes at different ages. Kim et al. [29] reported different responses to energy level with different strains of broilers. The results obtained here with slow-growing Chinese yellow chickens favor the increase in energy level over the control (2997 kcal/kg) and lower levels; the highest calorie intake occurred with the most energy-dense diet. Touchburn et al. [32] similarly noted that caloric intake increased as dietary ME level increased.

4.2. Carcass Characteristics

For the slow-growing Chinese yellow chickens studied here, dietary ME level had no significant effect ($p > 0.05$) on the dressing percentage, eviscerated and semi-eviscerated proportions, relative weights of breast muscle, thigh muscle nor abdominal fat. Supporting results were reported by Infante-Rodríguez et al. [4], who tested dietary energy levels (2960 to 3160) close to those used here; there was no influence on carcass weight, breast, drumstick and thighs, wings and back fat weight or carcass yields. Rosa et al. [33] used diets with 2950, 3200 and 3400 kcal/kg ME, but observed no effect on breast weight, carcass yield or back fat, despite the increase in energy concentration depressing the yield of thigh and drumstick and increasing abdominal fat. A preliminary study of Waldroup et al. [34] indicated no effect of dietary caloric level on growth performance or abdominal fat, although a higher energy level increased dressing percentage in females, but not in males. The present results with male chickens are consistent with that of the latter study. Others [35,36], similarly, found no effect of dietary energy level on carcass yield and abdominal fat. In contrast, Zhao et al. [37] found that dressing percentage, breast and thigh muscles, and abdominal fat content were greater with dietary energy and lysine levels higher than those in their controls. Marcu et al. [38] reported an improved growth performance and carcass yield for the main cuts of broiler chickens fed diets with high energy and protein contents. The preponderant previous findings on the effect of dietary energy level in broilers were inconclusive, but the results of the latter two studies showed that increased dietary energy along with increased CP or amino acids may result in a higher meat yield.

4.3. Composition of Whole Body, Breast and Thigh Meat

In the present study, dietary energy level did not influence the protein, fat or energy content in the whole body, but the fat content in thigh muscle increased linearly with increased caloric level, whereas the fat content showed a quadratic response and the highest value was obtained with the 2997 kcal/kg diet. Other studies showed similar results, with dietary energy level having no effect on the chemical composition of broiler's carcass muscles [39,40]. Ferreira et al. [3] indicated that using reduced dietary energy levels lowered the intramuscular fat in broilers. The present results are in partial agreement with those of Infante-Rodríguez et al. [4], who found that increased dietary energy had no effect on CP content in breast muscle, although the lower ME levels (2960 and 3040 kcal/kg) resulted in more lipids in breast meat than with higher caloric levels (3080 and 3160 kcal/kg). In another study, Marcu et al. [41] found that decreasing dietary ME level reduced CP and increased the lipid content of broiler breast and thigh muscles. The results here showed that the fat (Table 5) content in the whole body was not affected by the dietary energy. The latter results agree with our results, which suggest that increasing dietary energy content for broilers may not increase meat lipids in the thigh and pectoral muscles.

In commercial Ross 308 broilers, Rosa et al. [33] reported that increasing the dietary ME level reduced carcass CP and increased its lipid content. Marcu et al. [42] found that increasing dietary energy and protein levels increased breast weight and muscle mass, and reduced fat content, but reducing nutrient level decreased protein content and elevated fat content in pectoral muscle. The discrepant results could be attributable to using different strains in the previous studies. Díaz et al. [43] and Rosa et al. [33] reported different changes in meat quality and carcass composition among different genetic groups fed graded levels of dietary energy.

4.4. Breast Meat Quality

The color of raw broiler meat is highly affected by dietary nutrient factors [5]. Meat color is an important attribute for consumers; the greater a* score of meat indicates better meat quality and the lower L* and b* scores implies less pale meat. Boulianne and King [44] reported that pale fillets have higher L* and b* values, and a lower a* value than normal fillets. No available information could be found on possible effects of dietary energy level on breast meat color. The most important finding in the present study is related to the shear force measured on the breast muscle, which decreased in the 2897, 3095 and 3236 kcal/kg diets. Increased shear force is associated with increased connective tissue and decreased fat content in meat [45,46]. This implies a reduced content of connective tissue in the breast meat of birds fed the 2897, 3095 and 3236 kcal/kg diets. The measured fat contents for the 3095 and 3236 levels were consistent with this interpretation to some extent. The control level (2997) had relatively higher ($p > 0.05$) breast fat; however, it unexpectedly showed a high shear force value. The reason behind this increased shear force with this energy level is not clear, or it might imply a high content of connective tissue in this treatment. Low drip loss and shear force indicate higher meat quality. Higgins et al. [47] and Min and Ahn [48] reported that increased drip loss and decreased meat color a* score reflects lipid peroxidation, leading to loss of pigments and deterioration of meat quality. Drip loss, meat color, and meat pH were not affected here by the dietary caloric levels used.

4.5. Blood Biochemical Variables

The evaluation of blood biochemistry in poultry shows metabolic alterations due to a number of factors, such as the physiological status, feeding standards, weather change, genetic type, age, housing conditions, and exposure to diseases [49–51]. The modification of dietary nutrient concentration can initiate stresses that induce dramatic changes in blood biochemistry [51,52]. In the present study, the increase in dietary ME level led to a linear decrease in plasma UA concentrations, likely reflecting changes in protein catabolism in the body [52]. The plasma UA values obtained here were comparable to those of Wang et al. [53], with the same chicken breed. According to the regression model, the lowest plasma UA was obtained with the 3200 kcal ME/kg diet, suggesting therefore that this level was optimal

or most adequate for the efficient use of protein. This confirms that high caloric levels (3236 kcal/kg) are more adequate than the lower levels, which led to higher plasma UA content. This is consistent with Rosebrough, McMurtry, & Vasilatos-Younken [54,55], who found that reducing dietary energy increased broiler's plasma UA content, and partially agreed with Rezaei and Hajati [52], who found in broilers at 21 d of age that a 40% reduction in dietary nutrients increased the concentrations of plasma UA; in contrast, they also found reduced plasma concentrations of CHOL and TG.

5. Conclusions

The increase in dietary energy level showed some benefits, with lowered ADFI, FCR, plasma UA, and shear force; without any adverse effect on the other meat quality variables, i.e., meat yield, nutrient deposition, mortality rate, or abdominal fat content. Under the conditions of this study, the 3095 kcal/kg diet was adequate for the best feed intake, shear force, and plasma uric acid, and the 3236 kcal/kg diet tended to increase the body weight and daily gain and reduce the feed conversion ratio of Lingnan males between 9–15 weeks of age; further studies are still required for testing higher ME levels. The regression analyses revealed that the optimal dietary ME levels for plasma UA, fat content in breast muscle, and fat content in thigh muscle were 3200, 3047, and 3135 kcal/kg, respectively.

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Effects of Dietary Xylanase and Arabinofuranosidase Combination on the Growth Performance, Lipid Peroxidation, Blood Constituents, and Immune Response of Broilers Fed Low-Energy Diets

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Simple Summary: Arabinoxylans (AXs) constitute the major non-starch polysaccharides (NSPs) existent in maize and soybean meal, comprising about 52% and 65% of the total NSP. Previous works have illustrated that the incorporation of arabinofuranosidase (Abf; GH51) plus xylanase (Xyl; GH11) enhanced the dry matter digestibility of maize and wheat in vitro, in comparison with Xyl alone. In broilers, the combination of dietary Xyl and Abf (Rovabio® Advance) enhanced energy, fat, fiber, and protein utilizations. This study shows the effect of feeding low-energy diets with or without Rovabio® Advance, including high concentrations of Xyl and Abf, on the growth performance, nutrient digestibility, lipid peroxidation, blood constituents, and immune response of broilers. Our results confirm the improved growth, digestibility, and immunity obtained by enzymes supplementation. Furthermore, diets supplemented with enzymes caused a higher antibody titer against the Newcastle disease virus. Moreover, they enhanced plasma lipid profiles and antioxidation.

Abstract: The present study was conducted to examine that impact of dietary xylanase (Xyl) and arabinofuranosidase (Abf) supplementation on the performance, protein and fat digestibility, the lipid peroxidation, the plasma biochemical traits, and the immune response of broilers. A total of 480, un-sexed, and one-day-old broilers (Ross 308) were randomly divided into three treatments with eight replicates, where chicks in the first treatment were fed basal diets and served as the control, chicks in the second treatment were fed diets formulated with reductions of 90 kcal/kg, and chicks in the third treatment were fed the same formulated diets used in the second group as well as the Xyl and Abf combination (Rovabio® Advance). Feed intake was decreased by the low energy diet, leading to an enhancement in feed efficiency enzyme supplementation in the low energy diet ($p < 0.015$). Both protein and fat digestibility were improved ($p < 0.047$) due to enzyme supplementation. Moreover, enzyme supplementation increased muscle total lipids content and decreased muscle thiobarbituric acid retroactive substance content. Furthermore, diets supplemented with Xyl and Abf exhibited an increase in antibody titers against the Newcastle disease virus ($p < 0.026$). In addition, enzyme supplementation increased gene expression related to growth and gene expression related to fatty acid synthesis. It could be concluded that dietary Xyl and Abf supplementation had beneficial impacts on growth, nutrient digestibility, lipid peroxidation, immune response, and gene expressions related to growth and fatty acid synthesis in broiler chickens fed low-energy diets.

Keywords: xylanase; arabinofuranosidases; broilers; nutrient utilization; growth performance; immunity

1. Introduction

Nowadays, in the broiler production industry, the total price of energy ingredients is about 65–70% of the total costs of the broiler diets. Additionally, these ingredients are usually imported from outside Egypt. Therefore, diverse experiments have been conducted to decrease the cost by reducing the rate of these energy ingredients in broiler feed, along with animating the performance of broilers [1]. One scenario involves adding enzymes to the broiler diets, which promotes such growth performance parameters as feed efficiency and body weight gain [2]. Indeed, the presence of soluble non-starch polysaccharides (NSPs) has reduced nutrient utilization and consequently minimized growth performance in broilers. These carbohydrates cannot be digested by birds, as they do not have the capability to produce these enzymes. Therefore, NSP enzymes are functional when they are added to cereal-based diets, e.g., wheat, soybean, barley, and maize. Exogenous enzymes like xylanase (Xyl), amylase, and protease are produced using a microbial source [3]. Almirall et al. found that the feed conversion ratio (FCR) was enhanced by exogenous enzyme supplementation in broiler diets, and this effect was connected with improving the digestibility and minimizing the viscosity of intestinal contents [4]. Furthermore, Abdel-Latif et al. and Saleh et al. illustrated that the improvement of growth performance due to NSP enzyme addition might be explained by their participation in reducing the digesta viscosity and amendment of gut microbiota by improving the beneficial microbes [3,5]. Worldwide broiler production, including in Egypt, farmers depend on corn and soybean meal for feeding birds, as these are the available ingredients; however, the level of NSP is 29% in soybean and 9% in corn [6]. Broilers do not produce enzymes for the hydrolysis of these NSPs present in the cell walls of the grains [7,8]. Exogenous enzymes which can hydrolyze NSPs are abundantly found in the feed given to birds [9].

Arabinoxylans (AXs) exemplify the major NSP existent in maize and wheat, comprising about 4.7% and 7.3% of the dry matter and 65% and 52% of the total NSP, respectively [10]. Previous studies showed that a mixture of arabinofuranosidase (Abf) and Xyl improved the dry matter digestibility of maize and wheat in vitro, in comparison with Xyl alone [11]. In fact, endoxylanases support the degradation of AXs by hydrolyzing the xylan backbone. Additionally, arabinose substitutions minimized the activity of Xyl in yellow corn and its correlating byproducts [10]. Abf could split the xylose backbone in arabinose and give access to endoxylanase activity [12]. Moreover, Cozannet et al. illustrated that dietary Rovabio® Advance (including high concentrations of Xyl and Abf) had a positive effect on the energy utilization and digestibility of starch, protein, fat, and insoluble and soluble fibers [11]. Furthermore, Ravn et al. documented that the addition of an enzyme combination (Xyl and Abf) improved the growth performance and gut morphology in broilers [13]. It could be hypothesized that the supplementation of a combination of Xyl and Abf to broiler diets might be involved in improving the utilization of nutrients of a low-energy diet and could consequently enhance the growth performance and immune responsiveness of broilers. The objective of this study was to examine the impact of dietary Xyl and Abf (Rovabio® Advance) supplementation on the growth performance, fat and protein digestibility, lipid peroxidation, biochemical plasma traits, and immune response of broilers fed low-energy diets.

2. Materials and Methods

2.1. Animals and Experimental Design

The study was approved by the Ethics Committee of Local Experimental Animals Care Committee and conducted in accordance with the guidelines of Kaferelsheikh University, Egypt (Number 4/2016 EC). A total of 480 un-sexed one-day-old broilers (Ross 308) were housed in bins (stocking density

was 10 birds/m^2) and randomly divided into 3 experimental treatments with 8 replicates (20 birds each) to equalize the average body weight in each group. The control group was fed basal diets as commercial feed formulated according to the strain requirements, the second experimental group of chicks was fed diets formulated with reductions of 90 kcal/kg AME and 3% digestible amino acids, and the third experimental group was fed the same formulated diets used in the second group as well as Xyl and Abf. The composition and chemical analysis of the experimental diets (starter, grower, and finisher) are shown in Table 1. The Xyl and Abf (Rovabio® Advance) were kindly given by the Adisseo company, France S.A.S. Antony Parc 210, Place du Général de GaulleF-92160 ANTONY, France. ® This enzyme was industrially created by the fermentation of *Talaromyces versatilis* (IMI378536 and DSM26702; Adisseo France S.A.S. proprietary strains), and the main enzyme activity in Enz comes from Xyl and Abf. The enzyme was added to the premix mixture, which is one of the basic ingredients in the all diets. The diets were provided to the birds ad libitum; starter diets were in crumble form. However, the grower and finisher diets were pellet form. The trail was managed in an open-door house with a 23 h light–1 h dark cycle. Daily temperature and humidity inside the house were controlled at 24–26 °C and 60–70%, respectively. The experimental diets were offered from 1 day to 35 days of age. Bird body weight was measured individually every week. However, feed intake was measured daily (on a group basis per pen) throughout the experimental period. At 32 days, all birds were weighed individually and sorted from the smallest to the heavy weight. Then, 12 male birds/treatment have the same average weight were transferred to special batteries containing individual cages to enact the digestibility experiment. Then, these birds were slaughtered and dissected to gauge the weights of the breast muscle, thigh muscle, liver, gizzard, heart, spleen, abdominal fat, and bursa of Fabricius. All organs were weighed and described as a ratio of the body weight. Blood samples were collected from the wing vein immediately before slaughtering, gathered into heparinized test tubes, and then rapidly centrifuged (3000 rpm for 20 min at 5 °C) to separate the plasma. Plasma was stored at −20 °C pending analysis.

Table 1. Composition of the experimental starter, grower, and finisher diets.

Ingredient	Starter (1–10 days)		Grower (11–25 days)		Finisher (26–35 days)	
	Control	Low Energy	Control	Low Energy	Control	Low Energy
Yellow corn	507	543	548	584	578	613
Soybean meal, 46%	370	352	317	300	280	266
Corn gluten meal, 60%	38	39	50	50	50	47
Soya oil	37	17	41	22	51	32
Calcium carbonate	14.0	14.8	13.8	14.0	12.6	13.4
Dicalcium phosphate	20.0	20.0	17.5	17.5	16.0	16.0
Salt	2.3	2.3	2.4	2.4	2.3	2.3
Sodium sulfate	1.8	1.8	1.6	1.6	1.6	1.6
Dl Methionine, 99%	2.7	2.4	2.0	1.8	1.9	1.8
L-Lysine HCl, 98%	2.5	2.4	2.3	2.3	2.2	2.2
L-Threonine	1.1	1.0	0.7	0.7	0.6	0.6
Choline chloride, 60%	0.8	0.8	0.8	0.8	0.8	0.8
Premix *	2	2	2	2	2	2
Anticoccidia	0.2	0.2	0.2	0.2	0.2	0.2
Anticlostridia	0.1	0.1	0.1	0.1	0.1	0.1
Antimycotoxin biology	0.25	0.25	0.25	0.25	0.25	0.25
Silica	1	1	1	1	1	1
Chemical Analysis on DM basis						

Table 1. *Cont.*

Ingredient	Starter (1–10 days)		Grower (11–25 days)		Finisher (26–35 days)	
	Control	Low Energy	Control	Low Energy	Control	Low Energy
AME kcal	3000	2910	3100	3010	3200	3110
Crude protein, %	23.0	22.4	21.5	20.9	20.0	19.4
Fat, %	6.3	4.5	6.9	5.1	7.9	6.2
Digestible LYS, %	1.28	1.24	1.15	1.11	1.06	1.02
Digestible M and C, %	0.95	0.92	0.87	0.84	0.83	0.80
Digestible THR, %	0.86	0.83	0.77	0.74	0.71	0.68
Digestible ARG, %	1.37	1.33	1.25	1.20	1.14	1.11
Digestible ILE, %	0.90	0.87	0.85	0.82	0.77	0.74
Digestible LEU, %	1.87	1.83	1.84	1.78	1.74	1.68
Digestible VAL, %	0.96	0.93	0.91	0.87	0.84	0.81
Calcium, %	0.96	0.96	0.87	0.84	0.81	0.76
Available P, %	0.48	0.48	0.44	0.42	0.41	0.38
Sodium, %	0.16	0.16	0.16	0.16	0.16	0.16
Chloride, %	0.23	0.23	0.23	0.23	0.23	0.23

* Hero mix® (Hero pharm, Cairo, Egypt). Composition (per 3 kg): Vitamin A 12,000,000 IU, vitamin D3 2,500,000 IU, vitamin E 10,000 mg, vitamin K3 2000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1500 mg, vitamin B12 10 mg, niacin 30,000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid 10,000 mg, manganese 60,000 mg, zinc 50,000 mg, iron 30,000 mg, copper 4000 mg, iodine 300 mg, selenium 100 mg, and cobalt 100 mg. Diets ingredients and final feed diets were analyzed by chemical analysis in the Adisseo company lab, Antony, France.

2.2. Nutrient Digestibility

In the last three days of the experiment, excreta were gathered and weighted from 12 males per treatment, where broilers were housed individually in special metabolic cages (40 × 40 × 50 cm) for digestibility tests. During these three days, the birds and feed intake were weighted daily, and extracted faces were collected, weighted, and stored in a freezer. After the digestibility experiment period, all samples were dried in a drying oven at 60 °C for 24 h. The whole dried samples were then homogenized. Samples were taken and finely ground for analysis according to the Association of Official Analytical Chemists (AOAC) [14]. The crude protein concentration in the diet and excreta was gauged to determine nitrogen digestibility using the Kjeldahl method, and crude fat was gauged by the Soxhlet method (AOAC 945.38 F and 920.39 C, respectively). The calculation was as follows: Nitrogen digestibility (%) = (total nitrogen intake − total nitrogen excreted)/total nitrogen intake × 100.

2.3. Biochemical Analysis

Triglycerides (TG), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), glucose, creatinine, total protein, albumin, and globulin were measured colorimetrically using commercial kits (Diamond Diagnostics, Egypt) according to the procedure outlined by the manufacturer. Muscle total lipid content, fatty acid profile, and amino acid analysis were measured using gas liquid chromatography (GLC) according to the method of Saleh [15]. The muscle thiobarbituric acid retroactive substance (TBARS) concentration was measured by the process of Ohkawa et al. [16].

2.4. Serum Antibody Titers

Serum antibody titers against Newcastle disease (ND) and avian influenza (H9N1) were determined by means of the hemagglutination inhibition test using standard methods qualified by OIE [17].

2.5. RNA Analysis

Each breast muscle sample was homogenized, and the total RNA was extracted using a total RNA purification kit following the manufacturer's protocol (Fermentas, K0731, Thermo Fisher Scientific, Waltham, MA, USA). The extracted total RNA (5 μg per sample) was reverse transcribed into cDNA using Revert Aid H Minus Reverse Transcriptase as described by the manufacturer (Fermentas, EP0451, Thermo Fisher Scientific, Waltham, MA, USA). Following amplification, PCR products were electrophoresed, and the expression level of different bands was analyzed using the ImageJ gel analysis program [18].

2.6. Statistical Analysis

The differences between the experimental treatments and the control were analyzed with a General Liner model using SPSS Statistics 17.0 (Statistical Packages for the Social Sciences, SPSS Inc., Chicago, IL, USA, released 23 August 2008). Tukey's multiple comparison test was used to identify which treatment conditions were significantly different from each other.

3. Results and Discussion

One of the major objectives of the current study was to evaluate the impacts of feeding low-energy diets supplemented with or without Xyl and Abf enzymes on the growth performance, nutrient digestibility, lipid peroxidation, blood plasma biochemical traits, immune response, and gene expressions related to growth and fatty acid synthesis in broilers. The inclusion of Xyl and Abf enzymes in low-energy diets in the present study improved the growth performance in broilers, and this improvement might be related to the enhancement of nutrient digestibility by Xyl and Abf enzyme supplementation. This supposition is in harmony with Nortey et al., who suggested that dietary exogenous enzyme addition had a beneficial effect on nutrient digestibility in swine specimens [19]. Moreover, Slominski et al. reported that the inclusion of a debranching enzymes mixture improved the overall enzyme effectiveness and, consequently, enhanced the nutrient digestibility and the alleviation of the negative impacts of NSPs [20]. Recently, Ravn et al. stated that the addition of an enzyme combination (Xyl and Abf) improved duodenum villi length, which was probably involved in enhancing the growth performance, including the body weight and FCR, in broilers [13].

The data presented in Table 2 show that feeding low-energy diets decreased body weight gain significantly compared to the control group, while, dietary supplementation with Xyl and Abf enzymes increased body weight gain and improved FCR, crude protein digestibility, and crude fat digestibility significantly ($p < 0.05$). No significant differences were detected in the feed intake. These findings are in correspondence with Cozannet et al., who demonstrated that a dietary combination of Xyl and Abf had a positive effect on the energy utilization and digestibility of protein, starch, fat, and insoluble and soluble fibers [11]. Additionally, Cowieson and Ravindran reported improvements in crude protein and amino acid digestibility when a multiple enzyme mixture including protease, Xyl, and amylase was employed to supplement corn–soybean diets [21]. Similarly, Rutherfurd et al. found enhancements in crude protein and amino acid digestibility in broilers fed commercial diets supplemented with a multiple enzymes complex, including amylase, β-glucanase, and Xyl [22]. Cowieson and Ravindran reported that the mechanisms that enhanced the amino acid utilization due to the addition of exogenous enzymes are connected with minimizing endogenous losses related to a decreased secretion of endogenous enzymes [23]. Moreover, Meng et al. stated that dietary enzymes take off the nutrient encapsulating effect of NSP [24], thus enhancing the nutrient availability to endogenous enzymes and improving the overall nutrient digestibility and intestinal microbial environment [11,21].

Table 2. Effect of non-starch polysaccharide (NSP) enzyme supplementation on growth performance in broilers.

	Control	Low Energy	Low Energy and Enzymes	*p*-Value
Initial body weight, g	40.9 ± 0.5	40.6 ± 0.7	41.0 ± 0.5	0.29
Final body weight, g/35 d	2382 ± 52 ^{a}	2296 ± 57 ^{b}	2358 ± 56 ^{ab}	0.03
Body weight gain, g/35 d	2341 ± 21.9 ^{a}	2255 ± 27.2 ^{b}	2317.5 ± 16.4 ^{ab}	0.04
Feed intake, g/35 d	3485 ± 34.5	3500 ± 38.9	3410 ± 32.7	0.18
FCR, g/g	1.49 ± 0.02 ^{b}	1.55 ± 0.01 ^{a}	1.47 ± 0.02 ^{b}	0.02
Crude protein digestibility, %	72 ± 3.6 ^{a}	64 ± 5.4 ^{b}	71 ± 4.8 ^{a}	0.05
Crude fat digestibility, %	44 ± 2.4 ^{b}	41 ± 3.5 ^{b}	52 ± 4.3 ^{a}	0.05

^{a,b} Mean values with different letters in the same raw differ significantly at $p < 0.05$. Values are expressed as means ± standard error. The NSP enzyme used in this experiment was Rovabio® Advance. FCR: Feed conversion ratio.

The bursa of Fabricius relative weight was significantly increased by feeding low-energy diets supplemented with Xyl and Abf enzymes, while the abdominal fat relative weight was significantly decreased in both low-energy diets and control. However, the carcass, thigh, liver, gizzard, heart, and spleen relative weights were not affected by low-energy diets supplemented with or without a combination of Xyl and Abf enzymes (Table 3). These findings are in agreement with previous reports [25,26]. However, Farran et al. found that breast muscle, pectoralis major, thigh, and drum yields were not affected by the inclusion of enzyme preparations [27]. Garipoglu et al. reported that the dressing percentage was reduced but abdominal fat weight was not influenced by feeding diets supplemented with multienzymes [28]. Similarly, Kocher et al. found that found that a Xyl, amylase and protease addition to the corn–soybean meal did not affect abdominal fat weight [29]. Contrarily, Garcia et al. reported that the Xyl and β-glucanase supplementation of barley–wheat-based diets elevated the abdominal fat content in broilers [30]. In the present study (Table 3), the lower abdominal fat relative weight noted in low-energy diets, with or without enzyme supplementation, might be attributed to the fact that the lower energy diets caused less fattening and were also connected with reducing the feed intake.

Table 3. Effect of NSP enzyme supplementation on organ weights (g/100 g BW) in broilers.

	Control	Low Energy	Low Energy and Enzymes	*p*-Value
Carcass	64 ± 0.63	65 ± 0.90	63 ± 0.63	0.33
Breast muscle weight	23 ± 0.49 ^{ab}	22 ± 0.63 ^{b}	25 ± 1.11 ^{a}	0.07
Thigh muscle weight	18 ± 0.37	18 ± 0.34	17 ± 0.29	0.75
Liver weight	1.85 ± 0.07	1.72 ± 0.08	1.82 ± 0.17	0.70
Gizzard weight	1.28 ± 0.08	1.28 ± 0.08	1.19 ± 0.06	0.61
Heart weight	0.37 ± 0.02	0.39 ± 0.01	0.43 ± 0.03	0.27
Spleen weight	0.14 ± 0.02	0.11 ± 0.08	0.12 ± 0.02	0.46
Abdominal fat weight	1.18 ± 0.12 ^{a}	0.81 ± 0.09 ^{b}	0.89 ± 0.02 ^{b}	0.02
Bursa of Fabricius weight	0.13 ± 0.02 ^{b}	0.14 ± 0.02 ^{b}	0.20 ± 0.02 ^{a}	0.04

^{a,b} Mean values with different letters in the same raw differ significantly at $p < 0.05$. Values are expressed as means ± standard error. The NSP enzyme used in this experiment was Rovabio® Advance. BW: Body weight.

Table 4 shows the effect of low-energy diets supplemented with or without Xyl and Abf enzymes on blood biochemical parameters. Plasma globulin and HDL-cholesterol were significantly increased due to dietary supplementation in comparison with the control group. However, plasma total cholesterol was significantly reduced by the addition Xyl and Abf enzymes to low-energy diets compared with the control group; however, plasma GOT, GPT, albumin, triglycerides, glucose, LDL-cholesterol, and creatinine were not significantly affected. Interestingly, serum antibody titers against ND were significantly increased by the enzyme group, while the antibody titer against avian influenza (H9N1) was enhanced insignificantly in comparison with the control group ($p = 0.11$). However, there was an

insignificant increase in the antibody titer against avian influenza (H9N1) due to the dietary Xyl and Abf enzyme supplementation to low-energy diets. These results supported our findings of the bursa of Fabricius relative weight, which was significantly increased due to the dietary Xyl and Abf enzyme supplementation. These results are coincident with our previous findings, which noted that ND and infectious bronchitis disease (IBD) antibody titers were improved by enzyme addition to broiler diets, and this may be regarded as an improvement in protein digestibility because of these enzyme mixtures and peptide transporter 1(PEPT1) gene expression, which enhanced absorption [3]. Different authors have reported the impacts of supplementing AX or arabinoxylooligosaccharides in a broiler diet, and they have observed that oligosaccharides with a polymerization score of less than five encourage the propagation of beneficial bacteria and enhance microbiota variety [31–33]. The increment of arabinoxylooligo-saccharides (AXOS) presented in wheat or soybean AX improves the proliferation of bifidobacteria in ceca without influencing the body weight of birds. The inclusion of AXOS encourages beneficial bacteria and protects against pathogenic bacteria [34,35], which consequently enhances gut health. Lei et al. documented that dietary Xyl, Abf, and feruloyl esterase improved gut health [36]. Pettey et al. also indicated that adding 0.05% β-mannanase and arabinoxylooligosaccharides led to an improved blood IGF-I concentration in growing and finishing pigs [37]. It might be speculated that the inclusion of Xyl and Abf in low-energy diets had a positive effect on gut health and nutrient digestibility, leading to improved growth performance, lymphoid organs weights, and immune response in broilers.

Liver function indicators (plasma GOT and GPT) and the kidney function indicator (plasma creatinine) were not significantly affected. These results are in harmony with Ahmad et al., who evaluated the effect of dietary Xyl addition on plasma biochemical constituents in broilers and illustrated that Xyl might be safe in poultry rations without negative effects on vital organ functions [38]. Additionally, Saleh et al. reported that the serum concentrations of GOT, GPT, and creatinine were not significantly affected by dietary enzyme supplementation [3].

Table 4. Effect of NSP enzyme supplementation on plasma parameters in broilers.

	Control	Low Energy	Low Energy and Enzymes	*p*-Value
GPT, I/U	19 ± 2.33	18 ± 2.61	15 ± 1.05	0.38
GOT, I/U	399 ± 18.10	361 ± 24.70	374 ± 40.60	0.64
Total protein, mg/dL	2.83 ± 0.10	2.98 ± 0.09	3.21 ± 0.12	0.06
Albumin, mg/dL	1.58 ± 0.05	1.59 ± 0.09	1.56 ± 0.08	0.97
Globulin, mg/dL	1.26 ± 0.09 ^{b}	1.43 ± 0.04 ^{b}	1.7 ± 0.11 ^{a}	0.01
Total cholesterol, mg/dL	150 ± 5.3 ^{a}	135 ± 3.4 ^{b}	133 ± 2.3 ^{b}	0.02
Triglycerides, mg/dL	21 ± 2.18	25 ± 2.47	18 ± 3.02	0.20
HDL-cholesterol, mg/dL	56.98 ± 3.01 ^{b}	67.47 ± 3.82 ^{ab}	71.83 ± 4.44 ^{a}	0.04
LDL-cholesterol, mg/dL	79.28 ± 4.2	87.10 ± 11.9	75.81 ± 5.5	0.60
Glucose, mg/dL	193 ± 3.7	193 ± 4.8	199 ± 4.4	0.49
Creatinine, mg/dL	0.47 ± 0.04	0.50 ± 0.02	0.51 ± 0.02	0.49
ND, titer	2.75 ± 0.47 ^{b}	3.75 ± 0.47 ^{ab}	5.25 ± 0.62 ^{a}	0.03
H9N1, titer	0.25 ± 0.03	1.0 ± 0.04	1.25 ± 0.03	0.11

^{a,b} Mean values with different letters in the same raw differ significantly at $p < 0.05$. Values are expressed as means ± standard error. Glutamic oxaloacetic transaminase (GOT); glutamate pyruvate transaminase (GPT); high-density lipoprotein (HDL); low-density lipoprotein (LDL). International Units (I/U). The NSP enzyme used in this experiment was Rovabio® Advance.

The data presented in Table 5 illustrate the effect of a low-energy diet supplemented with or without Xyl and Abf enzymes on the muscle content of fatty acids, amino acids, total lipids, and TBARS. Muscle TBARS content was significantly decreased, while muscle total lipids content was significantly increased by feeding a low-energy diet supplemented with Xyl and Abf enzymes compared with the control group. However, the muscle contents of lysine, methionine, oleic, linoleic, and linolenic acids were not significantly influenced by dietary treatments. These findings are in agreement with Cowieson and Ravindran, who found enhancements in the digestibility of lysine, methionine, cysteine, and threonine when a multiple enzyme mixture possessing protease, Xyl, and amylase was used

to supplement corn-based diets, but these improvements did not affect the amino acid contents in muscle [21]. Furthermore, Head et al. reported that dietary α-linolenic acids in the form of linseed resulted in a significant increase of hepatic omega-3 poly unsaturated fatty acids (n-3PUFA) [39]; however, the inclusion of a multiple enzyme complex of Xyl and amylase in a linseed-based diet resulted in a reduction in the n-6PUFA-like linoleic acid, but oleic and linolenic acids were not affected. Regarding lipid peroxidation, the data illustrate that the muscle TBARS concentration was decreased by reducing the energy in diets, and this agreed with Cho and Kim, who observed that muscle malondialdehyde (MDA) concentration was decreased in low-energy density diets supplemented with or without β-mannanase and Xyl supplementation in pigs [9].

Table 5. Effect of NSP enzyme supplementation on lipid peroxidation, fatty acids, and amino acids in breast muscle of broilers.

	Control	Low Energy	Low Energy and Enzymes	*p*-Value
TBARS, nanomole/g	7.08 ± 0.71 ^{a}	5.03 ± 0.46 ^{b}	5.35 ± 0.34 ^{b}	0.05
Lysine, g/100 g protein	5.89 ± 0.43	5.91 ± 0.49	5.93 ± 0.50	0.99
Methionine, g/100 g protein	1.14 ± 0.04	1.20 ± 0.08	1.20 ± 0.01	0.56
Total lipids, g/100 g muscle	3.13 ± 0.07 ^{b}	3.66 ± 0.48 ^{ab}	4.98 ± 0.54 ^{a}	0.03
Oleic acid, mg/100 g fat	0.124 ± 0.008	0.152 ± 0.012	0.155 ± 0.005	0.96
Linoleic acid, mg/100 g fat	0.226 ± 0.009	0.777 ± 0.065	0.424 ± 0.025	0.63
Linolenic acid, mg/100 g fat	0.095 ± 0.003	0.083 ± 0.008	0.157 ± 0.007	0.70

^{a,b} Mean values with different letters in the same raw differ significantly at $p < 0.05$. Values are expressed as means ± standard error. Thiobarbituric acid reactive substance (TBARS). The NSP enzyme used in this experiment was Rovabio® Advance.

The mRNA expressions of the growth hormone receptor (GHR), insulin-like growth factor receptor (IGFR), and fatty acid synthesis (FAS) were significantly increased by adding Xyl and Abf enzymes to low-energy diets in comparison with the control group (Figure 1A–C). Meanwhile, acetyl-coA carboxylase (ACC) was increased by Xyl and Abf enzyme supplementation to a low energy diet but these differences were not significant in comparison with the control group (Figure 1D). These improvements are confirmed by the previous results of Guo et al., who demonstrated that the addition of Xyl upregulated the expression of the sodium–glucose cotransporter 1 and IGFR genes in broiler chickens [40]. Furthermore, Hosseini et al. reported that the inclusion of Xyl improved the expression of GHR and IGFR genes [41]. Moreover, ACC and FAS encode a biotin-dependent enzyme which is involved in the biosynthesis of fatty acids via the catalyzation of the irreversible carboxylation of acetyl-CoA for malonyl-CoA production [42]. In the present study, ACC and FAS gene expressions were significantly elevated in the low-energy diet supplemented with the Xyl and Abf enzyme combination. This impact was connected with the freedom of blocked macronutrients and, consequently, elevated lipogenesis due to the eternal energy adequacy [43]. Indeed, FAS and ACC, which play important roles in the lipogenic passage, are also key determinants for the maximal ability of a muscle tissue to synthesize fatty acid; they are extremely expressed in tissues such as those of the liver and muscles [3,44]. In common physiological cases, nutritional factors such as high-fat feed and hormones could organize the enzyme activity and gene expression of the FAS and ACC [39,45,46].

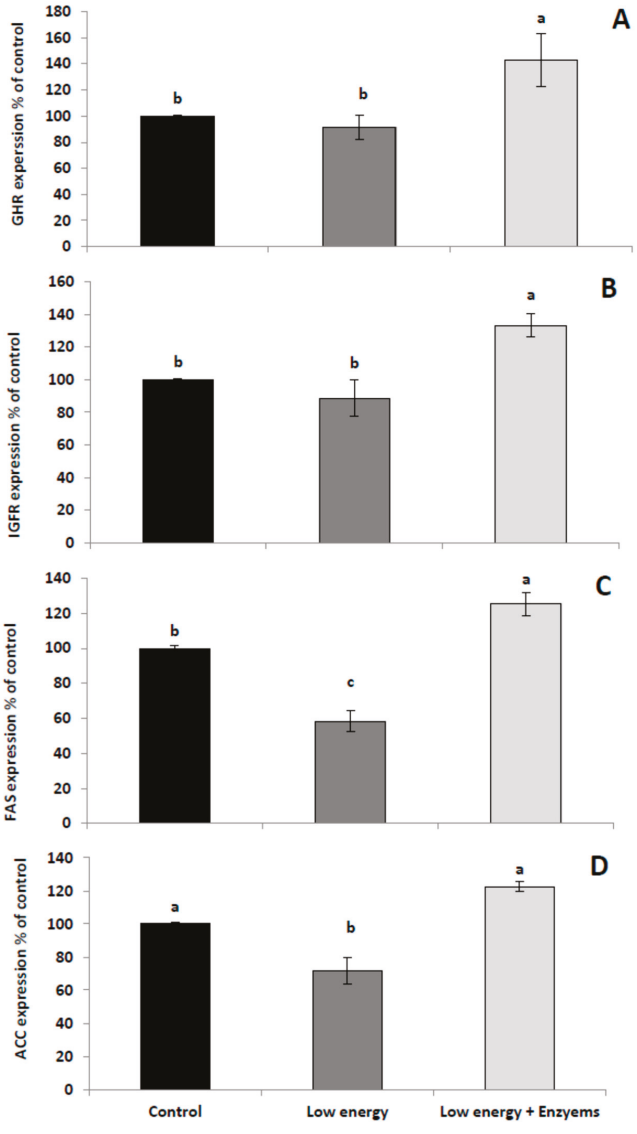

Figure 1. Effect of NSP enzyme supplementation on the gene expression of the growth hormone receptor (GHR) (**A**), insulin-like growth factor receptor (IGFR) (**B**), fatty acid synthesis (FAS) (**C**), and acetyl-coA carboxylase (ACC) (**D**) in broilers. [a,b] Mean values with different letters in the same column differ significantly at $p < 0.05$. Values are expressed as means ± standard error. The NSP enzyme used in this experiment was Rovabio® Advance.

4. Conclusions

It could be concluded that dietary Xyl and Abf (Rovabio® Advance) supplementation had positive effects on the growth performance, protein and fat digestibility, plasma lipid profiles, lipid peroxidation, immune response, and gene expression related to growth and fatty acid synthesis in broiler chickens fed low-energy diets.

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Does in Ovo Injection of Two Chicken Strains with Royal Jelly Impact Hatchability, Post-Hatch Growth Performance and Haematological and Immunological Parameters in Hatched Chicks?

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Simple Summary: The present investigation examined improvements in egg hatchability and the growth performance of hatched chicks of two strains upon injection with increasing concentrations of royal jelly (RJ). The results showed positive effects of RJ injection on all parameters. Limited impacts of the different chicken strains were observed on the tested parameters. The study revealed that varying the chicken strain could alter the response to the in ovo injection with RJ.

Abstract: The hypothesis of the present work was that the effects of in ovo injection may differ in different chicken strains. The influence of in ovo royal jelly (RJ) injection on hatching, growth and blood parameters in two chicken strains (Dokki-4 and El-Salam as example for different strains) was evaluated. A total of 1080 eggs were used. On the seventh day of incubation, the eggs were randomly allocated into six experimental groups in a 2×3 arrangement that included the two chicken strains and three concentrations of RJ (0, 0.25 and 0.5 mL RJ/egg). Injection with 0.5 mL RJ/egg improved hatchability compared to the other treatments. The El-Salam strain exhibited significantly higher body weight and body weight gain than the Dokki-4 strain. Injection with 0.5 mL RJ/egg significantly ($p < 0.05$) improved chicken body weight and daily weight gain compared to the control treatment. RJ injection decreased blood lipid profile parameters and the numbers of monocytes and eosinophils and increased total protein, globulin, haemoglobin (Hb) and lymphocyte levels compared to the control treatment. The Dokki-4 strain showed significantly higher antibody titres against avian influenza virus (AIV) ($p < 0.05$) and sheep red blood cells (SRBCs) ($p < 0.0001$) than the El-Salam strain and RJ injection enhanced antibody titres against AIV, Newcastle disease virus (NDV) and SRBCs. Therefore, the Dokki-4 strain was superior to the El-Salam strain for the tested parameters and injection with 0.5 mL RJ/egg produced the best hatching parameters, growth performance and health-related traits. RJ in ovo injection was much more effective in the Dokki-4 strain than in the El-Salam strain, which supported the hypothesis of the study that varying the chicken strain could alter the response to the in ovo injection with RJ.

Keywords: strain; in ovo injection; royal jelly; chicken; growth; hatchability; blood

1. Introduction

Embryonic growth in poultry can be manipulated through in *ovo* administration of nutrients and natural bioactive compounds [1–7]. In ovo injection of such substances influences the pre- and post-hatching physiological status of broiler embryos, leading to improved hatchability, superior nutritional status of hatchlings, greater vigour and higher post-hatch growth [8,9].

Royal jelly (RJ), a honeybee secretion fed to larvae and queen bees, is a rich dietary supplement for humans. RJ in fresh form consists of water (60–70%), protein (9–18%), carbohydrate (7–18%), fat (3–8%), mineral salts (calcium 1.5%), 10-hydroxy-2-decenoic acid (1.4%), fructose (3–13%), glucose (4–8%), sucrose (0.5–2.0%) and Ash (0.8–3.0%). While the lyophilised form contains <5% water, 27–41% protein, 22–31% carbohydrate and 15–30% fat [10,11].

RJ is the richest known natural source of vitamin B5 [12]. The other components of RJ include gamma globulins, mostly immunoglobulins, which powerfully strengthen the immune system; 10-hydroxy-Δ2-decanoic acid, which is a powerful antibacterial and anti-fungal agent [13] that keeps RJ sterile; gelatine, the precursor of collagen in skin, tendon, ligaments; and up to 1 mg/g acetylcholine, of which RJ is the richest natural source and which important in nerve transmission and the production and release of glandular secretions [14].

RJ has been reported to have several pharmacological properties, such as antioxidant [15], hypocholesterolaemic [16], anti-inflammatory [17], anti-malignant [18], antibacterial [19] and anti-ageing [20] properties, in animals. Additionally, RJ in ovo injection has been concluded to improve chick body weight [21], internal organ weight and luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion without adverse effects on hatchability [22]. Furthermore, in ovo injection of RJ has been found to promote feed intake in broiler chicks with no effect on immunity against Newcastle disease virus (NDV) [23], egg quality parameters or erythrocyte counts [24].

Different strains of chickens differ in their productivity, reproductive performance and immune responses [25–28]. Furthermore, strain differences affect feed intake, feed conversion ratios (FCRs) [29] and carcass traits [30,31].

Several studies have reported the effects of in ovo injection on the pre- and post-hatching performance of chickens; however, interactions between strain and in ovo injection have not been investigated. Therefore, the present study aimed to analyse the effects of in ovo injection of different levels of RJ, strain differences and interactions between strain and RJ treatment levels on hatching, growth rates, blood chemistry, haematology and immunological parameters.

2. Materials and Methods

This study was carried out at the Poultry Farm, Faculty of Agriculture, Kafr El-Sheikh University, Egypt. All procedures and experiments were performed in accordance with the ethical guidelines of the Committee of Local Experimental Animal Care and were approved by the Faculty of Agriculture, Kafr El-Sheikh University, Egypt (KFS2018-0078). All efforts were made to minimize the suffering of the animals.

A total of 1080 incubating eggs produced by two local chicken strains (Dokki-4 and El-Salam) were used. The Dokki-4 strain was developed by El-Itriby and Sayed [32] from a cross between a Fayoumi cock and Barred Plymouth Rock females (used as a dual purpose, meat and egg production) and the El-Salam strain was developed by Abd El-Gawad et al. [33] from crosses between Nichol sires and Mamourah dams (used as a dual purpose, meat and egg production).

The eggs were collected from the Experimental Research Station, Sakha, of the Animal Production Research Institute, Agricultural Research Centre, Ministry of Agriculture, Egypt. The eggs were stored at 15 ± 1 °C with 70–75% relative humidity for 3 days. The eggs were immediately cleaned with a dry, clean cloth; then, the surface of each egg was sprayed with a disinfectant solution and the shell was

wiped dry with clean paper. The eggs were incubated at 37.6 ± 1 °C with a relative humidity of 57 ± 2%. All eggs were set large end up in an automatic turner. The incubation equipment included an incubator (for the first 18 days of incubation) and a hatcher (for the remaining 3 days until hatching). The eggs from each experimental group were set in separate and marked containers in both the incubator and the hatcher.

On day 7 of incubation, the eggs were randomly divided into six groups in a 2 × 3 factorial design that included the two chicken strains (Dokki-4 and El-Salam) and three levels of in ovo injections (0, 0.25 and 0.5 mL/egg). Each group had six replicates of 30 eggs each. The RJ solution was prepared by dissolving 1 g of pure RJ (YS Organic Bee Farms "2774 N 4351st Rd, Sheridan, IL 60551, USA," item #69313, 3x freeze-dried RJ; equivalent to 1500 mg of fresh RJ) in 2 mL of normal saline solution in a water bath at 37 °C for 15 min.

A hole was made in the broad end of each egg using an automatic needle (syringe); 0.5 mL of RJ solution was then warmed to 30 °C and injected through the hole using a 23–gauge needle. The injection site was disinfected with 70% ethanol prior to injection. The pinhole was sealed with sterile paraffin wax immediately after injection and the eggs were returned to the incubator and set large end up to complete the incubation process. At the end of 18 days of incubation, the eggs were transferred to hatching trays at 37.2 °C under 70% humidity for the following 3 days or until hatching. After 21 day of incubation, the live hatched chicks were graded and counted, while the un-hatched eggs were broken to estimate the percentage of fertility. Hatchability was calculated as a percentage of fertile eggs using the following equations:

$$Fertility\ (\%) = (fertile\ eggs/total\ eggs) \times 100 \quad (1)$$

$$Hatchability\ of\ the\ fertile\ eggs\ (\%) = (hatched\ chick/fertile\ eggs) \times 100 \quad (2)$$

Twenty hatched chicks for each replicate were sexed, wing-banded and reared until 12 weeks of age and were supplied with standard feed for local chicken strains. The basal diet contained 19% crude protein (CP), 2834 kcal/kg metabolizable energy (ME), 3.019% ether extract, 3.906% crude fibre, 1.018% calcium, 0.348% available phosphorus, 0.857% lysine, 0.360% methionine and 0.699% methionine and cystine. The birds were vaccinated against most epidemic diseases in Egypt; they were vaccinated with Hitchner B1 (HB1) and Gumboro vaccines at 7 and 10 day of age, respectively, via eye drops and killed NDV, Reo, Gumboro and infectious bronchitis (IB) vaccines were injected intramuscularly at 13 day of age. Killed avian influenza virus (AIV; H5N2) vaccines were injected intramuscularly at 15 day of age, while Gumboro and Lasota vaccines were given at 22, 32 and 42 day of age via eye drops. Later, booster doses of Lasota vaccines were given at 50 day of age and then biweekly via eye drops. Feed and drinking water were offered ad libitum. All birds were reared under the same environmental, managerial and hygienic conditions. Body weight was recorded to the nearest 0.1 g. Feed intake was recorded at 0, 4, 8 and 12 weeks of age and the FCR was then calculated.

At 7 weeks of age, the birds received a single intramuscular injection of 0.1 mL of a 0.25% sheep red blood cell (SRBC) suspension. After 5 days, ten blood samples (1 mL each) from each group were collected from the wing vein with a sterile syringe and 0.5 mL of each sample was transferred into a heparinized tube. Plasma antibodies were measured by the microtitre haemagglutination method [34]. The titres are expressed as the log2 of the reciprocal of the highest dilution in which there was haemagglutination. The remaining 0.5 mL of each blood sample was allowed to coagulate in sterile tubes, after which the serum was collected to assess the antibody titre against NDV.

At 12 weeks of age, ten birds (5 males and 5 females) were selected randomly from each treatment, weighed and then sacrificed by decapitation. The carcasses and giblets (gizzard, heart and liver) were individually weighed to the nearest 0.1 g. The studied carcass traits were recorded as the percentage of the live body weight. Ten blood samples were collected in heparinized tubes to determine the complete blood count (CBC) and white blood cell (WBC) differentiation. After overnight clotting at 4 °C, the samples were centrifuged for 20 min at 4000× *g*.

The separated serum was transferred to a private laboratory for analysis of biochemical parameters. The total lipids (mg/dL), cholesterol (mg/dL), triglycerides (TGs; mg/dL), high-density lipoprotein (HDL, mg/dL), low-density lipoprotein (LDL, mg/dL), total protein (g/dL) and albumen (g/dL) levels were determined spectrophotometrically according to the methods of Akiba et al. [35], using commercial diagnostic kits from Biodiagnostic Company, Giza, Egypt. Additionally, the antibody titres against AIV and NDV were estimated.

The data were subjected to ANOVA with the generalized linear model (GLM) procedure of SAS software, USA [36] according to the following model:

$$X_{rtk} = \mu + O_r + B_f + I_{r\times t} + E_{rtk} \quad (3)$$

where X_{rtk} = the value of any observation, μ = the population mean O_r = the in ovo injection effect (of 0, 0.25 or 0.5 mL RJ/egg), B_f = the strain effect (El-Salam or Dokki-4), $I_{r\times t}$ = the interaction between the treatment and the chicken strain and E_{rtk} = random error.

3. Results

3.1. Hatching Parameters

As shown in Table 1, the Dokki-4 strain presented significantly higher fertility than the El-Salam strain ($p < 0.05$). No differences were recorded in the hatchability of the total eggs and fertile eggs ($p < 0.05$) between the two studied strains. Regarding the effect of in ovo RJ injection, the 0.5 mL RJ/egg dose significantly increased the hatchability of the total incubated eggs ($p < 0.0001$) compared to both the 0.25 mL RJ/egg dose and the control dose (0 mL RJ/egg) and improved the hatchability percentages of fertile eggs ($p < 0.05$) compared to the control dose.

Table 1. Fertility and hatchability percentages of two chicken strains (El-Salam and Dokki-4) injected in ovo with two different levels of royal jelly (RJ; 0.25, 0.5 mL/egg) compared to counterpart control strains (0 mL RJ/egg) and the interaction between strain and treatment level.

Items		Number of Replicates	Fertility (%)	Hatchability (%) (Based on Fertile Eggs)
Strain Effect				
El-Salam		18	84.09 [b]	81.09
Dokki-4		18	85.64 [a]	83.81
RJ in ovo Injection Effect (mL/egg)				
0		12	84.83	79.75 [b]
0.25		12	84.95	82.28 [ab]
0.50		12	84.82	85.32 [a]
Interaction Effect				
Strain	RJ in ovo Injection			
El-Salam	0	6	84.07	78.93
	0.25	6	84.2	81.13
	0.5	6	84	83.2
Dokki-4	0	6	85.6	80.57
	0.25	6	85.7	83.43
	0.5	6	85.63	87.43
SEM			0.76	1.86
p-Value				
Strain		-	0.028	0.097
In ovo injection		-	0.982	0.034
Strain × in ovo injection		-	0.995	0.771

SEM—standard error of the mean. In the same column and within the same effect, means with different superscripts ([a, b]) differ significantly ($p < 0.05$).

3.2. Growth Performance and Carcass Parameters

The results of chicken performance according to strain and in ovo injection level, as well as their interactions, are presented in Table 2. Daily weight gain was not significantly affected ($p \geq 0.05$) by strain. The results of the interaction between strain and in ovo inoculation were not significant ($p \geq 0.05$).

Table 2. Effect of in ovo injection of royal jelly (RJ) at two levels (0.25, 0.5 mL/egg) on growth performance and carcass parameters of two chicken strains (El-Salam, Dokki-4) as compared to counterpart control chicks (0 mL/egg) and the interaction of strain and treatment levels.

Items		Number of Replicates	Daily Weight Gain (DWG, g/bird)	Feed Consumption (FC, g)	Feed Conversion Ratio (FCR, g Feed/g Gain)	Carcass Parameters (% of Live Weight)	
			0–12 Weeks	0–12 Weeks	0–12 Weeks	Dressing	Giblets
Strain Effect							
El-Salam		18	35.38	56.53 ^{a}	1.62	71.50	5.53 ^{b}
Dokki-4		18	31.76	51.89 ^{b}	1.67	70.17	7.37 ^{a}
RJ in ovo Injection Effect (mL/egg)							
0		12	32.22	53.20	1.67	68.37 ^{b}	7.13
0.25		12	33.52	54.53	1.67	71.62 ^{ab}	6.93
0.50		12	34.98	54.90	1.60	72.52 ^{a}	5.93
Interaction Effect							
Strain			RJ in ovo Injection				
El-Salam	0	6	33.67	55.03	1.67	69.43	5.97
	0.25	6	35.6	57.3	1.63	71.8	5.37
	0.5	6	36.87	57.27	1.57	73.27	5.27
Dokki-4	0	6	30.6	51.37	1.67	67.3	8.3
	0.25	6	31.43	51.77	1.67	71.43	7.23
	0.5	6	32.83	52.53	1.6	71.77	6.6
SEM			2.12	0.68	0.12	1.01	0.46
p-value							
Strain			0.058	0.0001	0.65	0.273	0.004
In ovo injection			0.449	0.063	0.808	0.031	0.211
Strain × in ovo injection			0.954	0.411	0.947	0.823	0.751

SEM—standard error of the mean. In the same column and within the same effect, means with different superscripts ($^{a, b}$) differ significantly ($p < 0.05$).

Feed consumption and conversion ratios did not differ significantly between the El-Salam and Dokki-4 strains, except for feed consumption at 0–12 weeks of age ($p < 0.0001$), which were significantly improved in the El-Salam strain. No variation in dressing percentages was observed between strains ($p \geq 0.05$) but Dokki-4 had higher ($p < 0.01$) giblet percentages than the El-Salam Strain. For the in ovo RJ injection effects, chickens from eggs inoculated with 0.5 mL RJ consumed more feed ($p < 0.05$) compared to injection with 0.25 mL RJ or the control at 0–12 weeks of age; chickens from eggs inoculated with 0.5 mL RJ also displayed higher ($p < 0.05$) dressing percentages compared to the control group.

3.3. Serum Lipid and Protein Profiles

Strain clearly had no effect on serum lipid and protein profiles (Table 3). In contrast, in ovo injections with 0.5 mL RJ/egg decreased serum total lipids and increased ($p < 0.001$) globulin and total protein levels ($p < 0.05$) compared to the other injection. Both levels of RJ injection (0.25 and 0.5 mL RJ/egg) resulted in significantly reduced serum levels of cholesterol, TGs, HDL and LDL. No significant interactions between the strain and treatment effects were recorded for the serum lipid and protein profiles.

Table 3. Effect of in *ovo* injection of royal jelly (RJ) at two levels (0.25 and 0.5 mL/egg) on the serum lipid and protein profiles of two chicken strains (El-Salam and Dokki-4) compared to control saline injection (0 mL RJ/egg) and the interaction between strain and treatment level.

Parameter	Number of Samples	Total Lipids (mg/dL)	Cholesterol (mg/dL)	TGs (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TP (g/dL)	Albumen (g/dL)	Globulin (g/dL)	AG Ratio
Strain Effect										
El-Salam	30	231.89	114.32	103.64	44.52	80.19	4.63	1.93	2.69	0.72
Dokki-4	30	227.50	111.72	103.87	43.80	78.93	4.80	2.03	2.77	0.73
RJ in ovo injection effect (mL/egg)										
0	20	236.15 ^{a}	125.60 ^{a}	106.13 ^{a}	46.90 ^{a}	85.05 ^{a}	4.22 ^{b}	1.81	2.41 ^{c}	0.75
0.25	20	228.50 ^{a}	108.38 ^{b}	103.18 ^{b}	43.50 ^{b}	77.65 ^{b}	4.71 ^{ab}	1.99	2.72 ^{b}	0.73
0.5	20	224.43 ^{b}	105.08 ^{b}	101.95 ^{b}	42.08 ^{b}	75.98 ^{b}	5.21 ^{a}	2.14	3.07 ^{a}	0.70
Interaction Effect										
Strain	RJ in ovo Injection									
El-Salam	0	10	240.6	128.33	106.67	47.6	85.7	4.13	1.78	2.35	0.75
	0.25	10	229.77	109.27	102.77	43.8	78.5	4.77	1.98	2.79	0.71
	0.5	10	225.3	105.37	101.5	42.17	76.37	4.98	2.05	2.94	0.7
Dokki-4	0	10	231.7	122.87	105.6	46.2	84.4	4.32	1.86	2.46	0.75
	0.25	10	227.23	107.5	103.6	43.2	76.8	4.65	2	2.65	0.75
	0.5	10	223.57	104.8	102.4	42	75.6	5.44	2.23	3.21	0.69
SEM			3.39	2.36	3.39	2.36	1.18	1.5	3.31	0.28	0.21
p-Value										
Strain			0.139	0.201	0.82	0.567	0.65	0.468	0.601	0.4376	0.847
In ovo injection			0.014	0.0001	0.011	0.021	0.04	0.014	0.354	0.0005	0.72
Strain × In ovo injection			0.53	0.57	0.647	0.917	0.99	0.604	0.93	0.262	0.936

TGs—triglycerides; HDL—high-density lipoproteins; LDL—low-density lipoproteins; TP—total protein; AG—albumin/globulin; SEM—standard error of the mean. In the same column and within the same effect, means with different superscripts ($^{a, b, c}$) differ significantly ($p < 0.05$).

Table 4. Effect of in ovo injection of royal jelly (RJ) at two levels (0.25, 0.5 mL/egg) on complete blood count (CBC) of two chicken strains (El-Salam, Dokki-4) as compared to counterpart control chicks (0 mL/egg) and the interaction of strain and treatment levels.

Parameter		Number of Samples	RBCs (10^6/mm^3)	PCV (%)	Hb (g %)	WBCs (10^3/mm^3)	Heterophils (%)	Lymphocytes (%)	HL Ratio	Monocytes (%)	Eosinophils (%)	Basophils (%)
Strain Effect												
El-Salam		30	2.17	32.24	18.32	4.31	21.89	71.50	46.70	4.80	1.70	3.03
Dokki-4		30	2.19	32.08	18.61	4.42	21.82	71.91	46.87	4.72	1.63	2.80
RJ in ovo Injection Effect (mL/egg)												
0		20	2.05	31.50	16.49 ^{b}	4.27	22.39	68.81 ^{b}	45.60	6.42 ^{a}	1.91	3.30 ^{a}
0.25		20	2.23	32.33	18.93 ^{a}	4.28	21.76	72.56 ^{a}	47.16	4.61 ^{b}	1.59	2.88 ^{ab}
0.5		20	2.25	32.64	19.97 ^{a}	4.56	21.42	72.74 ^{a}	47.58	3.26 ^{c}	1.52	2.57 ^{b}
Interaction Effect												
Strain							RJ in ovo Injection					
El-Salam	0	10	2.04	31.61	16.31	4.16	22.46	68.84	45.65	6.27	1.97	3.5
	0.25	10	2.22	32.31	18.72	4.25	21.78	72.27	47.03	4.79	1.56	3.1
	0.5	10	2.25	32.8	19.92	4.53	21.43	73.39	47.41	3.33	1.57	2.5
Dokki-4	0	10	2.05	31.4	16.68	4.37	22.32	68.78	45.55	6.56	1.83	3.1
	0.25	10	2.24	32.35	19.13	4.31	21.74	72.85	47.29	4.42	1.61	2.65
	0.5	10	2.25	32.48	20.02	4.58	21.41	74.1	47.75	3.18	1.46	2.63
SEM			0.12	0.12	1.17	0.65	0.17	0.69	1.07	0.87	0.42	0.21
p-Value												
Strain			0.936	0.868	0.594	0.439	0.902	0.648	0.815	0.832	0.703	0.221
In ovo injection			0.194	0.613	0.0005	0.183	0.386	0.001	0.093	0.0001	0.196	0.022
Strain × in ovo injection			0.993	0.967	0.967	0.867	0.996	0.929	0.963	0.737	0.891	0.389

RBCs—red blood cells, PCV—packed cell volume, Hb—haemoglobin, WBCs—white blood cells, HL—heterophil/lymphocyte, SEM—standard error of the mean. In the same column and within the same effect, means with different superscripts ($^{a, b, c}$) differ significantly ($p < 0.05$).

3.4. Complete Blood Count (CBC)

The effects of strain, RJ in ovo injection level and their interaction on CBCs are listed in Table 4. Strain had no significant effect ($p > 0.05$) on red blood cell (RBC) count; packed cell volume (PCV); haemoglobin (Hb); WBC count; heterophil and lymphocyte numbers; the heterophil/lymphocyte (HL) ratio; or monocyte, eosinophil and basophil numbers. With regard to the in ovo RJ injection levels, both 0.25 and 0.5 mL RJ/egg resulted in increased Hb levels and lymphocyte counts compared to 0 mL RJ/egg, whereas significant reductions were observed in the numbers of monocytes and eosinophils. No significant interactions were recorded between the strain and treatment effects for any of the blood parameters analysed.

3.5. Immunological Parameters

As illustrated in Table 5, the Dokki-4 strain had significantly higher antibody titres against the AIV vaccine ($p < 0.05$) and SRBCs ($p < 0.0001$) than the El-Salam strain but no significant differences ($p > 0.05$) were recorded between the two strains for NDV titres. Regarding the impact of in ovo RJ injection on the immunity of the chickens, both levels of RJ (0.25 and 0.5 mL RJ/egg) increased ($p < 0.0001$) the antibody titre against the AIV vaccine, while 0.5 mL RJ/egg increased ($p < 0.0001$) the antibody titres against NDV and SRBCs compared to the other treatments. Regarding the interaction between the strain and in ovo RJ injection effects, no significant differences in antibody titres against AIV and NDV were recorded between the different groups; however, in ovo injection with 0.25 or 0.5 mL RJ/egg increased immunity ($p < 0.0001$) against SRBCs in the Dokki-4 strain compared to the El-Salam strain.

Table 5. Effect of in ovo injection of royal jelly (RJ) at two levels (0.25, 0.5 mL/egg) on Antibody titre against AIV, NDV and SRBCs of two chicken strains (El-Salam, Dokki-4) as compared to counterpart control (0 mL/egg) and the interaction of strain and treatment levels.

Parameter		Number of Samples	AIV 12 Weeks	NDV		SRBCs
				7 Weeks	12 Weeks	
Strain Effect						
El-Salam		30	5.77 ^{b}	6.50	6.70	6.18 ^{b}
Dokki-4		30	6.22 ^{a}	6.72	7.04	7.41 ^{a}
RJ in ovo Injection Effect (mL/egg)						
0		20	4.55 ^{c}	6.10 ^{b}	6.25 ^{b}	6.33 ^{b}
0.25		20	6.41 ^{b}	6.20 ^{b}	6.57 ^{b}	6.33 ^{b}
0.5		20	7.03 ^{a}	7.53 ^{a}	7.80a	7.74 ^{a}
Interaction Effect						
Strain		RJ in ovo Injection				
El-Salam	0	10	4.5	6	6.2	4.95 ^{d}
	0.25	10	5.99	6.1	6.3	6.23 ^{c}
	0.5	10	6.82	7.4	7.6	7.37 ^{b}
Dokki-4	0	10	4.6	6.2	6.3	6.43 ^{c}
	0.25	10	6.82	6.3	6.83	7.70 ^{ab}
	0.5	10	7.23	7.67	8	8.11 ^{a}
SEM			0.22	0.15	0.22	0.15
p-Value						
Strain			0.027	0.097	0.051	0.0001
In ovo injection			0.0001	0.0001	0.0001	0.0001
Strain × in ovo injection			0.283	0.968	0.538	0.0001

AIV—avian influenza virus; NDV—Newcastle disease virus; SRBCs—sheep red blood cells; SEM—standard error of the mean. In the same column and within the same effect, means with differ superscripts ($^{a, b, c, d}$) differ significantly ($p < 0.05$).

4. Discussion

The data listed in Table 1 shows that the in ovo RJ injection (0.5 mL RJ/egg) improved the hatchability percentages of fertile eggs ($p < 0.05$) compared to the other groups. The improvement in hatchability may be due to the enriched nutritive values of RJ, which contain vitamins and essential amino acid that enhance chick embryonic growth and hatchability. However, our results disagree with those obtained by Moghaddam et al. [21] who reported that in ovo RJ injection significantly decreased hatchability (46.7%) compared to saline injection (68.3%). Moreover, Moghaddam et al. [22] found significantly lower hatchability percentages with RJ compared to saline phosphate antibiotic injection. Conclusively, RJ in ovo injections (0.5 mL RJ/egg) improved hatchability percentage of chicken eggs.

Our results showed that in ovo RJ injection with 0.5 mL RJ/egg improved the hatchability percentages of fertile eggs ($p < 0.05$) compared to the other injections. The improvement in hatchability may have been due to the high nutritive value of RJ, which contains vitamins and essential amino acids that enhance chick embryonic growth and hatchability. However, our results disagree with those obtained by Moghaddam et al. [21], who reported that in ovo, RJ injection significantly decreased hatchability (46.7%) compared to saline injection (68.3%). Moreover, Moghaddam et al. [22] found significantly lower hatchability percentages in eggs injected with RJ than in eggs injected with a saline phosphate antibiotic. Conclusively, RJ in ovo injections (0.5 mL RJ/egg) improved the hatchability percentages of chicken eggs in this study.

The results presented in Table 2 indicate that the El-Salam strain had significantly greater body weight and daily weight gain ($p < 0.05$) than the Dokki-4 strain, which may be attributable to its genetic makeup [28]. Injecting eggs with 0.5 mL RJ/egg significantly ($p < 0.05$) improved chicken body weight and daily weight gain compared to injecting eggs with saline. In agreement with our results, Ahangari et al. [23] reported that in ovo RJ injection elicited a significant positive effect on the body weight of broiler chicks at 21 days of age. RJ plays an important role in bee colonies, stimulating and increasing larval growth and metabolism [37] and some RJ bioactive components can affect crucial physiological processes [13,38]. Additionally, increased body weight has been observed after injection or ingestion of RJ in experimental animals [37]. The differences in body weight between the two strains at 8 weeks of age can be explained by the fact that at this age, this quantitative trait in chickens is affected by complex physiological mechanisms and multiple genetic factors [39]. Overall, RJ in ovo injections (0.5 mL RJ/egg) had limited beneficial effects on the body weights and weight gain of the chickens.

Consistent with the findings of Rondelli et al. [29], our results revealed improved feed consumption at 8–12 weeks of age ($p < 0.05$) and total feed consumption (0–12 weeks of age) ($p < 0.0001$) in the El-Salam strain compared to the Dokki-4 strain. No significant improvement in the feed conversion ratio (FCR) was recorded upon in ovo RJ injection. Ahangari et al. [23] found that in ovo RJ injection increased feed consumption and reduced the FCR. Seven et al. [40] reported that propolis and RJ enhanced growth performance measured as body weight, feed intake and FCR; these effects could be attributed to enhanced intestinal health, digestion and absorption due to the antimicrobial effects of the RJ and propolis components. The significant increases in dressing percentage upon treatment with 0.5 mL RJ/egg are consistent with results obtained by Moghaddam et al. [21], who found that in ovo RJ injection significantly increased dressed carcass percentages and heart and liver weights compared to a control treatment. There are several possible explanations for these results. First, RJ can increase oxygen metabolism and animal activity by increasing the concentration and use of blood glucose [41] and can also promote respiration and oxidative phosphorylation, increasing tissue oxygen consumption and, consequently, performance and endurance [42]. Furthermore, RJ also exhibits antioxidant properties [42] in addition to containing many dietary proteins with a wide range of functional and biological properties; some of these properties are attributable to biologically active peptides (of 2–20 amino acid residues) that are inactive when part of a protein but are activated when digested in vivo [43]. Feed consumption and giblet percentages were substantially affected by strain differences but dressing percentages were increased significantly in the group treated with 0.5 mL RJ/egg compared to the other groups.

Our results showed that there were no strain effects on the tested serum parameters. However, in ovo RJ injection decreased lipid profile parameters and increased total protein and globulin content compared to control saline injection (Table 3). A hypocholesterolaemic effect of RJ has also been reported by Kashima et al. [44], who suggested that the major identified RJ proteins (MRJP1, MRJP2 and MRJP3), as bile acid-binding proteins, significantly decreased the micellar solubility of cholesterol. Pavel et al. [45] confirmed the ability of RJ to reduce blood cholesterol and several studies have demonstrated the efficacy of RJ in lowering and controlling blood TGs and cholesterol levels [46,47]. Vittek [48] showed that administration of 50–100 mg RJ/d lowered serum total cholesterol levels by 14% and total lipids by 10%. A different study reported that ingestion of 6 g RJ/d for 4 weeks led to reduced serum total cholesterol and LDL but had no effect on HDL or TGs content [16,49]. The increased total protein, albumen and globulin in the RJ-treated groups may be attributable to a direct promoting impact of RJ on haemopoietic tissue in addition to a stimulatory anabolic effect on liver tissues that favours protein synthesis. In addition, RJ has been proven to protect against degeneration of body protein [50]. Our results are similar to those recorded by Mahmoud [51], who found that feeding of RJ to Ross broilers under different stocking densities increased serum total protein, albumen and globulin levels. Collectively, RJ in ovo injection (0.5 mL RJ/egg) had a hypocholesterolaemic effect on chickens in addition to a role in increasing total serum protein.

The results presented in Table 4 show that in ovo RJ injection increased the Hb percentage and lymphocyte count and decreased monocyte and eosinophil numbers compared to control saline injection. The increased lymphocyte count indicates that in ovo RJ injection improved the chicken response to stress. However, contradictory results were obtained by Ahangari et al. [23], who reported that RJ injection resulted in decreased lymphocyte counts and increased heterophil counts and heterophil/lymphocyte (HL) ratios. Additionally, Rabie et al. [52] reported that dietary or drinking water supplementation of Cobb 500 broiler chicks with propolis significantly increased Hb concentrations compared with a control treatment. However, Morita et al. [53] reported that there is a lack of articles that interpret the effect of RJ on anaemia. Recently, Bhalchandra et al. [54] reported that intraperitoneal injection of RJ in rats improved Hb concentrations and mean corpuscular haemoglobin (MCH) and suggested that honey and RJ exert protective effects against blood cell damage via preservation of cellular integrity. Our findings suggest that RJ feeding might have an anti-anaemic effect.

The results revealed a higher antibody titre against AIV ($p < 0.05$) and SRBCs ($p < 0.0001$) in the Dokki-4 strain than in the El-Salam strain (Table 5). The better immune response of Dokki-4 chickens to AIV and SRBCs may be attributable to the genetic potential inherited from the Fayoumi strain, a pure Egyptian native strain known globally for its strong immunity [28]. The effects of in ovo RJ injection on chicken immunity were unique (Table 5). RJ contains several forms of free amino acids at levels ranging from 0.6–1.5% and most are L-series amino acids, such as lysine and proline [55]. Administration of these amino acids influences the immune responses of poultry against disease, during which body proteins are broken down and the resultant amino acids are used for critical protein synthesis rather than growth, with consequent enhancement of defence against certain diseases [56]. Ahangari et al. [23] reported non-significant effects of RJ injection on day 14 of the experiment, although a significant increase in antibody titre against NDV was observed on day 28 of the trial. Generally, the Dokki-4 strain had a better immune response against AIV and SRBCs than the El-Salam strain; moreover, RJ in ovo injection (0.5 mL RJ/egg) improved the immunity of chickens against AIV, NDV and SRBCs.

Finally, with regard to the feasibility of RJ application in poultry production, RJ can be injected into eggs at trace concentrations (not exceeding 0.5 mg/egg). Poultry production depends on both input and output; the marked improvements in bird growth characteristics in the present study as a result of RJ treatment suggest that even if the price of RJ is high, the economic return will cover the cost of its use and even yield a good profit margin for chicken keepers.

5. Conclusions

On the basis of our results, the study hypothesis was accepted that varying the chicken strain could alter the response to the in ovo injection with RJ (the Dokki-4 strain was superior to the El-Salam strain for the tested parameters). RJ injection into the yolk sac elicited significant positive effects on hatching parameters, growth performance, blood chemistry, haematology and immunological parameters. Among the injected doses, the 0.5 mL dose of RJ resulted in the best hatching parameters, growth performance and immune and health-related traits.

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Dietary Chitooligosaccharide Inclusion as an Alternative to Antibiotics Improves Intestinal Morphology, Barrier Function, Antioxidant Capacity, and Immunity of Broilers at Early Age

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Simple Summary: At early an age, broilers are susceptible to exterior stressors and therefore have a higher disease incidence rate. Antibiotic growth promoters have been forbidden in animal production by the European Union and other countries since their usage has caused potentially adverse effects such as antibiotic residues in livestock, environmental pollution, and the generation of drug-resistant bacteria. The search for safe and environmentally friendly alternatives to antibiotics to prevent disease and promote growth has become necessary in poultry production. Chitooligosaccharide (COS), a natural alkaline polymer of glucosamine with a number of bioactive groups, is easily obtained by chemical and enzymatic hydrolysis of chitosan, which is the second most abundant carbohydrate polymer in nature. Our results indicated that dietary supplementation with chitooligosaccharide, at a dosage of 30 mg/kg, enhanced the feed conversion ratio, benefited the intestinal morphology and barrier function, and improved antioxidant capacity and immunity in broilers at 21 days of age. These effects were similar with those observed as a result of chlortetracycline inclusion. Therefore, dietary COS supplementation can be used as a potential alternative to antibiotics in broilers.

Abstract: This study aimed to investigate the effects of chitooligosaccharide (COS) inclusion as an alternative to antibiotics on growth performance, intestinal morphology, barrier function, antioxidant capacity, and immunity in broilers. In total, 144 one-day-old Arbor Acres broiler chicks were randomly assigned into 3 groups and fed a basal diet free from antibiotics (control group) or the same basal diet further supplemented with either chlortetracycline (antibiotic group) or COS, for 21 days. Compared with the control group, inclusion of COS reduced the feed to gain ratio, the jejunal crypt depth, the plasma diamine oxidase activity, and the endotoxin concentration, as well as jejunal and ileal malondialdehyde contents, whereas increased duodenal villus height, duodenal and jejunal ratio of villus height to crypt depth, intestinal immunoglobulin G, and jejunal immunoglobulin M (IgM) contents were observed, with the values of these parameters being similar or better to that of the antibiotic group. Additionally, supplementation with COS enhanced the superoxide dismutase activity and IgM content of the duodenum and up-regulated the mRNA level of claudin three in the jejunum and ileum, when compared with the control and antibiotic groups. In conclusion, dietary COS inclusion (30 mg/kg), as an alternative to antibiotics, exerts beneficial effects on growth performance, intestinal morphology, barrier function, antioxidant capacity, and immunity in broilers.

Keywords: chitooligosaccharide; intestinal integrity; antioxidant capacity; immunity; broiler

1. Introduction

At an early age, broilers are susceptible to exterior stressors and, therefore, have a higher disease incidence because of their weak physiological status, including their small size, undeveloped organs, and poor immune function [1]. Antibiotics have excellent therapeutic effectiveness and growth promotion properties and were used as feed additives for livestock for several decades [2,3]. However, their usage has caused potential adverse effects, such as antibiotic residues in livestock, environmental pollution, and the generation of drug-resistant bacteria. The European Commission has therefore banned the use of antibiotics as growth promoters in animal production since 2006 [4]. A wide array of functional substances are currently being tested as substitutes for antibiotics to prevent disease and promote growth in livestock production, and these substrates include probiotics, prebiotics, plant extracts, and other agents [5,6]. Chitooligosaccharide (COS), as a functional prebiotic, is a natural alkaline polymer of glucosamine with a number of bioactive compounds and it is easily obtained by chemical and enzymatic hydrolysis of chitosan, which is the second most abundant carbohydrate polymer in nature [7]. Presently, many researchers tend to use chitosan in its oligosaccharide form since COS has a low molecular weight, good solubility, and low viscosity [8]. It is reported that COS could exert an antibacterial effect, regulate lipid metabolism, and promote antioxidant capacity and immunity in *in vitro* studies [9–11]. These properties of COS led to its application in livestock, especially pig production. Previous studies have shown that COS can be an alternative to antibiotics [5], promote growth [12,13], improve intestinal morphology and barrier function [14–17], and enhance antioxidant capacity and immunity in pigs [16,18]. In broilers, improved immunity and nutrient digestibility have been reported after inclusion of COS [19–21]. However, information is scarce concerning its effects on intestinal morphology and barrier function, as well as its antioxidant capacity, in broilers, although other functional oligosaccharides, such as fructooligosaccharide and mannan oligosaccharide could improve intestinal integrity and antioxidant ability in broilers [22–24]. In consideration of the similar biological functions among oligosaccharides and the application effects of COS in pigs, we hypothesized that dietary COS inclusion may be an alternative to antibiotics and may induce beneficial consequences in broiler chickens. Therefore, we investigated the effects of dietary COS supplementation, used as an alternative to antibiotics, on the growth performance, intestinal morphology, barrier function, antioxidant capacity, and immunity of broilers.

2. Materials and Methods

2.1. Animals, Diets, and Experimental Design

All procedures related with management and care of chickens in this experiment were approved by the Nanjing Agricultural University Animal Care and Use Committee (Certification No.: SYXK (Su) 2017-0007).

A total of 144 one-day-old male Arbor Acres broiler chicks with similar birth weights (42 ± 0.2 g) were used in this experiment. The chicks were randomly assigned to three dietary treatments of 6 replicate pens/cages per treatment, with eight broilers per pen. Broilers in the three treatments were fed a basal diet free from antibiotics (control group) or the same diet further supplemented with either 50 mg/kg of chlortetracycline (by effective content, antibiotic group) or 30 mg/kg of COS (COS group) for 21 days. The composition and nutrient contents of the basal diet are shown in Table 1. The broilers had free access to mash feed and water with continuous lighting in three-layer cages (120 cm × 60 cm × 50 cm) in a temperature-controlled room. The ambient temperature of the room was maintained at 32–34 °C for the first 3 days and then reduced by 2–3 °C per week to a final temperature of 26 °C. Body weight was recorded at 21 days of age after feed deprivation for 12 h and feed intake was determined from the difference between the offered and residual feed, on a cage basis, to calculate the average daily gain (ADG), the average daily feed intake (ADFI), as well as the feed/gain ratio (F/G). The COS dosage used in this study was according to the manufacturer's recommendation (Zhongkerongxin

Biotechnology Co., Ltd., Suzhou, Jiangsu, P.R. China). The average molecular weight of COS ranged from 1000 to 2000 Daltons (Da) and its purity was higher than 90%.

Table 1. Composition and nutrient level of the basal diet (g/kg, as fed basis unless otherwise stated).

Ingredients	1–21 Days
Ingredients	
Corn	576.1
Soybean meal	310
Corn gluten meal	32.9
Soybean oil	31.1
Limestone	12
Dicalcium phosphate	20
L-Lysine	3.4
DL-Methionine	1.5
Sodium chloride	3
Premix [1]	10
Calculated nutrient levels	
Apparent metabolizable energy (MJ/kg)	12.56
Crude protein	211
Calcium	10.00
Available phosphorus	4.60
Lysine	12.00
Methionine	5.00
Methionine + cystine	8.50

[1] Premix provided per kilogram of diet: Vitamin A (trans-retinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 3,000 IU; vitamin E (all-rac-α-tocopherol), 30 IU; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B12 (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulphate), 8.0 mg; Mn (from manganese sulphate), 110 mg; Zn (from zinc oxide), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

2.2. Sample Collection

On day 21, one bird per pen, that was close to the average body weight of the pen, was selected and weighed after a 12-h fasting. Whole blood samples were collected into both anti-coagulant tubes coated with EDTA and non-heparinized tubes via jugular venipuncture and kept at −20 °C until analysis. Serum was then obtained from the blood samples after centrifugation at 4450 × *g* for 15 min at 4 °C, and it was immediately stored at −20 °C for further determination. After blood collection, broilers were euthanized by cervical dislocation and necropsied immediately. Then, the immune organs including the thymus, spleen, and bursa of Fabricius were quickly excised and weighed to calculate the relative immune organ weight, which was expressed as g/kg live body weight. Approximately two-centimeter segments of the mid-duodenum, mid-jejunum, and mid-ileum were harvested, flushed several times with ice-cold phosphate-buffered saline (pH 7.4), fixed with 10% paraformaldehyde, and kept at 4 °C for evaluation of the mucosal morphology. The duodenum, jejunum, and ileum mucosa were scraped off using a sterile glass slide, which was frozen in liquid nitrogen rapidly and stored at −80 °C for further assessment.

2.3. Intestinal Morphological Examination

The preserved intestinal segments were dehydrated, cleared, and embedded in paraffin. Serial sections were performed at 5 μm thickness and stained with hematoxylin and eosin. The villus height and crypt depth were measured on the stained sections under a microscope with a Nikon ECLIPSE 80i light microscope equipped with a computer-assisted morphometric system (Nikon Corporation, Tokyo, Japan). A total of 10 well-oriented and intact villi were measured for each intestinal sample.

2.4. Evaluation of Serum Biomarkers of Intestinal Permeability

The activity of serum diamine oxidase (DAO) was determined by a corresponding reagent kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, P.R. China). Serum D-lactate acid levels were measured using a colorimetric assay kit (catalogue no. K667-100; BioVision Inc., Shanghai, China). Assays for serum endotoxin were carried out as described by the manufacturer's method of instruction (Xiamen Bioendo Technology Co., Ltd., Xiamen, Fujian, China).

2.5. Determination of Intestinal Antioxidant Capacity and Mucosal Immunity

Intestinal mucosal samples were homogenized (1:9, wt/vol) with ice-cold 154 mmol/L sterile sodium chloride solution using an Ultra-Turrax homogenizer (Tekmar Co., Cincinatti, OH, USA). Then, the mixture was centrifuged at 4450 × *g* for 15 min at 4 °C to obtain the supernatant, which was stored at −20 °C for the determination of the anti-oxidative and immune parameters. The anti-oxidative parameters, including total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and malondialdehyde (MDA) level, were assayed following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Immunoglobulin G (IgG), immunoglobulin M (IgM), and secretory immunoglobulin A (sIgA) were measured by enzyme-linked immunosorbent assay (ELISA) using chicken-specific IgG, IgM, and sIgA ELISA quantitation kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.6. Messenger RNA Quantification

The intestinal mucosal RNA was isolated using TRIzol reagent (Takara Biotechnology Co. Ltd., Dalian, Liaoning, China). Then, the RNA quality was analyzed in agarose gels stained with ethidium bromide and the total RNA concentration was determined from OD260/280 readings (ratio > 1.8) using a NanoDrop ND-1000 UV spectrophotometer (Nano Drop Technologies, Wilmington, DE). After determining the quality and purity, the resultant cDNA was synthesized using the PrimeScriptTM RT reagent kit (Takara Biotechnology Co. Ltd, Dalian, Liaoning, China), according to the manufacturer's instructions, and stored at −20 °C for real-time PCR. The primer sequences, including occludin (OCLN), claudin 2 (CLDN2), claudin 3 (CLDN3), and zonula occludens-1 (ZO-1), used real-time PCR and their gene bank ID numbers are presented in Table 2. The reaction mixture was prepared using a TB Green^{TM} Premix Ex Taq^{TM} kit (Takara Biotechnology Co. Ltd., Dalian, Liaoning, P.R. China) and gene expression levels were subsequently determined by a real-time quantitative PCR using an ABI PRISM 7500HT Detection System (Applied Biosystems, Foster City, CA, USA). The reaction was performed as follows: One cycle pre-run at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, and a 60 °C annealing step for 30 s. The expressions of relative genes were expressed as $2^{-\Delta\Delta CT}$ [25] and the results were normalized according to the expression of β-actin.

Table 2. Sequences for real-time PCR primers.

Genes [1]	Gene Bank ID	Primer Sequence	Product Size (bp)
OCLN	NM 205128.1	F: CCGTAACCCCGAGTTGGAT R: ATTGAGGCGGTCGTTGATG	214
CLDN2	NM 001277622.1	F: CCTGCTCACCCTCATTGGAG R: GCTGAACTCACTCTTGGGCT	145
CLDN3	NM 204202.1	F: CCCGTCCCGTTGTTGTTTTG R: CCCCTTCAACCTTCCCGAAA	126
ZO-1	XM 413773.4	F: TGTAGCCACAGCAAGAGGTG R: CTGGAATGGCTCCTTGTGGT	159
β-actin	NM 205518.1	F: TTGGTTTGTCAAGCAAGCGG R: CCCCCACATACTGGCACTTT	100

[1] OCLN, occludin; CLDN2, claudin 2; CLDN3, claudin 3; ZO-1, zonula occludens-1.

2.7. Statistical Analysis

A complete randomized design was used in this study and one-way ANOVA was performed using SPSS (Version 20.0, SPSS Inc., Chicago, IL, USA) with pen (cage) as the experimental unit. Differences among treatments were detected by Tukey's multiple range tests. Results were expressed as means with their pooled standard errors. Probability values less than 0.05 were considered significant.

3. Results

3.1. Growth Performance

The effect of dietary COS supplementation on growth performance in broilers is presented in Table 3. Broilers that received the COS supplemented diet had a lower F/G ($p < 0.05$) when compared with those offered the basal diet, with the value of F/G being similar between COS and antibiotic groups ($p > 0.05$). However, the ADG and ADFI were not affected by treatments ($p > 0.05$).

Table 3. Effect of COS supplementation on growth performance in broilers.

Growth Parameter [1, 2]	Control Group	Antibiotic Group	COS Group	SEM	*p*-Value
ADG (g/day)	29.1	30.7	30.4	0.5	0.343
ADFI (g/day)	46.3	47.5	46.4	0.7	0.764
F/G (g/g)	1.60 [a]	1.53 [b]	1.52 [b]	0.01	0.030

[a, b] Means within a row with different superscripts differ significantly at $p < 0.05$. [1] ADG: average daily gain; ADFI: average daily feed intake; F/G: feed to gain ratio. [2] Control group, basal diet; Antibiotic group, basal diet supplemented with 50 mg/kg chlortetracycline; COS group, basal diet supplemented with 30 mg/kg chitooligosaccharide; SEM, standard error of means ($n = 6$).

3.2. Relative Immune Organ Weights

Compared with the control group (Table 4), COS dietary supplementation tended to increase the thymus relative weight ($p = 0.095$) and the value of this parameter did not differ between the COS and antibiotic groups ($p > 0.05$). Relative spleen and bursa of Fabricius weights were similar among the groups ($p > 0.05$)

Table 4. Effect of COS supplementation on relative immune organ weight in broilers (g/kg).

Organ [1]	Control Group	Antibiotic Group	COS Group	SEM	*p*-Value
Thymus	3.46	4.40	4.37	0.20	0.095
Spleen	0.86	0.93	0.91	0.04	0.762
Bursa of Fabricius	1.81	2.32	1.94	0.14	0.333

[1] Control group, basal diet; Antibiotic group, basal diet supplemented with 50 mg/kg chlortetracycline; COS group, basal diet supplemented with 30 mg/kg chitooligosaccharide; SEM, standard error of means ($n = 6$).

3.3. Intestinal Morphology

Compared with the control group (Table 5), COS dietary supplementation increased duodenal villus height ($p < 0.05$) and the ratio of villus height to crypt depth ($p < 0.05$) in the duodenum and jejunum, whereas it caused the depression of crypt depth in jejunum ($p < 0.05$), with the values of these parameters being similar to antibiotic groups ($p > 0.05$). Moreover, the values of crypt depth in the ileum were also found to be decreased in response to antibiotic supplementation when compared with the control and COS groups ($p < 0.05$).

3.4. Serum Biomarkers of Intestinal Permeability

As was shown in Table 6, the activity of DAO and endotoxin concentration were found to decrease in response to COS or antibiotic supplementation ($p < 0.05$). Furthermore, the inclusion of COS or

antibiotic had a tendency to reduce the serum D-lactate level ($p = 0.061$). In addition, there were no significant difference between the COS and antibiotic groups regarding these parameters ($p > 0.05$).

Table 5. Effect of COS supplementation on intestinal mucosal morphology in broilers.

Intestinal Parameter [1]	Control Group	Antibiotic Group	COS Group	SEM	*p*-Value
Villus height (μm)					
Duodenum	1513[b]	1729 [a]	1723 [a]	35	0.006
Jejunum	1183	1198	1164	41	0.498
Ileum	835	739	866	44	0.492
Crypt depth (μm)					
Duodenum	212	177	195	7	0.084
Jejunum	289 [a]	209 [b]	210 [b]	13	0.004
Ileum	188 [a]	125 [b]	191 [a]	11	0.015
Villus height: crypt depth ratio					
Duodenum	7.24 [b]	9.80 [a]	8.63 [a]	0.31	<0.001
Jejunum	4.51 [b]	5.75 [a]	5.59 [a]	0.20	0.014
Ileum	4.54	6.31	4.66	0.35	0.064

[a, b] Means within a row with different superscripts differ significantly at $p < 0.05$. [1] Control group, basal diet; Antibiotic group, basal diet supplemented with 50 mg/kg chlortetracycline; COS group, basal diet supplemented with 30 mg/kg chitooligosaccharide; SEM, standard error of means ($n = 6$).

Table 6. Effect of COS supplementation on serum markers of intestinal permeability in broilers.

Serum Biomarker [1, 2]	Control Group	Antibiotic Group	COS Group	SEM	*p*-Value
DAO (U/L)	21.0 [a]	16.7 [b]	15.9 [b]	0.8	0.004
D-lactate (nmol/μL)	2.40	1.72	1.79	0.14	0.061
endotoxin (EU/mL)	0.0600 [a]	0.0358 [b]	0.0453 [b]	0.0032	0.001

[a, b] Means within a row with different superscripts differ significantly at $p < 0.05$. [1] DAO, diamine oxidase. [2] Control group, basal diet; Antibiotic group, basal diet supplemented with 50 mg/kg chlortetracycline; COS group, basal diet supplemented with 30 mg/kg chitooligosaccharide; SEM, standard error of means ($n = 6$).

3.5. Intestinal Antioxidant Capacity

Compared with the control and antibiotic groups (Table 7), dietary COS inclusion increased SOD activity in the duodenum of broilers ($p < 0.05$). Furthermore, broilers receiving the COS supplemented diet had a lower ileal MDA content ($p < 0.05$), when compared with those fed basal diet, and the value of this parameter was intermediate in the antibiotic group ($p > 0.05$). Additionally, the supplementation with COS caused depression of jejunum MDA content ($p < 0.05$), with the value of this parameter being similar between the COS and antibiotic groups ($p > 0.05$). However, the remaining values of anti-oxidative parameters were not affected by the incorporation of antibiotics or COS ($p > 0.05$).

Table 7. Effect of COS supplementation on intestinal oxidant status in broilers.

Parameter [1, 2]	Control Group	Antibiotic Group	COS Group	SEM	*p*-Value
Duodenum					
T-AOC (U/mg protein)	0.475	0.550	0.725	0.049	0.095
SOD (U/mg protein)	181 [b]	195 [b]	248 [a]	11	0.026
MDA (nmol/mg protein)	0.850	0.498	0.652	0.116	0.472
Jejunum					
T-AOC (U/mg protein)	0.550	0.629	0.633	0.026	0.350
SOD (U/mg protein)	181	190	188	3	0.518
MDA (nmol/mg protein)	1.10 [a]	0.54 [b]	0.48 [b]	0.09	0.001
Ileum					
T-AOC (U/mg protein)	0.99	1.17	1.01	0.07	0.614
SOD (U/mg protein)	165	183	186	7	0.522
MDA (nmol/mg protein)	1.65 [a]	1.30 [ab]	1.03 [b]	0.11	0.05

[a, b] Means within a row with different superscripts differ significantly at $p < 0.05$. [1] T-AOC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde. [2] Control group, basal diet; Antibiotic group, basal diet supplemented with 50 mg/kg chlortetracycline; COS group, basal diet supplemented with 30 mg/kg chitooligosaccharide; SEM, standard error of means ($n = 6$).

3.6. Immunoglobulin Concentration in the Intestine

The IgG levels (Table 8) of duodenum, jejunum, and ileum, as well as jejunal IgM level, were observed to be higher in response to COS supplementation ($p < 0.05$), with the values of these parameters being similar between COS and antibiotic groups ($p > 0.05$). Additionally, dietary supplementation with COS resulted in a higher duodenal IgM content when compared with the control and antibiotic groups ($p < 0.05$).

Table 8. Effect of COS supplementation on intestinal immunoglobulins in broilers (μg/mg protein).

Immunoglobulin [1, 2]	Control Group	Antibiotic Group	COS Group	SEM	*p*-Value
Duodenum					
IgG	126 [b]	134 [a]	134 [a]	1	0.019
IgM	7.38 [b]	7.85 [b]	8.12 [a]	0.12	0.029
sIgA	9.03	9.42	9.36	0.15	0.533
Jejunum					
IgG	132 [b]	145 [a]	144 [a]	2	0.023
IgM	7.92 [b]	9.04 [a]	9.02 [a]	0.20	0.021
sIgA	9.79	9.94	10.68	0.23	0.240
Ileum					
IgG	150 [b]	165 [a]	163 [a]	3	0.040
IgM	10.0	11.3	11.1	0.3	0.186
sIgA	12.0	11.6	11.6	0.3	0.858

[a, b] Means within a row with different superscripts differ significantly at $p < 0.05$. [1] IgG, immunoglobulin G; IgM, Immunoglobulin M; sIgA, secretory immunoglobulin A. [2] Control group, basal diet; Antibiotic group, basal diet supplemented with 50 mg/kg chlortetracycline; COS group, basal diet supplemented with 30 mg/kg chitooligosaccharide; SEM, standard error of means ($n = 6$).

3.7. Gene Expressions Related to Intestinal Barrier Function

Compared with control and antibiotic groups (Table 9), the supplementation of COS upregulated the mRNA expression of CLDN3 in the jejunum and ileum ($p < 0.05$). However, treatments didn't alter the mRNA abundance of intestinal OCLN, CLDN2, and ZO-1 ($p > 0.05$).

Table 9. Effect of COS supplementation on intestinal gene expression in broilers.

Intestinal Gene [1, 2]	Control Group	Antibiotic Group	COS Group	SEM	*p*-Value
Duodenum					
OCLN	1.00	1.10	1.00	0.07	0.822
CLDN2	1.00	1.09	1.22	0.10	0.703
CLDN3	1.00	1.22	1.25	0.16	0.823
ZO-1	1.00	1.04	1.01	0.06	0.965
Jejunum					
OCLN	1.00	1.09	1.08	0.07	0.863
CLDN2	1.00	1.06	1.16	0.06	0.605
CLDN3	1.00 [b]	1.08 [b]	1.51 [a]	0.09	0.050
ZO-1	1.00	1.11	1.11	0.05	0.655
Ileum					
OCLN	1.00	1.26	1.14	0.08	0.43
CLDN2	1.00	1.10	1.09	0.09	0.899
CLDN3	1.00 [b]	1.12 [b]	1.64 [a]	0.11	0.020
ZO-1	1.00	1.23	1.10	0.07	0.400

[a, b] Means within a row with different superscripts differ significantly at $p < 0.05$. [1] OCLN, occludin; CLDN2, claudin 2; CLDN3, claudin 3; ZO-1, zonula occludens-1. [2] Control group, basal diet; Antibiotic group, basal diet supplemented with 50 mg/kg chlortetracycline; COS group, basal diet supplemented with 30 mg/kg chitooligosaccharide; SEM, standard error of means ($n = 6$).

4. Discussion

4.1. Growth Performance

Previous studies have reported that the function of chitosan is closely associated with its molecular weight and Vila et al. [26] showed that chitosan with a molecular weight greater than 100000 Da

could only serve as an adhesive or a carrying agent. The COS could modulate immune responses and reduce establishment of pathogens in the intestine when its molecular weight is between 1000 to 10000 Da [27]. In the present study, the COS had an average molecular weight between 1000 and 2000 Da and our results indicated that broilers receiving the COS supplemented diet had a lower F/G, with the value being similar to antibiotic group. This finding suggests that the inclusion of COS could be used as an alternative to antibiotics for improving the growth performance of broilers. Consistent with our results, Huang et al. [21] observed that dietary COS supplementation improved the ADG and F/G in broilers by acting as an antibiotic. Likewise, Li et al. [28] reported that the inclusion of COS improved ADG, ADFI, and F/G in broilers. In weaning pig, both a higher ADG and feed conversion ratio (FCR) were observed in response to COS supplementation [16]. Additionally, Yin et al. [29] found that supplementation with COS improved the ADG and F/G in early-weaned piglets. There are several possible mechanisms that could explain the positive effect of COS on the growth performance in livestock, including enhancement of the nutrient digestibility [21,28], increment of growth hormone or IGF-1 concentration [30], and the improvement of intestinal integrity and antioxidant capacity, as well as immunity [16].

4.2. Relative Immune Organ Weights and Intestinal Immunoglobulin Levels

Relative organ weight could reflect the growth and development of organs in some degree [31]. The effects of COS on relative immune organ weight have already been studied, however, the results were inconsistent. Li et al. [32] found that COS promoted the development of immune organs in broilers. Similarly, Deng et al. [20] reported that COS supplementation increased the spleen, thymus, and bursa index of broilers on day 21. However, it has also been reported by Zhou et al. that COS did not affect the immune organ index in broilers [33]. The discrepancy is likely due to the degree of polymerization, deacetylation level, dosage, and purity of COS [32]. In the current study, supplementation with COS tended to increase the thymus relative weight, coupled with simultaneously increased IgG and IgM contents, which further indicated that dietary COS supplementation could exert a positive effect on immune function. These beneficial consequences are likely due to that COS could regulate cytokine secretion, promote the proliferation of T and B lymphocytes, and inhibit lymphocyte apoptosis in immune organs [20,32]. Huang et al. [19] also showed that supplementation with COS enhanced serum IgG and IgM contents of broilers. Similar results were found by Deng et al. [20], who demonstrated that broilers receiving the COS supplemented diet had a higher circulating IgM content. Additionally, Wu and Tsai [34] showed that COS improved the IgM secretion of human hybridoma HB4C5 cells, indicating that COS could improve immunity. These changes were likely attributed to the alteration in the microenvironment caused by COS supplementation [35] and were consistent with the results of intestinal morphology and barrier function observed in our study. Additionally, COS may also inhibit pro-apoptotic pathways via improving the capacity of free radical clearance in the immune organs, thus benefitting the immune function [32].

4.3. Intestinal Morphology and Barrier Function

The structure of the intestinal mucosa can reveal some information about gut health, and a shortening of villus height is associated with a decrease in the surface area for nutrient absorption [36]. On the other hand, a large crypt indicates fast tissue turnover and increased nutrient requirements for new tissue are needed, which contribute to poor nutrient absorption [37]. The ratio of villus height to crypt depth is a useful criterion to estimate the nutrient digestion and absorption capacity of the small intestine [38]. In the current study, dietary COS supplementation could exert a positive effect on intestinal morphology, as evidenced by the increased villus height and ratio of villus height to crypt depth, as well as the decreased crypt depth. Liu et al. [14] also reported that broilers fed a diet supplemented with COS had a higher villus height and ratio of villus height to crypt depth in the jejunum and ileum in weanling pigs. Similarly, it is reported that dietary COS supplementation could attenuate compromised intestinal morphology in weanling pigs challenged by *Escherichia coli* through

increasing villus height to crypt depth ratio [39]. Previous researches have shown that the N-acetyl glucosamine, the main component of COS, may bind to certain types of bacteria and therefore interfere with their adhesion to the gut tissue of host [40–42]. Additionally, Mourão et al. [43] reported that an increase in villus height in the ileum of weaned rabbits was correlated with a decreased intestinal microflora. The possible explanation for improved intestine structure in the present work was that the N-acetyl glucosamine abundance in COS may create a more favorable intestinal microbial environment.

DAO is an enzyme synthesized primarily in the gastrointestinal mucosal cells of mammalian species and distributed primarily in the cytoplasm and blood DAO levels are increased when the mucosa is damaged and DAO enter into the bloodstream [15]. Plasma D-lactate acid is produced by the intestinal microflora and the content of D-lactate acid in the serum may increase if the small intestine mucosa is injured as a result of dysfunction in the intestinal barrier [44]. Serum DAO activity and D-lactate acid level are useful biomarkers for evaluating the integrity of the gastrointestinal tract [45]. In the present study, dietary supplementation with COS decreased serum DAO activity and endotoxin content. Similarly, previous literature showed that COS dietary supplemented pigs had lower DAO activity and endotoxin concentration than pigs in the control group after 14 days of supplementation [16]. In addition, a lower DAO activity in the serum and a higher activity of DAO in the jejunum mucosa were found in piglets on day seven, postweaning, in response to COS supplementation [15]. Tight junction, the multi-protein complex, are made up of transmembrane proteins, peripheral membrane proteins and regulatory molecules including kinases, among which CLDN family proteins and ZO family proteins are crucial to tight-junction assembly [46]. It was reported that the permeability of the leak pathway can be acutely regulated by the cytoskeleton via mechanisms that involve ZO-1 and OCLN [47]. In the current study, the mRNA expression of CLDN3 was found to be higher in response to COS supplementation in broilers, coupled with the simultaneously decreased circulating DAO activity and endotoxin level, further indicated that COS could improve intestinal barrier function in broilers at an early age. Likewise, Alizadeh et al. [48] reported that the mRNA expressions of various tight junction proteins, including CLDN1, ZO-1, ZO-2, and OCLN, were up-regulated in the intestines of piglets fed a galacto-oligosaccharides diet. However, Xiong et al. [49] showed that the COS inclusion decreased the mRNA expressions of OCLN and ZO-1 in the intestines of weaned piglets, indicating that dietary COS supplementation compromised the intestinal barrier integrity in weaned piglets. These discrepancies may be also attributed to the polymerization level, purity, and dosage of COS, as well as the animal species. Further studies are necessary to examine the effects of COS on intestinal barrier function.

4.4. Intestinal Antioxidant Capacity

Oxidative stress is observed when production of reactive oxygen species (ROS) exceeds the capacity of cellular antioxidant defenses to remove these toxic species [6,50]. The SOD is an important antioxidant enzyme in scavenging the oxygen free radical [51] and the content of MDA is the main end product of lipid peroxidation by ROS [52]. In the present study, the supplementation of COS improved the activity of SOD, whereas it decreased the intestine lipid peroxidation biomarker MDA level. Likewise, Li et al. [53] reported that dietary COS supplementation enhanced the activities of T-AOC, glutathione peroxidase (GSH-Px), and SOD, whereas it decreased the MDA content of the ileum mucosa in broilers. Similar results were also observed by Zhao et al. [16], who demonstrated that the inclusion of COS enhanced circulating T-AOC and GSH-Px activities and decreased plasma MDA content, simultaneously, in weaned piglets. Available literature indicated that COS with average molecular weight below 5000 Da can be regarded as a potential antioxidant due to its ROS scavenging properties [10,54]. It can be concluded that the improved antioxidant capacity observed in our research is primarily attributed to the antioxidant characteristics of COS. In addition, the improved antioxidant capacity may also be closely related with the improved intestinal integrity and immunity observed in this study.

5. Conclusions

The results of our study indicated that dietary supplementation with COS at a dosage of 30 mg/kg can improve FCR, benefit the intestinal morphology and barrier function, and improve antioxidant capacity and immunity in broilers at an early age. These effects were similar with that observed after dietary chlortetracycline inclusion. Therefore, dietary COS supplementation can be used as a potential alternative to antibiotics in broilers.

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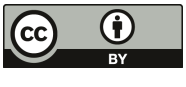

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Effects of Dietary Betaine on Growth Performance, Digestive Function, Carcass Traits, and Meat Quality in Indigenous Yellow-Feathered Broilers under Long-Term Heat Stress

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Simple Summary: Heat stress, one of the major problems in tropical and subtropical regions, adversely affects poultry production. This study was designed to evaluate the effects of dietary betaine on growth performance, digestive function, carcass traits, and meat quality in indigenous yellow-feathered broilers subjected to long-term heat stress. The results demonstrated that long-term heat exposure reduced the growth performance, digestive function, and carcass yield, and dietary betaine supplementation partially alleviated the adverse effects of heat stress on these parameters. These findings are useful for development of anti-heat stress feed additives in indigenous yellow-feathered broilers.

Abstract: Heat stress has a profound effect on poultry health and productivity. The present study evaluated whether feeding betaine could ameliorate long-term heat stress-induced impairment of productive performance in indigenous yellow-feathered broilers. A total of 240 five-week-old male broilers were randomly allocated to five treatments with six replicates of eight broilers each. The five treatments included a thermoneutral zone control group (TN, fed basal diet), a heat stress control group (HS, fed basal diet), and an HS control group supplemented 500, 1000, 2000 mg/kg betaine, respectively. The TN group was raised at 26 ± 1 °C during the whole study, HS groups exposed to 32 ± 1 °C for 8 h/day from 9:00 am to 17:00 pm. The results showed that heat stress decreased the body weight gain (BWG) and feed intake of broilers during 1–5, 6–10, and 1–10 weeks ($p < 0.05$). Dietary betaine tended to improve the BWG and feed intake of broilers under 5 weeks of heat stress (linear, $p < 0.10$), and betaine supplementation linearly increased the BWG and feed intake during 6–10 and 1–10 weeks ($p < 0.05$). Additionally, nitrogen retention was reduced by 5 weeks and 10 weeks of heat stress ($p < 0.05$), whereas dietary betaine could improve nitrogen retention in heat stressed broilers after both 5 and 10 weeks of heat stress (linear, $p < 0.05$). Moreover, this study observed that the trypsin activity of jejunum was decreased by 5 weeks of heat stress ($p < 0.05$), whereas betaine supplementation had quadratic effects on trypsin activity of jejunum in heat stressed broilers ($p < 0.05$). Furthermore, 10 weeks of heat stress induced a reduction of villus height of the duodenum and jejunum ($p < 0.05$), and decreased the villus height to crypt depth ratio of the jejunum ($p < 0.05$). Supplementation with betaine ameliorated the adverse effects of heat stress on these parameters ($p < 0.05$). Compared with the TN group, 10 weeks of heat stress reduced carcass and breast yield ($p < 0.05$) and betaine supplementation improved carcass and breast yield of heat stressed broilers (linear, $p < 0.05$). In conclusion, dietary supplementation of betaine could reduce the

detrimental effects of long-term heat stress on growth performance, digestive function, and carcass traits in indigenous yellow-feathered broilers.

Keywords: broilers; digestive function; heat stress; indigenous yellow-feathered breed

1. Introduction

With global warming, the deleterious effects of heat stress induced by high ambient temperature on poultry productivity have been of great concern all over the world, especially in tropical and subtropical regions. Heat stress has a profound effect on broilers' health and production, and leads to multiple physiological disturbances, such as endocrine disorders, systemic immune dysregulation, and electrolyte imbalance [1]. Heat stress also causes a disruption in the intestinal structure and function, including reduced regeneration and integrity of the intestinal epithelium [2,3], which in turn suppresses the growth rate and feed efficiency of birds. In addition, heat stress impairs carcass traits and meat quality through affecting energy-substance metabolism and redox status, resulting in decreased meat yield and increased abdominal fat rate in broilers [4,5]. Reducing the house temperature to the thermoneutral zone is a direct strategy to eliminate heat stress of poultry, and the thermoneutral zone can maximize the growth potential [1]. However, the cost of cooling equipment is relatively high in broiler production. It has previously been reported that nutritional manipulation could be a viable option to minimize the adverse impacts of heat stress on broilers [6], including supplementation of functional feed additives, such as probiotics, prebiotics, and natural active substances.

In recent years, special attention has been paid to the use of natural plant extracts in animal science. Betaine is a trimethyl derivative of the amino acid glycine and widely found by a variety of plants in nature. There is increasing evidence that it is a highly valuable feed additive and can produce positive effects on animal performance [7–9]. Betaine is known to have two major functions in the body, as a methyl group donor and an organic osmolyte. On the other hand, betaine has been shown to protect cells from osmotic pressure and allow them to continue normal metabolic activities under conditions that inactivate cells [10]; thus, the use of betaine may improve broiler tolerance to heat stress. Furthermore, it has been suggested that betaine could be used as a natural antioxidant and had the ability to improve meat quality of broilers [11]. Based on the above properties of betaine, previous studies have demonstrated that dietary betaine could improve the heat stressed broilers' growth performance, physiology, carcass criteria [12], lipid metabolism [13], immune response [14], and intestinal barrier function [15]. However, the findings of one study were inconsistent [16], which revealed that betaine supplementation had no significant effects on carcass traits and intestinal morphology of broilers under heat stress. The variable results suggested that further research and development is still required in this regard. Meanwhile, due to the good meat quality, yellow-feathered broilers are increasingly favored by Chinese consumers. Huaixiang chicken is a famous Chinese indigenous yellow-feather broiler breed and is widely farmed in southern China [17]. However, there is extremely limited information about the effects of betaine on these indigenous yellow-feathered broilers under long-term heat stress. Therefore, the current experiment was conducted to investigate the adverse effect of long-term heat stress on growth performance, digestive function, carcass traits, and meat quality in indigenous yellow-feathered broilers (Huaixiang chicken), and to evaluate whether feeding betaine could ameliorate long-term heat stress-induced impairment of these parameters.

2. Materials and Methods

2.1. Animal Ethics

The present study was carried out at the College of Agriculture, Guangdong Ocean University, Zhanjiang, China. The protocol of this experiment was approved by the Animal Care Committee, College of Agriculture, Guangdong Ocean University, Zhanjiang, China (SYXK-2018-0147).

2.2. Experimental Design, Animals, and Diet

A total of two hundred and forty 5-week-old yellow-feathered male broilers (indigenous breed, Huaixiang chickens) were randomly allocated to five treatments, each of which was replicated six times with eight broilers per replicate. The experimental period lasted 10 weeks. The five treatments were thermoneutral zone control group (TN, fed basal diet); heat stress control group (HS, fed basal diet); heat stress treatment group 1 (basal diet +500 mg/kg betaine); heat stress treatment group 2 (basal diet +1000 mg/kg betaine); heat stress treatment group 3 (basal diet +2000 mg/kg betaine). The betaine was obtained from a commercial Chinese company (anhydrous betaine, 99% purity, Shandong Jianchuan Biotechnology Co., Ltd., Shandong, China). The ingredient composition and nutrient content of basal diets are presented in Table 1. Basal diets were formulated to meet or exceed requirements suggested by the Chinese Chicken Feeding Standard (NY/T33-2004) [18]. The diet was given to the birds in the form of mash, and betaine was mixed into the diet before feeding. To ensure that the betaine was thoroughly mixed into the diet, firstly, betaine was mixed with 1 kg of feed by hand, and then the premix was mixed with the remaining feed by using a blender. The crude protein, lysine, cystine, methionine, calcium, and phosphorus of the diet were determined according to the methods of AOAC (2000) [19]. Birds had free access to feed and water. The broilers in TN group were raised at 26 ± 1 °C during the whole study. Other groups, designed as HS groups, were subjected to cyclic heat stress by exposing them to 32 ± 1 °C for 8 h/day from 9:00 am to 17:00 pm, the temperature of rest time is consistent with TN groups. Relative humidity was controlled at 65–75% among all groups during the entire experimental period. The birds in the TN and HS groups were housed in different facilities, the temperature and relative humidity of the TN and HS groups were measured three times a day. Continuous artificial light was used to illuminate the interior space for the whole period. The chicken houses were equipped with environmental control equipment, and the size of cage is 90 (length) × 70 (width) × 40 (height) cm.

2.3. Sampling and Measurements

The cage was considered as the experimental unit. Broilers were weighed on a cage basis (n = 30) initially and after 5 and 10 weeks of heat stress. The feed consumption was recorded weekly based on the cage (n = 30). Body weight gains (BWG), feed intake, and feed conversion ratio (FCR) were then calculated using this information for each phase.

After 5 and 10 weeks of heat stress, one bird from each replicate was randomly selected and moved to metabolic cages for metabolic testing (one bird per cage). The metabolic test lasted 4 days, and the nutrient retention was analyzed as average data by cage during the 4 days (n = 30). The total excreta collection method was used for determination of nutrient retention. During the test, feed intake and excrements were recorded daily, and the excreta were collected. The nitrogen, ash, gross energy, Ca, and phosphorus contents in the feed and excreta were then analyzed based on the method of AOAC (2000) [19]. The crude fat contents were analyzed by using a fat analyzer (Hua Bei Experimenting, Co., Ltd., Hebei, China) based on the Soxhlet extraction method, and ether was used as the solvent. The nutrient retention was calculated by the following formula:

$$\text{Nutrient retention (\%)} = (\text{feed intake} \times \text{Nf- excretion amount} \times \text{Ne})/(\text{feed intake} \times \text{Nf}) \times 100$$

where Ne = nutrient concentration in excreta (% DM), Nf = nutrient concentration in feed (% DM).

After 5 and 10 weeks of heat stress, six birds per treatment (one bird per replicate was randomly selected) were slaughtered (n = 30) by severing the jugular vein, respectively. Small intestine was then separated and samples of the contents of the duodenum, jejunum, and ileum were immediately collected for the determination of digestive enzyme activity by using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Subsequently, approximately 2 cm segments of the duodenum, jejunum, and ileum at the middle position were collected immediately. The intestinal samples from each section were fixed in 10% buffered formalin until analyzed. Each intestinal segment was embedded in paraffin. A 7 μm section of each sample was placed onto a glass slide and stained with alcian blue/haematoxylin and eosin for examination with a light microscope. Villus height and crypt depth of the small intestine were measured at 40× magnification using computer software (Sigma Scan, Jandel Scientific, San Rafael, CA, USA), then villus height to crypt depth ratio was calculated.

Table 1. Basal diet composition (as-fed basis).

Item	Contents (%)
Ingredients	
Corn	67
Soybean meal	23
Wheat bran	4.0
Fish meal	3.0
Limestone	1.5
$CaHPO_4$	1.0
Premix [1]	0.5
Nutrient levels [2]	
ME (MJ/kg)	11.94
Crude protein (%)	18.2
Ca (%)	0.98
Met (%)	0.32
Cystine (%)	0.31
Lys (%)	0.90
Total phosphorus (%)	0.51

[1] Premix provided per kilogram of diet: 5000 IU of vitamin A, 1000 IU of vitamin D_3, 10 IU of vitamin E, 0.5 mg of vitamin K_3, 3 mg of thiamin, 7.5 mg of riboflavin, 4.5 mg of vitamin B_6, 10 μg of vitamin B_{12}, 25 mg of niacin, 0.55 mg of folic acid, 0.2 mg of biotin, 500 mg of choline, and 10.5 mg of pantothenic acid. 60 mg of Zn, 80 mg of Mn, 80 mg of Fe, 3.75 mg of Cu, and 0.35 mg of I. [2] Nutrient levels on DM basis; except for metabolic energy (ME), others are measured values; ME calculated according to Chinese feed ingredient database.

At the end of the experiment (after 10 weeks of feeding trial), the carcass traits and meat quality of the slaughtered broilers were determined (one bird per replicate was randomly selected, n = 30). The carcass traits, included: slaughter rate (%) = (slaughter weight/live weight) × 100; semi-eviscerated carcass rate (%) = (semi-eviscerated weight/live weight) × 100; eviscerated carcass rate (%) = (eviscerated weight/live weight) × 100; leg muscle yield (%) = (leg muscle weight on both sides/live weight) × 100; breast muscle yield (%) = (breast muscle weight on both sides/live weight) × 100; abdominal fat rate (%) = (abdominal fat weight/live weight) × 100. Subsequently, cooking loss was measured by using approximately 20 g of meat sample from the left breast and leg muscle according to the method described by Honikel [20]. The shear force of breast and leg muscle was detected by using C-LM3 digital display tenderness meter (kgf, Northeast Agricultural University, Harbin, China). Duplicate pH values of leg and breast muscle for each sample at 45 min and 24 h after slaughtered were measured using a pH meter (PHSJ-5, Leici, Shanghai Yidian Scientific Instrument Co., Ltd., Shanghai, China).

2.4. Statistical Analysis

All data were analyzed by using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The growth performance was analyzed during 1–5 weeks, 6–10 weeks, and 1–10 weeks of heat stress. Nutrient

retention, digestive enzyme activity, and intestinal morphology were analyzed after 5 and 10 weeks of heat stress. Carcass traits and meat quality were investigated after 10 weeks of heat stress. Data were expressed as means. Differences among means were tested by using Tukey's test. Orthogonal polynomial contrasts were used to test the linear, quadratic, and cubic effects of the increasing levels of dietary betaine among HS groups. Variability in data was expressed as standard error of means (SEM), $p < 0.05$ was considered to be statistically significant, $0.05 \leq p < 0.10$ was considered to be a tendency.

3. Results

3.1. Growth Performance

The results of growth performance were shown in Table 2. During 1–5 weeks, heat stress reduced the BWG and feed intake ($p < 0.05$), whereas it increased the FCR ($p < 0.05$). Dietary betaine supplementation tended to improve the BWG (linear, $p = 0.078$) and feed intake (linear, $p = 0.075$) of broilers under heat stress. During 6–10 weeks, heat stress decreased the BWG and feed intake ($p < 0.01$). Supplementation of graded levels of betaine improved the BWG and feed intake (linear, $p < 0.05$) of broilers under heat stress. During the whole experimental period (1–10 weeks), heat stress reduced the BWG and feed intake ($p < 0.01$) and dietary inclusion of betaine increased BWG and feed intake (linear, $p < 0.05$) of broilers under heat stress.

Table 2. Effects of heat stress and dietary betaine on growth performance of yellow-feathered broilers *.

Dietary Betaine Levels (mg/kg)	Temperature	Initial BW, g	1–5 Weeks			6–10 Weeks			1–10 Weeks		
			BWG, g	FI, g	FCR	BWG, g	FI, g	FCR	BWG, g	FI, g	FCR
0	TN	404	426	1441	3.42	459	1729	3.88	992	3170	3.22
0	HS	393	328	1345	4.14	299	1248	4.28	720	2593	3.69
500	HS	399	369	1312	3.59	424	1575	3.84	849	2887	3.46
1000	HS	406	371	1429	3.88	363	1616	4.60	828	3046	3.70
2000	HS	391	385	1404	3.81	449	1671	3.66	939	3075	3.30
SEM		9.9	20.5	30.4	0.187	35.9	83.1	0.362	57.9	85.5	0.214
Contrast							*p*-value				
TN vs. HS		-	0.004	0.042	0.013	0.005	0.001	0.435	0.004	0.001	0.142
Linear		-	0.078	0.075	0.468	0.024	0.037	0.236	0.042	0.026	0.276
Quadratic		-	0.581	0.917	0.279	0.581	0.516	0.288	0.885	0.222	0.589

* BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; TN, thermoneutral zone; HS, heat stress; SEM, standard error of means; TN vs. HS, TN group vs. HS control (0 mg/kg betaine) group.

3.2. Nutrient Retention

Nitrogen retention was significantly reduced by 5 weeks of heat stress (Table 3, $p < 0.05$), and 5 weeks of heat stress tended to decrease the P retention ($p = 0.065$). Supplementation of betaine increased the nitrogen and P retention (linear, $p < 0.05$). After 10 weeks of heat stress, decreased nitrogen retention was observed ($p < 0.05$). Dietary betaine could improve nitrogen retention in heat stressed broilers (linear, $p < 0.05$).

Table 3. Effects of heat stress and dietary betaine on nutrient retention of yellow-feathered broilers *, %.

Dietary Betaine Levels (mg/kg)	Temperature	Nitrogen	CF	Energy	Ash	Ca	P
After 5 Weeks Heat Stress							
0	TN	79	86	77	47	59	50
0	HS	70	83	79	45	61	37
500	HS	77	84	81	47	57	46
1000	HS	79	80	79	54	63	55
2000	HS	79	85	82	58	62	52
SEM		2.7	3.1	2.3	5.1	4.3	4.7
Contrast				*p*-value			
TN vs. HS		0.032	0.579	0.554	0.786	0.843	0.065
Linear		0.027	0.932	0.486	0.092	0.686	0.045
Quadratic		0.215	0.419	0.905	0.828	0.828	0.250
After 10 Weeks Heat Stress							
0	TN	70	84	82	42	60	50
0	HS	64	85	81	45	59	43
500	HS	69	84	84	46	58	42
1000	HS	66	82	81	52	57	51
2000	HS	70	85	79	50	65	47
SEM		1.5	2.8	2.4	4.1	3.5	3.3
Contrast				*p*-value			
TN vs. HS		0.019	0.808	0.619	0.576	0.817	0.178
Linear		0.046	0.894	0.894	0.644	0.393	0.271
Quadratic		0.740	0.428	0.428	0.711	0.287	0.741

* CF, crude fat; TN, thermoneutral zone; HS, heat stress; SEM, standard error of means; TN vs. HS, TN group vs. HS control (0 mg/kg betaine) group.

3.3. Digestive Enzyme Activity

As presented in Table 4, after 5 weeks of heat stress, the trypsin activity of the jejunum was decreased by heat stress ($p < 0.05$). Detary supplementation of betaine had quadratic effects on trypsin activity of jejunum in heat stressed broilers ($p < 0.05$). Additionally, after 10 weeks of heat stress, dietary betaine supplementation improved the trypsin activity of the duodenum in heat stressed broilers (linear, $p < 0.05$).

Table 4. Effects of heat stress and dietary betaine on digestive enzyme activity of yellow-feathered broilers*, U/mg protein.

Dietary Betaine Levels (mg/kg)	Temperature	Duodenum		Jejunum		Ileum	
		Trypsin	Lipase	Trypsin	Lipase	Trypsin	Lipase
After 5 Weeks Heat Stress							
0	TN	248	1.76	351	1.69	346	1.71
0	HS	179	1.30	247	1.33	315	1.63
500	HS	172	1.72	361	2.68	380	2.25
1000	HS	196	1.05	310	1.36	335	1.65
2000	HS	189	1.36	271	1.40	333	1.55
SEM		33.1	0.518	32.6	0.491	54.0	0.462
Contrast		*p*-value					
TN vs. HS		0.151	0.537	0.035	0.605	0.686	0.904
Linear		0.590	0.845	0.898	0.653	0.975	0.695
Quadratic		1.000	0.926	0.038	0.239	0.559	0.461
After 10 Weeks Heat Stress							
0	TN	225	0.89	414	1.61	293	0.64
0	HS	116	0.55	313	0.91	243	0.60
500	HS	183	1.23	264	1.31	216	0.50
1000	HS	201	0.43	418	2.35	293	0.54
2000	HS	250	0.99	258	1.85	263	0.81
SEM		40.4	0.382	56.7	0.703	41.3	0.178
Contrast		*p*-value					
TN vs. HS		0.105	0.528	0.296	0.488	0.406	0.865
Linear		0.044	0.767	0.972	0.249	0.480	0.287
Quadratic		0.849	0.879	0.327	0.539	0.978	0.339

* TN, thermoneutral zone; HS, heat stress; SEM, standard error of means; TN vs. HS, TN group vs. HS control (0 mg/kg betaine) group.

3.4. Intestinal Morphology

As shown in Table 5 and Figures 1 and 2, after 5 weeks of heat stress, as compared with the TN group, the heat stress control group had lower villus height ($p < 0.05$) and tended to decrease the villus height to crypt depth ratio of duodenum ($p = 0.057$). Supplemental betaine had a tendency to increase the villus height to crypt depth ratio (linear, $p = 0.057$), and had a trend of quadratic effect on villus height ($p = 0.084$) and villus height to crypt depth ratio ($p = 0.056$) of the duodenum in heat-stressed treatments. After 10 weeks of the feeding trial, heat stress induced reduction of villus height of the duodenum and jejunum ($p < 0.05$), and decreased the villus height to crypt depth ratio of the jejunum ($p < 0.05$). Supplementation with betaine improved the villus height and villus height to crypt depth ratio of the jejunum (linear, $p < 0.05$) and had quadratic effects on villus height and villus height to crypt depth ratio of the duodenum ($p < 0.05$) in heat stressed broilers.

Table 5. Effects of heat stress and dietary betaine on intestinal morphology of yellow-feathered broilers *.

Dietary Betaine Levels (mg/kg)	Temperature	Duodenum			Jejunum			Ileum		
		Villus Height, μm	Crypt Depth, μm	VH:CD	Villus Height, μm	Crypt Depth, μm	VH:CD	Villus Height, μm	Crypt Depth, μm	VH:CD
After 5 Weeks Heat Stress										
0	TN	523	65	8.09	397	49	8.25	215	45	4.83
0	HS	415	68	6.14	335	46	7.28	204	42	4.99
500	HS	474	63	7.68	337	49	7.02	233	42	5.58
1000	HS	559	60	9.50	330	48	7.12	248	50	4.94
2000	HS	479	64	7.88	338	53	6.43	231	47	5.00
SEM		34.7	3.9	0.679	25.8	3.1	0.803	21.9	4.0	0.352
Contrast		*p*-value								
TN vs. HS		0.039	0.599	0.057	0.102	0.511	0.403	0.728	0.605	0.745
Linear		0.122	0.387	0.057	0.983	0.162	0.456	0.340	0.165	0.708
Quadratic		0.084	0.253	0.056	0.918	0.706	0.773	0.299	0.586	0.483
After 10 Weeks Heat Stress										
0	TN	566	105	5.54	318	66	4.95	192	59	3.26
0	HS	480	106	4.78	212	68	3.27	177	58	3.16
500	HS	540	87	6.42	265	60	4.55	228	58	4.15
1000	HS	555	102	5.84	287	63	4.72	209	58	3.60
2000	HS	529	109	5.03	283	61	4.99	189	60	3.13
SEM		22.9	8.4	0.508	21.9	6.0	0.434	23.9	5.9	0.378
Contrast		*p*-value								
TN vs. HS		0.036	0.975	0.370	0.003	0.781	0.028	0.664	0.885	0.850
Linear		0.114	0.541	0.751	0.037	0.405	0.037	0.882	0.822	0.717
Quadratic		0.046	0.155	0.033	0.232	0.640	0.347	0.179	0.898	0.080

* VH:CD, villus height to crypt depth ratio; TN, thermoneutral zone; HS, heat stress; SEM, standard error of means; TN vs. HS, TN group vs. HS control (0 mg/kg betaine) group.

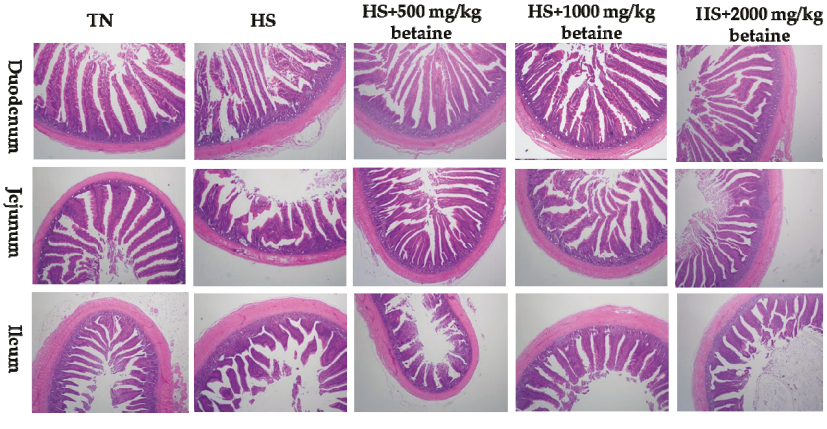

Figure 1. Photomicrographs of the effects of dietary betaine on intestinal morphology of yellow-feathered broilers after 5 weeks of heat stress (Stained with hematoxylin and eosin; TN, thermoneutral zone; HS, heat stress).

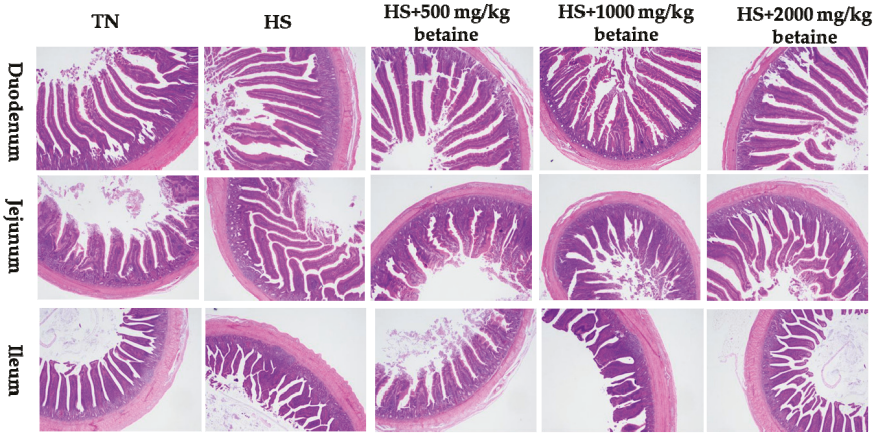

Figure 2. Photomicrographs of the effects of dietary betaine on intestinal morphology of yellow-feathered broilers after 10 weeks of heat stress (Stained with hematoxylin and eosin; TN, thermoneutral zone; HS, heat stress).

3.5. Carcass Traits

After 10 weeks of heat stress, broilers in the heat stress control group had lower eviscerated carcass rate and breast muscle yield than those in TN group (Table 6, $p < 0.05$). Additionally, heat stress tended to reduce semi-eviscerated carcass rate ($p = 0.085$). Dietary betaine improved the semi-eviscerated carcass rate, eviscerated carcass rate, and breast muscle yield of heat stressed broilers (linear, $p < 0.05$).

Table 6. Effects of heat stress and dietary betaine on carcass traits of yellow-feathered broilers *, %.

Dietary Betaine Levels (mg/kg)	Temperature	Slaughter Rate	Semi-Eviscerated Carcass Rate	Eviscerated Carcass Rate	Leg Muscle Yield	Breast Muscle Yield	Abdominal Fat Rate
0	TN	92.5	85.0	61.5	14.0	8.5	1.05
0	HS	91.0	83.3	58.5	14.6	7.4	1.47
500	HS	91.8	84.4	60.0	14.1	8.3	1.05
1000	HS	90.7	84.0	59.3	15.2	8.3	1.10
2000	HS	92.3	85.4	62.9	15.7	8.7	1.29
SEM		0.82	0.66	0.99	0.80	0.33	0.249
Contrast				*p*-value			
TN vs. HS		0.197	0.085	0.044	0.575	0.047	0.255
Linear		0.511	0.048	0.019	0.223	0.014	0.702
Quadratic		0.662	0.592	0.328	0.485	0.956	0.304

* TN, thermoneutral zone; HS, heat stress; SEM, standard error of means; TN vs. HS, TN group vs. HS control (0 mg/kg betaine) group.

3.6. Meat Quality

The results of meat quality were presented in Table 7. Heat stress challenge had no effects on cooking loss, shear force, and pH of breast and leg muscle ($p > 0.05$). In heat stress treatments, dietary betaine supplementation had no significant effects on investigated meat quality parameters ($p > 0.05$).

Table 7. Effects of heat stress and dietary betaine on meat quality of yellow-feathered broilers *.

Dietary Betaine Levels (mg/kg)	Temperature	Breast Muscle				Leg Muscle			
		Cooking Loss, %	Shear Force, kgf	pH45min	pH24h	Cooking Loss, %	Shear Force, kgf	pH45min	pH24h
0	TN	38.2	2.83	5.75	5.30	40.3	1.99	5.78	5.71
0	HS	36.3	2.92	5.59	5.37	38.2	1.85	5.82	5.47
500	HS	32.5	2.46	5.87	5.55	35.0	1.55	5.76	5.64
1000	HS	38.4	2.20	5.74	5.45	39.1	1.72	6.00	5.50
2000	HS	34.8	2.77	5.58	5.45	38.4	2.12	5.97	5.40
SEM		2.51	0.259	0.143	0.130	2.15	0.264	0.102	0.139
Contrast		*p*-value							
TN vs. HS		0.545	0.809	0.726	0.413	0.509	0.698	0.233	0.818
Linear		0.913	0.521	0.773	0.802	0.618	0.438	0.153	0.605
Quadratic		0.969	0.052	0.107	0.522	0.558	0.215	0.930	0.396

* TN, thermoneutral zone; HS, heat stress; SEM, standard error of means; TN vs. HS, TN group vs. HS control (0 mg/kg betaine) group.

4. Discussion

4.1. Growth Performance

It has been well documented that heat stress causes a series of drastic changes in broilers' physiological function, including decreasing the feed intake, disturbing the intestinal function and electrolyte balance, and adversely affecting blood metabolites and hormonal secretions, which results in impairment of productive performance [2,4]. In this study, expectedly, heat stress induced a reduction in BWG and feed intake of indigenous yellow-feathered broilers. This was in agreement with the reports by Zhong et al. [21,22], who observed that heat stress suppressed the average daily gain of yellow-feathered broilers under similar experimental conditions. The lower growth rate in heat stressed broilers may be attributed to the decreased feed intake, which is a defense mechanism to reduce the heat increment of bodies [23]. In addition, the heat stressed broilers consume more energy to adapt to high ambient temperature, thereby reducing energy for growth and leading to a lower growth performance [1].

Betaine is a functional active substance from a variety of plants, which can act as methyl group donor and organic osmolyte, and has the ability to improve growth performance in animals [7–9]. Meanwhile, according to the previous studies, betaine could be used as an effective antistress additive in broilers. For instance, He et al. [13] demonstrated that betaine improved the BWG and feed intake of Arbor Acres broilers under 32 °C heat stress. Chand et al. [14] found that dietary supplementation of 1.5% and 2% betaine increased the feed intake and BWG and reduced the FCR of fast-growing broilers exposed to heat stress. Similar findings have been reported in the studies of Sakomura et al. [16] and Singh et al. [24], who observed a significant increase in feed intake and BWG of heat stressed Cobb broilers fed with diet contained betaine. However, the studies related to the effects of betaine on indigenous slow-growing broilers are very scarce. Attia et al. [12] reported that 0.5 or 1.0 g/kg betaine supplementation improved the BWG and feed intake, whereas it decreased the FCR of slow-growing white-feathered broilers under heat stress. To the best of our knowledge, no research has been reported to study the effect of dietary betaine on Chinese indigenous yellow-feathered broilers. Our data showed that dietary supplementation of betaine could mitigate the adverse effects of heat stress on feed intake and BWG in indigenous yellow-feathered broilers, indicating that betaine has potential as an anti-heat stress additive for slow-growing yellow-feathered broilers. Regarding the mechanism of action, it was assumed that the beneficial effects on growth performance of heat stressed broilers might be due to the osmoregulatory, methyl group donors, and antioxidative properties of betaine.

4.2. Digestive Function

During heat stress, the intestinal epithelial cells of broilers are subjected to osmotic stress, as high ambient temperature may lead to water imbalance and cell permeability changes through dehydration [25]. Additionally, the fluid transport in the gastrointestinal tract during heat stress may also cause changes in intestinal structure and digestive function [23]. Indeed, in the current study, heat stress groups had lower nitrogen retention and trypsin activity. This was supported by previous findings of Attia et al. [12] and Chen et al. [26], who demonstrated that heat stress decreased the nitrogen digestibility and digestive enzymes activity of broilers. The present study showed that dietary supplementation of betaine ameliorated the nitrogen retention and trypsin activity of the duodenum and jejunum in heat stressed yellow feather broilers. Similarly, Attia et al. [12] reported that 0.5 or 1.0 g/kg betaine supplementation recovered the crude protein digestibility coefficients from the adverse effects of heat stress on slow-growing chicks. Eklund et al. [27] revealed that supplementation of betaine in broilers' diet could improve the apparent nutrient digestibility, including protein, methionine, and crude fat. However, because the available data regarding the effect of betaine on nutrient digestibility and digestive enzymes activity in heat stressed yellow-feathered broilers is limited, no more comparisons could be made. On the other hand, according to the results obtained by Wang et al. [28], betaine supplementation increased the activities of amylase, lipase, trypsin, and chymotryps of the small intestine in stressed rats. Pollard and Wyn Jones [29] also found that betaine protected against stress inhibition of enzymes. It has been suggested that betaine could promote the activity of key cellular enzymes, and the effects of betaine involved universal water–solute–macromolecule interactions [28]. Betaine possesses an osmotic effect and attaches to the surface of biopolymers and helps proteins fold more compactly [30]. In the same study, they also noted that this protective effect may be limited to certain enzymes. This was in agreement with our results, which only show an increase in the activity of trypsin.

The intestinal villus height and villus height to crypt depth ratio were decreased by heat stress in our experiment, suggesting that heat stress induced deterioration of intestinal morphology. These findings were in accordance with previous reports of Quinteiro-Filho et al. [2] and Burkholder et al. [3]. Animals have mechanisms to regulate body temperature as well as changes in physiological status. When the ambient temperature exceeds the thermoneutral zone, the body temperature raises, and peripheral blood flow increases as a response to heat stress, meaning that the blood flow of turbinate, nasal mucosa, myocardium, and respiratory muscles is higher than that of the intestine [2]. Ischemia and hypoxia of the intestine can cause epithelial shedding, leading to a deeper crypt depth and shorter villus height [3]. This study showed that the intestinal epithelial morphology was revived by the inclusion of betaine. Several possible mechanisms could explain the positive response of heat stressed broilers to dietary betaine: the methyl group donor nature of betaine might promote the proliferation of intestinal epithelial cells; the osmotic effect of betaine could improve the intestinal environment; and the antioxidant activity of betaine could alleviate intestinal oxidative damage induced by heat stress [28]. However, most studies to date have only investigated the effects of betaine on intestinal morphology of fast-growing broilers or rats. For instance, Kettunen et al. [31] discovered that dietary betaine supplementation increased the villus-crypt ratio of intestine in broilers. Eklund et al. [27] reported that betaine could maintain gut villi integrity and consequently promote better nutrient digestibility and absorption in broilers. Wang et al. [28] also demonstrated that betaine supplementation enhanced villus heights and villus height to crypt depth ratio of the duodenum, jejunum, and ileum in stressed rats. One study on the effects of betaine on heat stressed broilers obtained a contrary finding [16], revealing that the morphometrics of the intestinal crypts and villi in heat stressed broilers were not influenced by supplementation of betaine. The extent and duration of heat stress, species of broilers, growth stages, and the type of diet could help to explain these inconsistencies. Overall, betaine favorably affected the intestinal structure and digestive function could account for the boosted growth performance in this study.

4.3. Carcass Traits and Meat Quality

The present study discovered that the eviscerated carcass rate and breast muscle yield were reduced in response to heat exposure. This was supported by previous findings of Lu et al. [32], who reported that the carcass parameters were negatively affected by chronic heat stress in broilers. However, Sakomura et al. [16] did not find any significant impacts of heat stress on carcass, leg, and breast yield. The possible reasons of these results might be due to the experimental conditions and genetic background of broilers. In that study, thermoneutral zone groups were held at 28 °C from day 22 to day 45, and they used fast-growing (Cobb) broilers; these differences could lead to inconsistent findings. Betaine is often considered as a carcass modifier due to methyl group donor property, which causes a higher availability of methionine and cystine for protein deposition, thus contributing to improving the carcass lean percentage [33]. In this study, when supplemented with betaine in heat stressed broiler groups, the carcass traits were subsequently improved. Consistent with our results, Attia et al. [12] observed an improvement in the carcass traits of heat stressed slow-growing chicks by dietary betaine. Nofal et al. [34] found that inclusion of 0.2% betaine increased carcass weight and breast muscle yield in growing broilers under heat stress conditions. Similar results in thermoneutral conditions were obtained by Rao et al. [7] and Zhan et al. [35], who reported that dietary betaine supplementation enhanced the breast muscle yield of male broiler chickens.

Regarding meat quality, even though some studies have suggested that chronic heat stress had adverse effects on the meat quality of broilers, such as drop loss, cooking loss, shear force, pH, and meat color [4–6], our study failed to show any significant impacts of heat stress on cooking loss, shear force, or pH of breast and leg muscle. The different broiler breeds used might explain the difference of these results. Feeding betaine ameliorated heat stress-induced impairment of meat quality according to Attia et al. [12], who suggested that dietary betaine improved dry matter composition and water holding capacity of meat in slow-growing broilers. Also, Alirezaei et al. [11] indicated that betaine could act as an antioxidant agent and improve broilers' meat quality. However, there were no significant differences in meat quality criteria between treatments in this study. This might be because the number of observations was insufficient, or the detected criteria of meat quality were limited. Increasing the sample size of the experiment, and investigating other criteria related to meat quality, such as TBARS, intramuscular fat, lactic acid, etc., are essential in future studies.

5. Conclusions

To summarize, the current results indicated that long-term heat stress induced inferior growth performance, injured digestive function, and lower carcass yield in indigenous yellow-feathered broilers. Dietary supplementation of betaine was effective in improving growth performance, digestive function, and carcass traits in indigenous yellow-feathered broilers subjected to heat stress.

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Responsiveness Expressions of Bitter Taste Receptors Against Denatonium Benzoate and Genistein in the Heart, Spleen, Lung, Kidney, and Bursa Fabricius of Chinese Fast Yellow Chicken

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Simple Summary: In chickens, bitter taste is the most significant biological taste disrupter; it is believed to protect chickens against consuming poisonous/toxic materials and considered a warning signal prior to ingestion. The bitter taste receptors' extraoral expression information is deficient in chicken, and denatonium benzoate is extensively used as a bitter taste receptor agonist in different cells. Our results found that qRT-PCR showed a high level of dose-dependent expressions of ggTas2Rs in the starter and grower stages in the heart, spleen, lungs, and kidneys, while the dose-dependent expressions were lower in the bursa Fabricius. The growth performance of the selected organs significantly (and unexpectedly) improved upon the administration of denatonium benzoate 5 mg/kg and genistein 25 mg/kg treatments, while the gains in organ weights were impaired in the groups given denatonium benzoate 20 mg/kg and 100 mg/kg, respectively.

Abstract: The present study was conducted to investigate the responsiveness expressions of ggTas2Rs against denatonium benzoate (DB) and genistein (GEN) in several organs of the Chinese Fast Yellow Chicken. A total of 300 one-day-old chicks that weighed an average of 32 g were randomly allocated into five groups with five replicates for 56 consecutive days. The dietary treatments consisted of basal diet, denatonium benzoate (5 mg/kg, 20 mg/kg, and 100 mg/kg), and genistein 25 mg/kg. The results of qRT-PCR indicated significantly ($p < 0.05$) high-level expressions in the heart, spleen, and lungs in the starter and grower stages except for in bursa Fabricius. The responsiveness expressions of ggTas2Rs against DB 100 mg/kg and GEN 25 mg/kg were highly dose-dependent in the heart, spleen, lungs, and kidneys in the starter and grower stages, but dose-independent in the bursa Fabricius in the finisher stage. The ggTas2Rs were highly expressed in lungs and the spleen, but lower in the bursa Fabricius among the organs. However, the organ growth performance significantly ($p < 0.05$) increased in the groups administered DB 5 mg/kg and GEN 25 mg/kg; meanwhile, the DB 20 mg/kg and DB 100 mg/kg treatments significantly reduced the growth of all the organs, respectively. These findings indicate that responsiveness expressions are dose-dependent, and bitterness sensitivity consequently decreases in aged chickens. Therefore, these findings may improve the production of new feedstuffs for chickens according to their growing stages.

Keywords: denatonium benzoate; genistein; chicken; ggTas2Rs; bitter taste receptors

1. Introduction

In chickens, bitter taste is one of the most significant senses for choosing and consuming feeds, alongside their olfactory and visual senses [1–3]. Taste signals have been associated to food recognition

and avoidance, as well as feed or liquid intake in different species of animals [4–7]. Bitter taste provokes an aversive reaction and is assumed to protect chickens against consuming poisons and harmful toxic substances. The age effects in humans were found to be almost exclusively generic and taste sensitivity was found to decline with age, although the level of bitterness differs depending on taste quality and is never compound-specific within a taste [8]. Chickens demonstrate bitter taste sensitivity despite having only three bitter taste receptors: ggTas2R1, ggTas2R2, and ggTas2R7. It has been shown that chickens have a well-developed sense of taste and only three of the aforementioned bitter receptors have been investigated [9]. In chickens, an insufficient number of studies have been performed to investigate growth-related taste loss and its subsequent effect on the animal's production. Additionally, behavioral experiments were conducted and found that day-old and immature chicks were more susceptible against salt and sour taste qualities than the adults [10]. It is meaningful to elucidate the bitter taste sensitivity of chicken because of the different nutrient requirements and prerequisites during their growing stages. Bitter molecules detected by the ggTas2R family of G-protein-coupled receptors (GPCRs) were involved in perceiving potentially toxic compounds [11–13]. The commercial feed factories produce several categories of chicken feed according to their growth stages such as starter, grower, and finisher. When the taste buds are counted, birds have few compared to humans and other mammals. For example, humans have approximately 9000 taste buds, chickens have between 250–350 taste buds, and pigeons have only 37–75 [14–16]. Furthermore, in chickens, the gustatory and extra-gustatory mechanisms of involving taste signaling have been shown recently [1,17–19].

Denatonium benzoate (DB) is intensely bitter and non-toxic, which can be detected by human taste receptors [20]. Denatonium benzoate has been demonstrated widely as a bitter taste agonist and used to activate bitter taste receptors on many cell types, including tastes cells [21]. A previous study indicated that denatonium increased cholecystokinin release through Ca^{2+} influx in enteroendocrine STC-1 cells [22]. After oral glucose administration in diabetic mice, a prior gavage of denatonium attenuated blood glucose levels through the increased secretion of glucagon-like peptide-1 [23]. In addition, exposure to denatonium quickly suppressed the ongoing intake and delayed gastric emptying in rodents [24,25]. Apparently, genistein is mainly derived from soy products, which contain a phytochemical with isoflavone structure that is found in an extensive variety of foods, legumes, animal forages, and particularly in soybeans. Genistein (GEN) has protective effects against atherosclerosis, cardiovascular risk, and type 2 diabetes, which are attributed to its antioxidant activity; furthermore, dietary GEN can enhance the growth performance of livestock [26–29].

In recent years, the majority of reports on the expression of extra-gustatory taste receptors obviously suggested that their role is not restricted to taste perception in the mouth and gastrointestinal tract. Taste receptors have additionally been recognized in the respiratory system [30,31] and gastrointestinal tracts of mammals, in the male reproductive system, and in the brain, as well as in the heart [32,33]. Currently, the direct involvement of the human bitter taste receptor TAS2R38 in the detection of minimum bacterial sensing molecules was suggested [34]. One of the most recent additions of scientific investigations is the expression of bitter taste receptors in human and animal hearts [32,35]. Remarkably, the whole heart cDNA of neonatal rats analyzed by qRT-PCR, the seven bitter taste receptors genes, and two genes encoding the umami receptor subunits, Tas1R1 and Tas1R3, were identified as expressed in hearts. However, many research studies on the influence of bitter taste receptors materials on cardiac tissue observe need to be warranted [36].

Taste sensitivity in chickens is lower compared to that of mammals. It has been reported that chickens respond to several tastants, and these responses are conserved from post-hatching until adulthood [10,37–41]. Nevertheless, the relationship between taste sensitivity and number of taste buds in various chicken breeds remain unclear. It has been clarified recently that Tas2Rs receptors are also expressed in the extraoral tissues of the chicken [35,42]. The molecular mechanism of bitter molecules by their receptors is slightly complicated and less studied. However, bitter taste receptors (Tas2Rs or T2Rs) are developing as novel regulators of native immunity in the respiratory tract [43].

Recent studies findings indicate that T2Rs are extensively expressed in several parts of the human body, and have been identified to be involved in respiratory system physiology, the gastrointestinal tract, and the endocrine system, and T2Rs may play regulatory roles in the mentioned areas of the body [35,41,44,45]. In contrast, GPCRs facilitate the sensations of bitter, sweet, and umami tastes in mammals and chickens [46]. Moreover, the gene expression compilation collection (http://www.ncbi.nlm.nih.gov/geo) also confirmed that T2Rs are widely expressed in other human tissues such as the heart, brain, skeletal muscle, endometrium, liver, omental adipose tissue, nasal cavity, lung, and different cell types (chemosensory cells, smooth muscle cells, endothelial cells, epithelial cells, and inflammatory cells) [47].

The detection of taste thresholds and their identification is crucial for studying the potential effects of Tas2Rs on chicken feeding behavior. Interestingly, the chicken's genome contains only three bitter taste receptors, which are responsible for bitterness identification, as described previously [48]. The presence of a minimum lower number of bitter taste receptors makes the chicken a significant minimalistic model for an understanding of vertebrate taste necessities [49]. However, there is limited knowledge about the expressions of bitter taste receptors, and no study has yet investigated the bitter taste responsiveness expressions in the extra-gustatory organs of chickens. Therefore, the objectives of this research were to investigate the bitter taste receptors' (ggTas2Rs) responsiveness expressions against different doses of denatonium benzoate, genistein and compare the ggTas2Rs mRNA expressions levels among different organs in the starter, grower, and finisher stages of Chinese Fast Yellow Chicken.

2. Materials and Methods

2.1. Chemicals

Denatonium benzoate 98% was purchased from Adamas Reagent Co Ltd. (Nanjing, China), Genistein 98% was purchased from Kai Meng. Co Ltd. (Xi'an, China) and stored at room temperature. RNAose (phenol 38%), trichloroethane, isopropyl alcohol, alcohol absolute, and DNA/RNA-ase free water were purchased collectively for RNA extraction from TAKARA BIO INC (Nojihigashi 7-4-38, Kusatsu, Shiga Japan), while PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, Cat. # RR047A v201710Da) and TB Green™ Premix Ex Taq™ (Tli RNase H Plus, Cat. # RR420A v201710Da) were both purchased from TaKaRa (Dalian, China).

2.2. Birds and Procedures

The experimental protocol and procedures were designed and approved in accordance with the Guidelines for the Care and Use of Animals prepared by the Institutional Animal Care and Ethical Committee for Nanjing Agricultural University, Nanjing, China (Permit Number: SYXK (Su) 2019–0036). A total of three hundred (300), 1-day-old Chinese Fast Yellow chicks at the average weight of 32 ± 5 g were randomly allocated into five (5) groups with five replicates of 12 chicks in each. In order to find the dose-dependent comparison expressions of bitter taste receptor genes against DB, we designed the experiment with different doses of denatonium benzoate from low levels to high levels; the experiment groups were as follows: the control, denatonium benzoate 5 mg/kg (Low Dose), denatonium benzoate 20 mg/kg (Medium Dose), denatonium benzoate 100 mg/kg (High Dose), and genistein 25 mg/kg (GEN 25 mg/kg). All were reared under the ventilated chicken house in which the light remained 16-h light: 8-h dark, humidity was approximately 40–45% and formulated feed (Table 1) was offered ad libitum with freely available tap water for 56 consecutive days.

Table 1. Feed formulation for the entire period of the experiment (d 1–56).

Item	Diet	
	D 1 to 28	**D 28 to 56**
Ingredient (%)		
Corn	61.52	74.00
Soybean meal	29.00	12.00
Soybean oil	2.44	2.60
Corn gluten meal	2.00	7.32
Dicalcium phosphate	1.68	1.02
Premix	1.50	1.00
Limestone	1.15	1.05
Lysine sulfate	0.51	0.80
Methionine	0.20	0.21
Total	**100**	**100**
Calculation of nutrients		
Metabolizable energy, MJ/kg	11.92	12.13
Crude protein, %	21.00	19.00
Lysine, %	1.10	0.97
Methionine, %	0.46	0.40
Methionine + cystine, %	0.80	0.72
Calcium, %	1.00	0.90
Available phosphorus, %	0.70	0.65

Provided the following % per kilogram in completed diet: vitamin A, 12,500 IU; vitamin D3, 2500 IU; vitamin E, 30 IU; vitamin K3, 2.65 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; nicotinic acid, 50 mg; pantothenic acid, 12 mg; vitamin B_6, 4 mg; folic acid, 1.25 mg; vitamin B_{12}, 0.025 mg; biotin, 0.25 mg; Fe, 50 mg; Zn, 75 mg; Mn, 100 mg; Cu, 8 mg; I, 0.35 mg; Co, 0.2 mg; and Se, 0.15 mg.

2.3. Feed Formulation and Mixing Procedure

We purchased the basal diet from ADM factory (Nanjing, China) with respective ingredients (Table 1); then, we mixed the basal diet feed with the different treatments through an electric feed mixing machine available in the Nanjing Agricultural University animal house. According to the experimental design, five (5) types of treatments were prepared: the basal diet (Control), denatonium benzoate 5 mg/kg (Low Dose), denatonium benzoate 20 mg/kg (Medium Dose), denatonium benzoate 100 mg/kg (High Dose), and genistein 25 mg/kg. The chemicals and organic materials were mixed appropriately with the help of an electric mixer and provided ad libitum for the feeding of chicken until the end of the experiment, respectively.

2.4. Organs Weight Measurements

A total of 10 chickens in each group (two chickens/replicate) were used in each stage of killing to collect and measure the heart, spleen, lung, kidney, and bursa Fabricius weights at Day 7 (starter stage), Day 28 (grower stage), and Day 56 (finisher stage), respectively (Table 3).

2.5. Sample Collection and RNA Extraction

On days 7, 28, and 56 (the starter, grower, and finisher stages) tissues from the heart, spleen, lung, kidney, and bursa Fabricius were collected and kept in an −80 °C freezer until RNA extraction. Afterward, the total RNA for RT-PCR and real-time PCR was extracted and purified from frozen collected tissues using RNAose (TAKARA BIO INC, Nojihigashi 7-4-38, Kusatsu, Shiga Japan), which includes gDNA Eraser (Perfect Real Time) for the elimination of genomic (g) DNA according to the manufacturer's protocols.

2.6. Primer Design and RT-PCR

Initially, first-strand cDNA was synthesized by reverse transcription (RT) with the application of 2.0 ug of total RNA with or without reverse transcriptase using the PrimeScript™ RT reagent Kit with

gDNA Eraser (Perfect Real Time, Cat. # RR047A v201710Da) in accordance with the manufacturer's instructions. Gene-specific primers for ggTas2R1 (Accession no. AB249766.1), ggTas2R2 (Accession no. AB249767.1), ggTas2R7 (Accession no. NM_001080719.1) and the housekeeping gene (β-actin) (Accession no. NM_205518.1) were generated with the aid of the nucleotide database of The National Center for Biotechnology Information (NCBI) [50], according to their published cDNA sequences (Table 2). The target genes and the housekeeping gene were synthesized by the Sangon company and applied for real-time PCR (Table 2). Amplicon lengths for real-time PCR were between 102–162 bp. The PCR mixture consisted of 2 µL of a cDNA template diluted in a ratio of 1:3, 10 µL of TB Green premix Ex Taq (Tli RNase H Plus) (2×), 0.4 µL of forward primer (10 µM), 0.4 µL of reverse primer (10 µM), 0.4 µL of ROX Reference Dye 1 or Dye 2 (50×) and 6.8 µL of DNA/RNA enzyme-free water in a final volume of 20 µL (TaKaRa, Dalian, China). Entire PCR reactions were performed in 96-well reaction plates on a 7500 Real-time PCR instrument (Applied Biosystems, ABI, USA), and all the genes were repeated six times under the following conditions: ha old stage (95 °C for 30 s), a PCR stage (40 cycles of 95 °C/2 min, 60 °C/34 s) apparently to verify the amplification of a single product, while a stage with a temperature increment (melt curve stage) was conducted to generate a melting curve under the following conditions: (95 °C/15 s, 60 °C/1 min), followed by a temperature increment of 95 °C/15 s.

Table 2. Primers used for real-time PCR analysis of genes expressions.

Target	Accession No	Forward Primer	Reverse Primer	Product Size (bp)
ggTas2R1	AB249766.1	GGTGCCATCAAGACAGTCTTCTC	ACAGGCAGCCACTACAACAACA	135
ggTas2R2	AB249767.1	GCGATGATTCCATGGCTGC	CGTTGACCTGCAGAGGTAGG	102
ggTas2R7	NM_001080719.1	TGGCAGAGCAGCACAACACAAC	TACAAGACGCAGCCACAATGAA	111
β-actin	NM_205518.1	CCAGCCATGTATGTAGCCATCCAG	ACGGCCAGCCAGATCCAGAC	162

gg–Gallus Gallus; TasR-Taste Receptor, β-actin-Housekeeping gene.

2.7. Housekeeping Gene for Internal Control (β-actin)

We investigated the Ref-Finder online database (http://www.leonxie.com/rferencegene.php) to choose the most constant housekeeping gene as an internal control for the real-time PCR analysis. The mentioned database consists of various computational programs (geNorm, Norm-finder, Best-Keeper, and the comparative ΔΔCt method). We calculated the relative gene expression (arbitrary units) utilizing the $2^{-\Delta\Delta Ct}$ method and normalized the relative abundance to tested candidate reference genes. Promising housekeeping genes were statistically tested for significant differences among various tested tissues, developmental time points, and their interaction using JMP Pro 10 software (SAS Institute, 2006, Cary, NC) [49,51]. The geometric average of β-actin was found to be the most stable and significant reference gene with no significant differences ($p > 0.05$) among the target organs (heart, spleen, lung, kidney, and bursa Fabricius) on days 7, 28, and 56 using one-way ANOVA for analysis.

2.8. Determination of mRNA Expression by Real-Time PCR Using the Comparative ΔΔCt Method

To confirm and validate the target gene expressions for the first time, the data were subjected to new ΔΔCt fold-change calculations [52], and statistical analysis were carried out to compare the expression of the ggTas2Rs target genes in heart, spleen, lung, kidney, and bursa Fabricius in the starter, grower, and finisher stages of the chicken. Finally, the efficiencies of all the tested genes and the reference gene were calculated. Cycle threshold (Ct) values for every sample were calculated using the ΔCt (Δ cycle threshold) procedure [53]. Gene expression was normalized against the geometric average of β-actin. Changes in mRNA determination were analyzed by comparing the relative expression among the genes in the heart, spleen, lung, kidney, and bursa Fabricius in different denatonium benzoate treatments, GEN, and a control group. Each stage's relative expression data were analyzed separately, and consequently, each target gene in a single selected organ was compared across all of the growing stages, respectively. Primers and gene accession numbers are described in Table 3, as described in detail in previous publications [49,54].

Table 3. Live body weight and organ weights in the starter, grower, and finisher stages.

Stages	Treatments	Heart (g)	Spleen (g)	Lungs (g)	Kidneys (g)	Bursa Fabricius (g)	Live Body Weight (g)
Starter	Control	0.64 ± 0.04^{b}	0.32 ± 0.05^{a}	$0.52 \pm 0.01^{b,c}$	0.72 ± 0.17^{a}	0.21 ± 0.30^{a}	70.94 ± 0.79^{b}
	DB-Low Dose	0.66 ± 0.02^{b}	0.25 ± 0.03^{b}	$0.52 \pm 0.02^{b,c}$	0.70 ± 0.10^{b}	0.09 ± 0.03^{b}	76.34 ± 0.41^{a}
	DB-Medium Dose	0.51 ± 0.04^{c}	0.22 ± 0.01^{c}	0.63 ± 0.04^{a}	$0.68 \pm 0.08^{b,c}$	0.09 ± 0.04^{b}	63.64 ± 0.88^{c}
	DB-High Dose	0.43 ± 0.02^{d}	$0.19 \pm 0.03^{c,d}$	0.47 ± 0.09^{d}	0.65 ± 0.08^{c}	0.09 ± 0.02^{b}	60.02 ± 0.98^{d}
	GEN	0.69 ± 0.07^{a}	0.34 ± 0.04^{a}	0.57 ± 0.01^{b}	0.73 ± 0.05^{a}	0.09 ± 0.01^{b}	63.70 ± 0.99^{c}
Grower	Control	$2.09 \pm 0.28^{b,c}$	0.58 ± 0.24^{a}	1.75 ± 0.17^{b}	$2.46 \pm 0.08^{a,b}$	0.55 ± 0.02^{b}	255.00 ± 1.33^{a}
	DB-Low Dose	2.13 ± 0.13^{b}	0.56 ± 0.15^{b}	1.31 ± 0.13^{c}	2.14 ± 0.09^{b}	0.57 ± 0.03^{a}	220.50 ± 2.14^{b}
	DB-Medium Dose	1.71 ± 0.24^{c}	0.37 ± 0.13^{c}	1.84 ± 0.23^{a}	2.13 ± 0.04^{b}	0.47 ± 0.02^{c}	197.10 ± 3.12^{d}
	DB-High Dose	1.63 ± 0.27^{d}	0.31 ± 0.14^{d}	1.29 ± 0.07^{d}	$2.11 \pm 0.06^{c,d}$	0.48 ± 0.02^{c}	197.40 ± 2.13^{d}
	GEN	2.37 ± 0.19^{a}	$0.45 \pm 0.19^{b,c}$	1.79 ± 0.11^{b}	2.56 ± 0.04^{a}	0.41 ± 0.02^{d}	208.30 ± 1.91^{c}
Finisher	Control	6.41 ± 0.02^{b}	2.24 ± 0.17^{b}	$4.75 \pm 0.11^{b,c}$	8.58 ± 0.03^{a}	$2.26 \pm 0.09^{b,c}$	888.20 ± 2.12^{a}
	DB-Low Dose	6.92 ± 0.02^{a}	2.36 ± 0.25^{a}	3.97 ± 1.13^{c}	7.87 ± 0.02^{b}	2.60 ± 0.05^{a}	687.00 ± 2.13^{c}
	DB-Medium Dose	5.29 ± 0.03^{c}	1.79 ± 0.19^{c}	4.86 ± 1.31^{a}	7.77 ± 0.06^{c}	$2.18 \pm 0.08^{d,e}$	725.30 ± 1.93^{b}
	DB-High Dose	$5.27 \pm 0.08^{c,d}$	1.59 ± 0.32^{d}	3.66 ± 0.16^{d}	7.22 ± 0.02^{d}	2.21 ± 0.03^{c}	686.50 ± 1.75^{d}
	GEN	$6.68 \pm 0.03^{a,b}$	2.32 ± 0.16^{a}	$4.77 \pm 0.50^{a,b}$	8.62 ± 0.04^{a}	2.20 ± 0.02^{c}	620.50 ± 2.12^{e}
P-Value	Starter	<0.001	<0.001	<0.001	<0.001	0.004	0.0047
	Grower	0.005	0.004	0.005	0.004	0.005	0.0038
	Finisher	0.001	0.005	0.001	0.003	0.004	0.0044
	Feed (Treatments)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Feed × Stages	0.183	0.181	0.111	0.094	0.917	<0.001

Heart, spleen, lung, kidney, and bursa Fabricius weight unit (g, n = 10); the table shows different treatments, DB 5 mg/kg (Low Dose), DB 20 mg/kg (Medium Dose), DB 100 mg/kg (High Dose), and GEN 25 mg/kg, values shown are mean ± SE. (Standard Errors), $^{a-e}$ means in a row with different superscript differ significantly ($p < 0.05$). The table also shows the interaction between stages and feed. DB: denatonium benzoate; GEN: genistein.

2.9. Statistical Analysis

2.9.1. Organ Weight Measurements

Weight measurements for five (5) selected organs were described previously [51]. Significant differences between treatment groups and the control group were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's post hoc test. A $p < 0.05$ was considered as statistically significant, and subsequently marked with (a, b, c, d). Value = MEAN ± SEM, weight unit (g).

2.9.2. Gene Expression

For relative gene expression analysis of the genes (ggTas2R1, ggTas2R2, and ggTas2R7), for each organ compared the chosen control gene (β-actin) in all the tissues (heart, spleen, lung, kidney, and bursa Fabricius) in different growing stages using one-way ANOVA. In addition, multiple comparison among means of ggTas2R1, ggTas2R2, ggTas2R7, and the β-actin gene in each group were calculated using Dunnett's test (marked with a, b, c, d, and e), and $p < 0.05$ was considered significant, as shown in the figures, respectively. An alpha level of 0.05 was set for all the tests. These statistical analyses were conducted with GraphPad Prism 6 and IBM SPSS Statistics, version 20 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Organ Weight Measurements

The results on the heart weight gained showed that the GEN and DB 5 mg/kg (Low Dose) groups significantly ($p < 0.05$) gained more weight compared to other DB and control groups (Table 3). Conversely, the live body weight and organ weights gained for the heart in the denatonium benzoate 100 mg/kg (High Dose) significantly ($p < 0.05$) decreased in the starter and grower stages, but not in the finisher stage (Table 3). However, no significant differences were observed among other treatments (Table 3). Meanwhile, the live body weight and spleen weight gained in the GEN group in all the growth stages significantly ($p < 0.05$) increased compared to the medium dose of DB 20 mg/kg. However, the

spleen weight in the denatonium benzoate 20 mg/kg (Medium Dose) group increased in the grower and finisher stages compared to the DB High-Dose group, respectively (Table 3). However, the spleen weight of the control group increased in the grower stage among all the groups (Table 3). Lung weight gaining also significantly ($p < 0.05$) increased in the GEN and DB Medium-Dose groups in the starter, grower, and finisher stages, while it significantly decreased in the DB High-Dose group in the starter stage and surprisingly increased in the DB Low-Dose group compared to the DB High-Dose group at the grower and finisher stages (Table 3). Furthermore, the kidney weight in the GEN group in all the growing stages increased significantly ($p < 0.05$) and decreased in the DB 100 mg/kg (High-Dose) group in the grower and finisher stages among the groups, while it increased in the DB 5 mg/kg (Low-Dose group) in the starter and grower stages compared to the other DB doses, respectively (Table 3). Finally, the weight gained for bursa Fabricius remarkably increased in the DB 5 mg/kg (Low-Dose group) in the grower and finisher stages, while it decreased in the GEN and DB 20 mg/kg (Medium-Dose) groups in all three stages of growing, respectively (Table 3). The interaction between feed and stages was declared in data analysis; there was lower interaction in the starter and grower stages, while higher interaction was observed in the finisher stage. Meanwhile, the live body weight and organ weights subsequently increased, while taste sensitivity decreased, respectively (Table 3).

3.2. Detection of ggTas2Rs Responsiveness Expressions Against Denatonium Benzoate and Genistein

3.2.1. mRNA Responsiveness Expressions of ggTas2Rs in Chicken Heart

Real-time PCR analysis showed that the expressions of ggTas2R1, ggTas2R2, and ggTas2R7 in chicken hearts was significantly ($p < 0.05$) higher in the starter and grower stages in all the treated doses of denatonium benzoate and genistein compared to the finisher stage (Figure 1A–C). While comparing different growing stages, the ggTas2R1, ggTas2R2, and ggTas2R7 genes were dose-dependent and highly expressed in GEN and DB 100 mg/kg (High Dose) in the starter and grower stages among other treatments and consequently similarly lower expressed in the finisher stage (Figure 1A–C). However, the expressions of ggTas2R1, ggTas2R2, and ggTas2R7 in the DB 5 mg/kg (Low Dose), DB 20 mg/kg (Medium Dose), and GEN groups were almost similar in the starter and grower stages, except for ggTas2R2 in the GEN group in the grower stage (Figure 1B and Figure 1A–C). The expressions of ggTas2Rs in all the groups were lower in the finisher stage, and no significant differences were observed among them.

3.2.2. mRNA Responsiveness Expression of ggTas2Rs in the Spleen of Chicken

The ggTas2Rs expressions in the spleens of chickens were quite highly and significantly ($p < 0.05$) expressed, and the responsiveness expressions were dose-dependent in the starter stage among all the growth stages, respectively (Figure 2A–C). For the comparison of the individual treatments, the ggTas2R1 and ggTas2R2 in DB 100 mg/kg (High Dose) in the starter stage was highly expressed among the other groups and growing stages (Figure 2A,B), whereas ggTas2R7 was highly expressed in the DB Medium-Dose group in the starter stage among all the other treatments in all the stages of growing, respectively (Figure 2C). Expressions of ggTas2Rs were dose-dependent to different DB doses and GEN in the starter stage (Figure 2A–C). However, it was also observed that the ggTas2R7 receptor was highly dose-dependent to the medium dose of DB in the grower stage, and the expressions for all the treatments subsequently decreased in the grower and finisher stages, respectively (Figure 2A–C). Furthermore, ggTas2Rs were gradually less expressed in the DB High-Dose and GEN groups in the grower stage and surprisingly increased in the finisher stage compared to other treatments within the stage. Finally, all the responsiveness expressions were absolutely descendant in the finisher stage, respectively (Figure 2A–C).

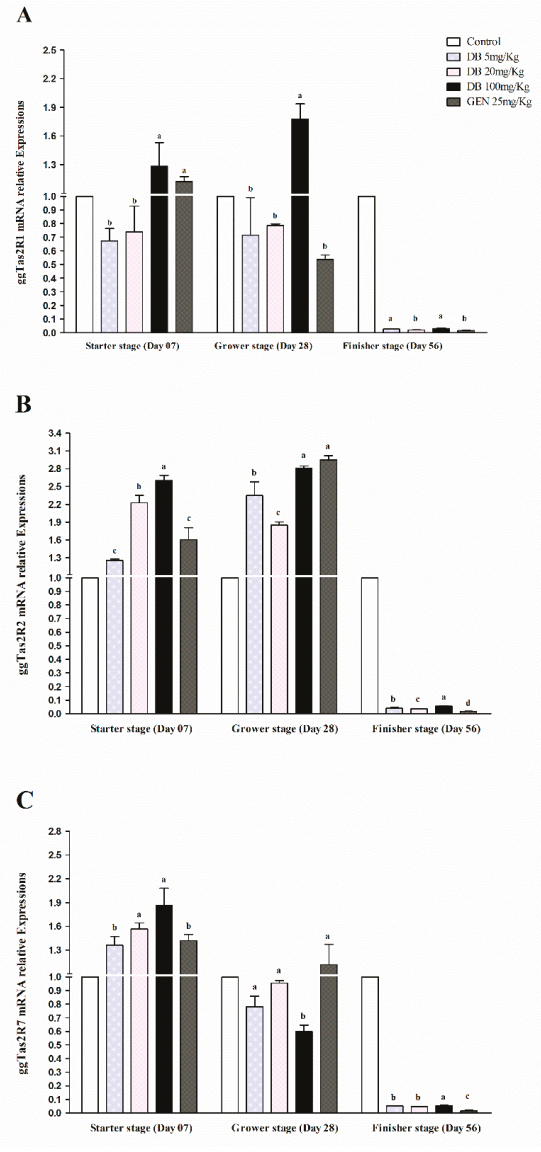

Figure 1. Real-time PCR analysis of (**A**) ggTas2R1, (**B**) ggTas2R2, and (**C**) ggTas2R7 of bitter taste receptors showing their relative mRNA expressions against different doses of DB and GEN in the hearts of chickens in different stages of growth (Day 7, Day 28, and Day 56). The relative mRNA abundance of ggTas2Rs in different growth stages with the heart serving as the control (relative expression set to 1; n = 6). Values are presented as the mean of relative expressions ± SEM. Differences between groups within a gene means those without a common letter differ significantly ($p < 0.05$); differences between the tested tissue (heart of chicken) and the control tissue (heart) within a gene means that those with marks (a, b, c) differ significantly ($p < 0.05$) from the control group by ANOVA.

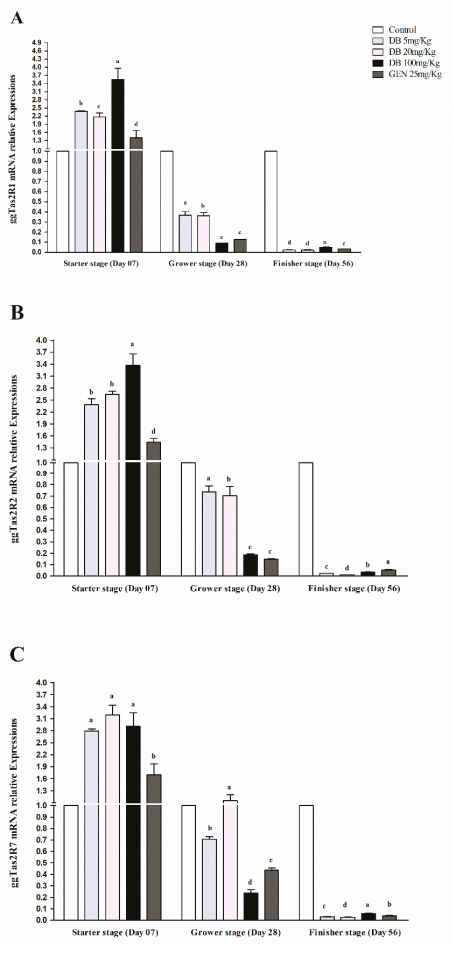

Figure 2. Comparing expressions of bitter taste receptors ((**A**) ggTas2R1, (**B**) ggTas2R2, and (**C**) ggTas2R7), showing their relative mRNA expressions against different doses of DB and GEN in chicken spleens in different growth stages (Day 7, Day 28, and Day 56). The relative mRNA abundance of ggTas2Rs in different growth stages with the spleen serving as the control (relative expression set to 1; n = 6). Values are presented as the mean of relative expressions ± SEM. Differences between groups within a gene mean that those without a common letter differ significantly ($p < 0.05$); differences between the tested tissue (spleen) and the control tissue (spleen) within a gene means that those with marks (a, b, c) differ significantly ($p < 0.05$) from the control group by ANOVA.

3.2.3. mRNA Responsiveness Expressions of ggTas2Rs in Chicken Lungs

The expressions of ggTas2Rs receptors in chicken lungs were significantly ($p < 0.05$) higher in the starter stage than in the grower and finisher stages, respectively (Figure 3A–C). The ggTas2R1 receptor in the DB 100 mg/kg group in the starter stage had potentially higher expression among the groups (Figure 3A). However, ggTas2R2 and ggTas2R7 were significantly ($p < 0.05$) comparably expressed in the grower and finisher stages within the treatments (Figure 3B,C). The responsiveness expressions of ggTas2Rs against DB different doses and GEN in three growing stages of Fast Yellow Chicken was almost equal and dose-dependent with negligible high expressions and variations in the group given

DB 100 mg/kg (High-Dose). Therefore, the expressions subsequently decreased in the finisher stage for all the groups, respectively (Figure 3A–C).

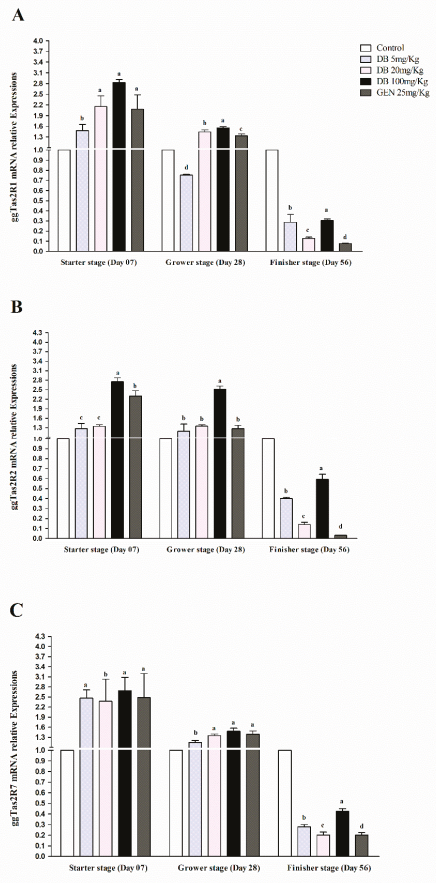

Figure 3. Comparing expressions of bitter taste receptors ((**A**) ggTas2R1, (**B**) ggTas2R2, and (**C**) ggTas2R7), which are showing their relative mRNA expressions against different doses of DB and GEN in chicken lungs in different growth stages (Day 7, Day 28, and Day 56). The relative mRNA abundance of ggTas2Rs in different growth stages with the lung serving as the control (relative expression set to 1; n = 6). Values are presented as mean of relative expressions ± SEM. Differences between groups within a gene mean that those without a common letter differ significantly ($p < 0.05$); differences between the tested tissue (lung of chicken) and the control tissue (lung) within a gene mean that those with marks (a, b, c) differ significantly ($p < 0.05$) from the control group by ANOVA.

3.2.4. mRNA Responsiveness Expressions of ggTas2Rs in Chicken Kidneys

The expressions of ggTas2Rs in chicken kidneys showed significantly ($p < 0.05$) high expressions against the GEN and DB Medium-Dose group with the exception of ggTas2R7, which was higher expressed in the DB High-Dose group in the starter stage, and then gradually less expressed than the other ggTas2Rs in the grower and finisher stages, respectively (Figure 4A–C). These results showed that ggTas2R1, ggTas2R2, and ggTas2R7 have contrary expressions levels in kidneys at different chicken growth stages (Figure 4A–C). For instance, it was found that the responsiveness expressions of ggTas2Rs were dose-dependent against DB different doses and GEN in the starter stage, while ggTas2R1

expressions were highly dose-dependent to DB 20 mg/kg in the starter stage and dose-independent in all the groups in the finisher stage (Figure 4A). However, ggTas2R2 response was highly dose-dependent to GEN in the starter stage and dose-independent in the grower and finisher stages for the mentioned gene (Figure 2B). The ggTas2R7 expression was approximately dose-dependent to all the treatments in the starter stage, but only to GEN in the grower stage (Figure 4C). In the finisher stage, neither of the expressions were dose-dependent and showed dose-independent responsiveness against all the treatments, respectively (Figure 4A–C).

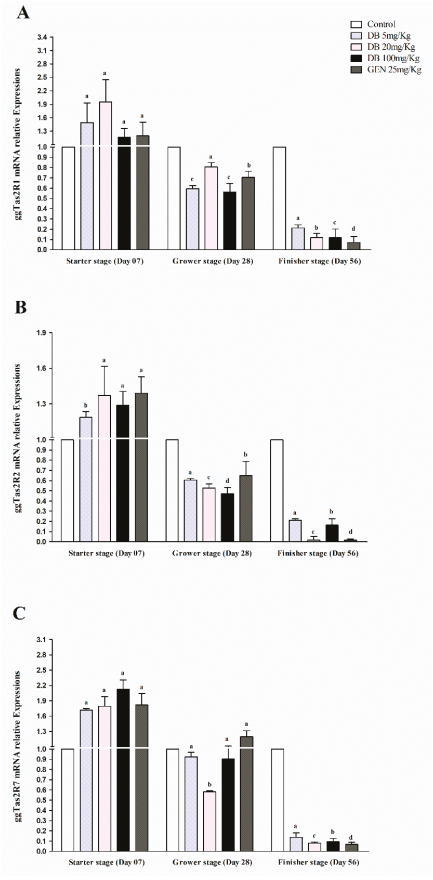

Figure 4. Comparing expressions of bitter taste receptors ((**A**) ggTas2R1, (**B**) ggTas2R2, and (**C**) ggTas2R7) showing their relative mRNA expressions against different doses of DB and GEN in chicken kidneys in different growth stages (Day 7, Day 28, and Day 56). Relative mRNA abundance of ggTas2Rs in different growth stages with the kidney serving as the control (relative expression set to 1; n = 6). Values are presented as the mean of relative expressions ± SEM. The differences between groups within a gene mean that without a common letter differ significantly ($p < 0.05$); differences between the tested tissue (chicken kidney) and the control tissue (kidney) within a gene means that those with marks (a, b, c) differ significantly ($p < 0.05$) from the control group by ANOVA.

3.2.5. mRNA Responsiveness Expressions of ggTas2Rs in Chicken Bursa Fabricius

The expressions of ggTas2Rs in the bursa Fabricius of Fast Yellow Chicken were significantly ($p < 0.05$) lower expressed than the control group in different growth stages (Figure 5A–C). Regarding each gene's individual expressions level, the mRNA expressions in the GEN group were significantly

($p < 0.05$) highly expressed in all the growing stages except for ggTas2R1, which was highly expressed in DB 20 mg/kg (the Medium-Dose group) in the starter stage. The responsiveness expression against dose was independent in bursa Fabricius and had a relatively low response in the starter and grower stages, but all of the expressions subsequently decreased in the finisher stage (Figure 5A–C). The dose-dependent responsiveness in bursa Fabricius at the starter/grower stages was a result of the subjection to GEN (Figure 5A–C).

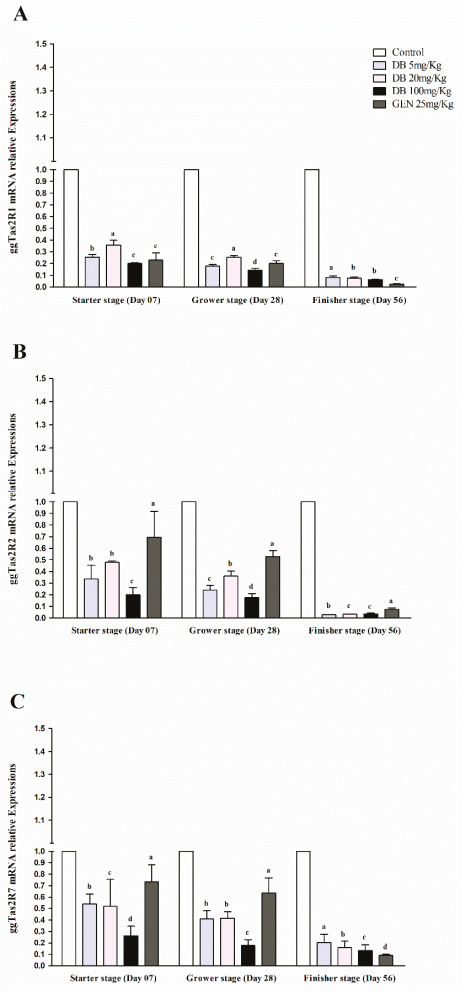

Figure 5. Comparing expressions of bitter taste receptors ((**A**) ggTas2R1, (**B**) ggTas2R2, and (**C**) ggTas2R7), and showing their relative mRNA expressions against different doses of DB and GEN in the bursa Fabricius of chicken at different growth stages (Day 7, Day 28, and Day 56). Relative mRNA abundance of ggTas2Rs in different growth stages with the bursa Fabricius serving as the control (relative expression set to 1; n = 6). Values are presented as mean of relative expressions ± SEM. Differences between groups within a gene mean that those without a common letter differ significantly ($p < 0.05$); differences between the tested tissue (chicken bursa Fabricius) and the control tissue (bursa Fabricius) within a gene mean that those with marks (a, b, c) differ significantly ($p < 0.05$) from the control group by ANOVA.

4. Discussion

Over the past years, tremendous progress has been made investigating the wide-ranging expression of bitter taste receptors (Tas2Rs) inside the vertebrate's various tissues and their bitter taste perception. Interestingly, numerous tissues in addition to gustatory and non-gustatory tissues have been identified to express taste receptor molecules. These findings bear imperative implications for the roles that taste receptors fulfill in vertebrates, which are currently envisioned much broader than previously thought [44]. The sense of taste facilitates the recognition of beneficial or potentially poisonous and harmful food ingredients prior to ingestion [55]. Bitter taste perception in vertebrates relies on the Tas2R genes, ranging from only three in chicken to over 50 in frogs. Possessing a low repertoire of Tas2Rs makes the chicken an appropriate candidate for a model animal in the study of different aspects regarding bitter taste. Furthermore, the agricultural reputation for finding bitter tastants in chicken feedstuff is countless, since their nutrition may be enhanced due to lack of aversiveness [17,43].

To our knowledge, this is the first research to evaluate the ggTas2Rs expression responses against different doses of denatonium benzoate and genistein in the heart, spleen, lung, kidney, and bursa Fabricius of chickens. Several studies reported that in vertebrates, the sensors for bitter compounds are taste receptors (T2Rs or Tas2Rs); basically, they are distributed in the taste receptor cells of taste buds of an oral cavity belonging to the G-protein-coupled receptors super family (GPCRs). In chickens, taste sensing research has mostly focused on taste bud morphological distribution, development, and various tastants' thresholds [17,35,43,45,46]. However, in mammals, it is well established that the expressions of Tas2Rs and their downstream signaling molecules and taste-related genes have been found in various extraoral systems such as the respiratory, digestive, and genitourinary systems, as well in brain and immune cells. These receptors are functional in different body locations with varied biological regulatory mechanisms [36]. The extra-gustatory Tas2Rs receptors have been concerned in diverse functions, representing cellular responses to poisons and toxins [56]. This suggests that bitter composition sensing has a physiological role beyond food evaluation and consumption. Furthermore, in chickens, the gustatory and extra-gustatory mechanisms of involving taste signaling have been recently shown [57]. In this study, the expression levels of different DB doses with GEN were varied, and a high dose of DB was comparatively higher expressed among different doses. Therefore, the study demonstrated the responsiveness expressions of ggTas2Rs (ggTas2R1, ggTas2R2, and ggTas2R7) against DB and GEN in five (5) essential organs of the local Chinese Fast Yellow Chicken.

Several studies reported that the expressions of taste-related genes demonstrated their involvement in gustatory and extra-gustatory tissues; furthermore, the expression was also evaluated as a taste transduction gene in chickens [58–60]. In the current experiment, these bitter taste-related genes, which were determined by qRT-PCR, were found at different expression levels in all the organs in the starter, grower, and finisher stages with varied responsiveness expressions against different doses of DB and GEN over 56 consecutive days of growing. Furthermore, the expressions were sufficiently higher at the starter and grower stages of growing, and then consequently decreased in the finisher stage. However, the organ weight gained adequately improved for all the treatments as predicted; beyond seven days, the chicken organ weights increased collaboratively with feed consumption, and bitterness sensitivity subsequently decreased. Therefore, the bitterness sensitivity in chickens is dependent on age. It was reported earlier that in chickens, bitterness susceptibility is dependent on the age of the chicken, as bitter taste receptors were highly expressed in zero to one-week-old chicks and dependently decreased in aged chickens, and these behavioral responses were conserved since hatching to the maturing period [19,61]. However, insufficient research has been done to investigate growth-related taste perceptions and their subsequent effect on the animal's growth [61,62]. Bitter molecules detected by the ggTas2R family of G-protein-coupled receptors (GPCRs) were involved in chickens perceiving potentially toxic compounds [63]. As described earlier, chickens in the initial period of growing are more susceptible against salt, sour, and bitterness than those entering the maturing stage [64,65]. Therefore, we also found that chicks in the starter/grower stages were more sensitive than those in the finisher stage, and the responsiveness expressions of bitter receptors were correlatively high in the

starter/grower stages than in the finisher stage of growing. In addition to this, it has been reported that the human bitter taste receptor hTAS2R39 seems to be a bitter receptor agonist for many dietary compounds, such as isoflavones from soy bean [66] and many other flavonoids from several plant sources and synthetic denatonium benzoate [67,68]. The birds fed with 40 to 80 mg/kg of genistein revealed the greater relative weight gains of thymus and bursa Fabricius; however, the spleen weight was not affected. Genistein supplementation not only improved growth performance, it also could beneficially affect immunological responses in broiler chicks [69]. Additionally, studies have shown that genistein improves kidney function and weight gain [70]. Therefore, our study also indicated that genistein improved the organ growth performance and may have a potential influence on the regulation of bursal immunity and kidney function.

In recent years, several reports on the extra-gustatory expression of taste receptors obviously suggested that their role is not only limited to taste perception [17,36,45,71]. Meanwhile, the expression of taste receptor genes and functions have been identified in the gastrointestinal and respiratory tracts of mammals, in the male reproductive system, as well as in the brain and heart [19,72–74]. Seemingly, the responsiveness expressions of the ggTas2Rs bitter receptors in the chicken organs were also found with distinguished expression levels in different stages of growth; these contributions have received the most attention from researchers in recent years. Bitter taste receptor expression is not restricted to the upper respiratory tract; it extends into the lower respiratory tract [75]. It should be acknowledged that Tas1r gene expression has been detected in the respiratory system of rodents [76]. qRT-PCR analyses showed that rat neonatal whole heart cDNA, the seven bitter taste receptor genes, as well as two genes encoding the umami receptor subunits, Tas1r1 and Tas1r3, were found to be expressed. Moreover, samples of ventricular tissue of failing hearts were tested and revealed the expression of more than half of all human Tas2Rs genes [13,32]. Remarkably, the mRNA responsiveness expressions of the chicken bitter taste receptor Tas2Rs against DB different doses and GEN, which are known to be extensively represented in the heart, spleen, and lungs of chickens. In comparison, the different DB doses with GEN, the responsiveness expressions against DB 100 mg/kg (High Dose) and GEN were highly dose-dependent in the heart, spleen, lung, kidney, and bursa Fabricius in the starter and grower stages. However, dose-independent lower responsiveness expressions were found in the finisher stage with some exceptions in bursa Fabricius. In summary, the results of the present study indicate the significantly higher expression of bitter taste responsive genes in the starter and grower stages among all the organs except for bursa Fabricius. The Tas2Rs receptors were highly expressed in the heart, spleen, and lung, but lower in the bursa Fabricius in the experimental period among the organs. These findings prove and suggest that bitterness sensitivity decreases as chickens age. However, baby chicks were found to be more sensitive to bitterness than adults. The mentioned findings may be useful in the production of new feedstuffs for chicken depending on their growth stages. Hereafter, further research studies are required to investigate bitter receptors' varied expressions in body organs, the physiological and functional effects of bitter taste receptors in the non-gustatory organs of the chicken, and the molecular mechanism pathways involved in bitter responsiveness and their role involved in the regulation of bronchodilation, heart functions, kidney functions, and the immunity of bursa Fabricius in chickens.

5. Conclusions

Our study demonstrated that the responsiveness expressions of ggTas2Rs (ggTas2R1, ggTas2R2, and ggTas2R7) against denatonium benzoate at different doses were higher in the lungs, spleen, and heart, but lower in bursa Fabricius among the organs. The responsiveness expressions were highly dose-dependent in the starter and grower stages of the heart, spleen, lungs, and kidneys, but dose-independent in the bursa Fabricius in different growing stages of local Chinese Fast Yellow Chicken; bitterness sensitivity decreased subsequently. However, organ weight gains were impaired in the group that received a high dose of denatonium benzoate, and the researchers observed that chickens have a lower tolerance for high-dose denatonium benzoate feed. These findings are valuable

for clinicians and pharmacologists because of ggTas2Rs-wide extraoral expressions, as taste biology is directly correlated to diseases, and may affect kidney and heart functions, the regulatory mechanisms of lungs, and the immunity pathways of bursa Fabricius. It may also help with nutritionist and benefit feed industries to improve the production of new feedstuffs for chicken according to their growing stages.

Author Contributions: Conceived and design the Experiment: the work was conceived by H.E. Performed the experiment: E.H., J.J., Z.S. & Z.L. Analyzed the data: data was analyzed by E.H. and R.M.M. Contributed reagents/materials/analysis tools: J.J., D.Y., Z.L., Q.W. and Z.S. Manuscript writing: Manuscript writing was performed by E.H. All authors reviewed and approved the manuscript.

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animals

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Review

Use of Licorice (*Glycyrrhiza glabra*) Herb as a Feed Additive in Poultry: Current Knowledge and Prospects

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Simple Summary: The present review updates the current knowledge about the beneficial effect of licorice supplementation in poultry diets, particularly its positive effect on the treatment of high-prevalence diseases of the immune system, liver, and lungs.

Abstract: Supplementation of livestock and poultry diets with herbal plants containing bioactive components have shown promising reports as natural feed supplements. These additives are able to promote growth performance and improve feed efficiency, nutrient digestion, antioxidant status, immunological indices, and poultry health. Several studies have used complex herbal formulas with the partial inclusion of licorice. However, the individual use of licorice has been rarely reported. The major problem of the poultry industry is the epidemiological diseases, mainly confined to the respiratory, digestive, and immune systems. Licorice has certain bioactive components such as flavonoids and glycyrrhizin. The roots of this herb contain 1 to 9% glycyrrhizin, which has many pharmacological properties such as antioxidant, antiviral, anti-infective and anti-inflammatory properties. Licorice extracts (LE) have a positive effect on the treatment of high-prevalence diseases such as the immune system, liver, and lung diseases. Studies showed that adding LE to drinking water (0.1, 0.2, or 0.3 g/L) reduced serum total cholesterol ($p < 0.05$) of broiler chickens. Moreover, LE supplementation in poultry diets plays a significant role in their productive performance by enhancing organ development and stimulating digestion and appetite. Along with its growth-promoting effects, licorice has detoxifying, antioxidant, antimicrobial, anti-inflammatory, and other health benefits in poultry. This review describes the beneficial applications and recent aspects of the *Glycyrrhiza glabra* (licorice) herb, including its chemical composition and role in safeguarding poultry health.

Keywords: licorice; *Glycyrrhiza glabra*; beneficial effects; pharmaceutical; poultry; health

1. Introduction

Medicinal plants have gained great popularity for their several beneficial applications in animals, poultry, and humans [1,2]. Nowadays, the addition of feed additives and nutritional supplements, including prebiotics, plant extracts, and probiotics, in the diets of birds are gaining wide attention owing to their multiple beneficial applications while enhancing growth performances and production as well as safeguarding the health of poultry [1,3–5]. This review focuses on the use of herb licorice (*Glycyrrhiza glabra*) as a feed additive in poultry, a popular traditional medicinal plant that belongs to the legume family Fabaceae [6]. It is broadly used in the medicine sector, as a flavouring and food preservative agent and also for commercial purposes [7]. It is derived from the sweet root of various species of *Glycyrrhiza*; however, the cultivation and harvesting practices modify the composition of various biologically important components of the *Glycyrrhiza* plant [8]. Phytochemical analysis showed that the major fraction of licorice extract (LE) consisted of triterpene saponins (e.g., glycyrrhizin, glycyrrhetinic acid, and licorice acid), flavonoids (e.g., liquiritin, isoflavonoids, and formononetin), sugars, starch, amino acids, ascorbic acid, tannins, choline, coumarins, phytosterols, and some other bitter principles [7,9]. Importantly, numerous pharmacological effects have been described for LE and its isolated active principles in humans and animals [7]. Licorice represents a replacement candidate reported to be useful for its multiple beneficial health effects including immunomodulatory, antimicrobial, antioxidative, anti-inflammatory, antidiabetic, hepatoprotextive, antiviral, anti-infective, and radical-scavenging activities [7,8,10]. This review describes the beneficial applications and recent aspects of the licorice herb, including its chemical composition, health benefits, and useful applications for nutritionists, physiologists, scientists, pharmacists, veterinarians, pharmaceutical industries, and poultry breeders. Therefore, we can safely assess and get a new vision for further research on licorice benefits in poultry nutrition and its effects on the growth and productive performance and immune and antioxidant status of poultry.

2. Chemical Composition and Structure

Licorice is also known as Radix Glycyrrhizae or Liquiritiae Radix. It is the root of *Glycyrrhiza uralensis* Fisch. ex DC., *G. glabra* L. or *G. inflata* Bat., Leguminosae [10,11]. The roots of *G. glabra* (Figure 1) are widely used in preparing several pharmaceutical preparations. Phytochemical analysis of licorice root extract exhibited that it contained flavonoids (isoflavonoids, formononetin, and liquiritin), saponin triterpenes (liquirtic acid and glycyrrhizin), and other components such as sugars, coumarins, amino acids, starch, tannins, phytosterols, choline, and vitamins (e.g., ascorbic acid) [7,9,12]. Previous reports have shown that more than 20 triterpenoids and 300 flavonoids have been procured from licorice [13]. Glycyrrhizin constitutes up to 25% of the licorice root extract [14]. Glycyrrhizin consists of glucuronic acid (two molecules) and glycyrrhetinic acid (one molecule) [15]. Badr et al. [16] analyzed the raw form of licorice chemically and summarized its contents as follows: carbohydrate (47.11%), fiber (24.48%), protein (9.15%), silica (3.56%) and low fat content (0.53%). Moreover, the ash and moisture content values of the licorice root were found to be 7.70 and 6.80%, respectively. Additionally, the same authors reported that the calcium and phosphorus content values of the raw LE were 1720 and 78 mg/100 g, respectively, and the major components of amino acids that were found in LE were proline (1.02%), aspartic (0.88%), alanine (0.51%) and glutamic acid (0.50%).

Figure 1. Pictorial representation of the *Glycyrrhiza glabra* herb, its root and extracts.

The licorice root color is yellow because of its flavonoid components such as hispaglabridins and glabridin [17]. Additionally, the dried aqueous extracts of licorice contain approximately 4–25% glycyrrhizinic acid [18]. The main active ingredients of licorice are liquiritin, isoliquiritigenin, liquiritigenin, and glycyrrhetinic acid, glycyrrhiza polysaccharide, and this herb is rich in flavonoids and syringic, abscisic, trans-ferrulic, 2,5-dihydroxy benzoic, abscisic, and salicyclic acids [7,9,19]. Pharmacological activities are contributed to by glycyrrhizin, 18β-glycyrrhetinic acid, glabrin A and B, and isoflavones of *Glycyrrhiza glabra* Linn [7].

3. Beneficial Role of Licorice

In ancient times, *G. glabra* was used as a medicine and flavouring herb. It is a soothing herb that enhances various body functions, protects the liver and is used in various conditions, such as mouth ulcers and arthritis, and as a potent anti-inflammatory, immunomodulatory, hepatoprotective, detoxifying, anti-cancer, anti-aging, antioxidant, antimicrobial, with growth promoting effects [6,8,9]. The licorice herb has several biological activities and health promoting effects, as are discussed in the following sections.

3.1. Antioxidant and Anti-Inflammatory Activities

Previous phytochemical analyses have revealed that the bioactive components of the licorice root include flavonoids (isoflavonoids and liquiritin), glycyrrhizic acid, liquiritigenin, triterpenes (glycyrrhizin), and saponins, which have anti-inflammatory and antioxidant properties [20–23]. Various modes of action with regards to antioxidant and anti-inflammatory properties of licorice can be narrated as: licorice extracts inhibits the lipid peroxidation of mitochondria, decreases the oxidative rate and reactive substance formation of thiobarbituric acid; protects from scavenging free radicals; stimulates antioxidant enzyme activities; inhibits the activity of phospholipase A2 that acts as a critical enzyme in various inflammatory processes; Licochalcone inhibits lipopolysaccharide- induced inflammatory responses; Lico A derived from the licorice root inhibits the lipopolysaccharide-induced inflammatory responses in a dose-dependent manner by suppressing the activation of NF-κB and p38/ERK MAPK signaling; licochalcone A prevents cellular oxidation; licorice flavonoids renders a pro-inflammatory action; flavonoids might target the NF-κB signaling pathway to prevent the secretion of inflammatory cytokines; glycyrrhizic acid, liquiritigenin, and liquiritin can reduce the expression levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the liver, and block the generation of several inflammatory mediators created by activated macrophages; glycyrrhizic acid directly inhibits prostaglandin E2 formation and cyclooxygenase activity and indirectly inhibits platelet aggregation and inflammatory factors [8,14,23–25]. Glycyrrhetinic acid might lead to the delayed secretion of cortisol with subsequent high levels of oxidation that resulted in increased heart weight in hens [26]. The proven and potent anti-inflammatory and antioxidant properties of the licorice herb need to be studied in poultry for the safeguarding of the heath of birds in poultry production.

3.2. Immunomodulatory and Antiviral Effects

Herbs have proven potent immunomodulatory and antiviral activities [2,25,27]. The extracts of licorice have a positive effect on the immune system of poultry. It can be used to optimize their immune response and improve the productive performance. The dietary supplementation of 0.1% LE improved the humoral immunity in broilers by inducing antibody titres against non-specific and specific antigens. An experiment was conducted to assess the effect of the supplementation of licorice root extracts on the immune profile of 54 commercial broiler chicks. The serum biochemical parameters, such as serum total protein, albumin, globulin, and albumin/globulin ratio, were estimated from three groups of chicks. Chicks were categorized into three groups; one in which the birds were provided with 1% *G. glabra* crude extract powder, the second one in which 0.1% *G. glabra* extract powder was given, and the third one in which no *G. glabra* extract was given. Humoral immunity was assessed by measuring the hemagglutination inhibition (HI) titre against the Ranikhet disease virus (LaSota) and the hemagglutination antibody (HA) titre against sheep red blood cells (SRBC) antigens, while the cell-mediated immune response was measured by estimating the total and differential white blood cell (WBCs) counts. The results of the study revealed that the chicks supplemented with 0.1% licorice extract powder showed considerable improvement in their immune responses [28]. Also, natural feed supplements are used as immunity enhancers because it increases WBC counts and ultimately boosts interferon levels [29]. Furthermore, Dorhoi et al. [30] stated that the addition of LE (50 μg/mL) to the diet of laying hens had some beneficial effects on their cellular immunity. The glycyrrhiza polysaccharide has a sturdy immune action and is widely involved in some features of immune regulation [31]. Additionally, LE increased the phagocytic capacity of mononuclear cells and granulocytes of chicken [30].

Notably, the addition of licorice in broiler diets improved the weight of immune organs, such as the spleen or bursa, thereby promoting immune efficacy and the situation of livability and health [32]. Glycyrrhetinic acid has several favourable pharmacological properties, such as immunomodulation and production of interleukins [1,2,12] with subsequent production of antibodies, gamma interferon, and T-cells, which indicates its antiviral activity [33]. However, Hosseini et al. [34] reported that the supplementation of the broiler diet with LE (2.5 and 5 g/kg diet) had no effect on immune organ weights.

The active components of licorice and its extracts have anti-inflammatory, immunomodulatory and antiviral functions, and thus can augment the immunity of poultry by modulating both humoral and cell-mediated immune responses, prevent viral diseases, and render supplementary treatment for viral diseases [35,36]. Omer et al. [35] reported that LE (60 mg/100 mL *Glycyrrhiza* extract) when used as a phytogenic feed additive exhibited antiviral activity against the Newcastle disease virus (NDV). Moreover, the broilers treated with glycyrrhizic acid (GRA) at the concentration of 60 μg /mL drinking water showed higher antibody titers against the ND virus as well as an enhanced cellular immune response, as indicated by an increase in blood lymphocyte and thrombocyte counts [36]. In an in vivo antiviral study, a dose of 300 μg/mL of *G. glabra* extract showed potent antiviral action against NDV. Survival rates were higher in embryonated egg groups inoculated with NDV and treated with extract, and no virus was recovered in allantoic fluids in such groups which indicated the effective control of the virus by the herbal extract [5]. Dziewulska et al. [37] stated that dietary LE (10% extract) supplementation inhibited paramyxovirus type 1 (PPMV-1) replication in pigeons, and the copy number of viral RNA in some organs, such as the kidney and liver, of the pigeons fed LE was lower compared with the control pigeons, suggesting that LE has antiviral effects. In an aqueous solution of LE when administered at a dose rate of 300 or 500 mg/kg body weight to pigeons inoculated with ppmV-1 for 7 days, the expression of the IFN-γ gene was found to be increased in all ppmV-1 inoculated and herbal extract treated pigeons. Expression of the CD3 gene was lowest at 7 dpi in treated birds. CD4 gene expression was higher in uninoculated and treated pigeons but was lower in extract treated pigeons inoculated with ppmV-1. The CD8 gene also showed a non-significant difference in inoculated and extract treated pigeons, and the percentage of IgM^+ B cells was also not affected [38]. The immunomodulatory and antiviral effects of licorice observed in pigeons also reveals

its potent application to be explored and applied in poultry and other avian species to counter viral diseases. However, Moradi et al. [39] reported that the antibody titres against Newcastle disease (ND) and avian influenza (AI) viruses, as well as liver and lymphoid organ (e.g., bursa of Fabricius, thymus and spleen) weights, were not affected by LE supplementation in broiler drinking water. Glycyrrhizin has been reported to act as an immune stimulant for ducklings, inhibits the cytopathic effect of duck hepatitis virus (DHV) in VERO cells, potentiates the production of higher antibody titer in a DHV vaccinated group, and demonstrates a pronounced lymphocytic proliferation response, indicating its antiviral effects [40]. Glycyrrhizin has also been reported to inhibit influenza A virus uptake into the cell, mediated by its interaction with the cell membrane, which, in turn, results in reduced endocytotic activity and decreased virus uptake [41]. Thus, several studies have confirmed the positive impact of licorice on the immune potential and anti-viral effects in poultry; however, further studies are recommended to optimize the inclusion levels of LE in poultry diets and to determine their possible physiological and protective effects, as well as economic value.

Regarding the protective role of licorice against aflatoxicosis in broilers, Al–Daraji et al. [42] reported that the addition of licorice to the aflatoxin (AF)-contaminated diet (at the concentrations of 150, 300, or 450 mg licorice/kg of diet) significantly recovered the adverse effects of AF on most carcass traits.

Saponins from *G. glabra* in combination with antigens from *Eimeria tennela* have shown the ability to serve as immune-stimulating complexes (ISCOMs) and provides immunity against avian coccidiosis, caused by *E. tennela* [43]. They protected birds from the experimental challenge of *E. tennela* and additionally, the antibody titer (IgG and IgM) against a homologous challenge was found to be increased. Saponins have also been identified to act as effective delivery units for antigens for vaccine development, have shown no toxicity and provided stronger immunity [44]. Being natural constituents of plants, saponins of *G. glabra* are unlikely to have any side effects and are comparable to *Aesculus hippocastanum*, *Gipsophila paniculata* or Quil-A saponins [43,44]. More recently, saponins derived from *G. glabra* (Glabilox) have shown better adjuvant potential and the ability to be used as immunostimulatory complexes along with antigens for vaccine purposes. *G. glabra* derived saponins were not found to be toxic or hemolytic as compared to Quil-A saponins, and could produce stable immunostimulatory complexes, hence were preferable as safe and effective vaccine adjuvants [33]. Glabilox induced strong humoral and cellular immune response against H7N1 influenza virus antigens on subcutaneous inoculation and provided 100% protection against homologous infection in chickens [33]

3.3. Effect of Licorice on Some Blood Chemistry

Broiler chickens given drinking water supplemented with LE (0.1, 0.2, or 0.3 g/L) showed reduced serum glucose, LDL cholesterol, and total cholesterol levels ($p < 0.05$) as well as reduced gall bladder weight [45]. The inclusion of *G. glabra* extract (0.5%) in broiler diets induced an increase in serum globulin concentration, which, in turn, led to an improvement in the humoral immune status [46]. However, the broilers fed 0.5 and 1 g licorice/kg during their growing period showed an increase in the number of WBCs ($p < 0.05$) compared to the control. Furthermore, dietary licorice supplementation (0.5, 1, and 2 g/kg) did not have significant effects on the lymphocyte (L), heterophil (H), and monocyte percentages, heterophil to lymphocyte (H/L) ratio, and proliferation of red blood cells [47]. Moreover, the heterophil and lymphocyte percentages and H/L ratio were not affected by LE supplementation (0.1, 0.2, and 0.3 mg/L) in drinking water [39]. The licorice root enclosed phytoestrogens that boosted the rate of erythrocyte sedimentation and decreased the number of erythrocytes [48]. Additionally, the injected LE stimulated cell cycle and activity in lymphocytes [49]. Furthermore, Sharifi et al. [50] clarified that the licorice root supplementation in broiler diets (2 mg/kg diet) reduced some serum components, such as triglycerides, cholesterol, and LDL, and increased the high-density lipoprotein (HDL) levels. In another study, Sedghi et al. [47] concluded that the concentrations of cholesterol and LDL significantly declined in the birds fed diets containing licorice (0.5, 1, and 2 g/kg) compared to the control. This might be attributed to the inhibition of lipid peroxidation and lipoxygenase and cyclooxygenase

enzyme activities as well as reduction of LDL oxidation by licorice. The cholesterol-lowering effects of LE are attributed to the high secretion of cholesterol, bile acids, neutral sterols, and improvement in the content of hepatic bile acid. Besides, the active components of licorice (saponin) are able to reduce the levels of LDL-associated carotenoids, inhibit the formation of lipid peroxides, and enhancement of the rate of conversion of cholesterol to bile acids with subsequent hepatic clearance. However, the feeding of licorice (0.5, 1, and 2 g/kg) in the study of Sedghi et al. [47] did not have a significant influence on the concentrations of triglycerides, HDLs, VLDLs, and glucose in the blood serum of broilers. Al-Daraji [51] concluded that the high levels of LE (150 to 450 mg/L in water) augmented glucose concentrations in the serum of broiler chickens under heat stress.

The dietary supplementation of LE increased the HDL concentration and HDL/LDL ratio in serum because of its high concentrations of flavonoids and ascorbic acid [45]. Moreover, the inclusion of 0.4% LE in the drinking water of broilers increased the plasma HDL levels, but reduced the level of alanine aminotransferase (ALT) ($p < 0.05$) [52]. However, Shahryar et al. [53] concluded that the serum blood parameters of the laying hens supplemented with different concentrations of licorice powder (0.5, 1.0, 1.5, and 2.0%) did not significantly ($p > 0.05$) vary compared to the control group. Thus, the presence of saponins and phytosteroids in LE could be essential for removing cholesterol and increasing the content of hepatic bile acid in animals fed LE diets. *G. glabra* produced lower abdominal fat percentage ($p < 0.05$) in broilers given 0.3 g/L of LE in drinking water [6]. Moreover, the supplementation of LE at levels 0.1, 0.2, and 0.3% in drinking water decreased the concentrations of serum LDL, total cholesterol, and glucose ($p < 0.05$) [45].

3.4. Effect of Licorice on Some Growth Parameters and Performance

Currently, it is well established that the growth and laying performances of poultry are usually improved via supplementation of feed additives or growth promoters, which have a positive influence on their general health state and growth performance [1,3]. The inclusion of 0.4% LE in the drinking water of broilers increased ($p < 0.05$) the feed intake at 21 and 42 days, but did not affect the body weight at different ages [51]. However, Jagadeeswaran and Selvasubramanian [28] found that the inclusion of 1% LE to the basal diet of the broiler chickens improved their body weight and FCR at 42 days of age in comparison to the control group. In Japanese quails [54] it was reported that the inclusion of 200 ppm of licorice root extract containing 1% probiotic supplement to the quail diet improved the amount of daily feed intake and body weight gain. Furthermore, LE had positive effects on the productive performance of heat-stressed broiler chickens [55,56]. *G. glabra* diet supplementation in poultry positively affected their growth performance by enhancing the development of their organs. Furthermore, the digestion and appetite improved in broilers fed diets supplemented with 2.5 g/kg *G. glabra*. Moreover, the inclusion of up to 0.5% *G. glabra* in poultry diets during the pullet growing period enhanced the performance of laying hens [6].

Concerning the use of glycyrrhizic acid (GRA), the broilers supplemented with GRA (60 μg/mL in water) had higher body weight gain (BG), final body weight, better FCR, and the lowest mortality rate compared to the non-treated controls [36]. The feed intake of the laying hens fed 0.5, 1.0, 1.5, and 2.0% of licorice powder added to the basal diet was not affected [52]. Simultaneously, Hosseini et al. [34] used 5 g licorice/kg broiler diet and found no significant effect ($p > 0.05$) on body weight, feed intake, FCR, livability, and production index. Additionally, Moradi et al. [39] concluded that the inclusion of 0.1, 0.2, and 0.3 mg LE/L drinking water for broiler chicks had no significant effect on their body weight, feed intake, and FCR compared to the control group. Moreover, Sedghi et al. [47] used 0.5, 1, and 2 g LE/kg broiler diet and reported no effect on broiler weight, feed intake, and FCR compared to the non-supplemented group. However, another study reported different results when the percentage of licorice was modified and given in combination. This study was performed to determine an improvement in the productive traits of 180 one-day-old Ross 308 broiler chicks fed diets supplemented with different concentrations of licorice and garlic mixture powders. It was concluded that the diet supplemented with a mixture of garlic and licorice (at 0.25, 0.50, and 1%

concentrations) improved the productive performance of broiler birds [57]. Another study carried out on 480 one-day-old male broiler chicks (Ross 308) showed the beneficial effects of 1% LE on the growth performance, immune system, and blood parameters of broilers, when supplemented along with the extracts of other plants, such as German chamomile, yarrow, eucalyptus, Iranian caraway, and garlic, and one antibiotic virginiamycin [58]. One more study performed on 400 unsexed (Cobb 500) broiler chicks advocated that LE reduced the abdominal fat of chicks without illustrating any adverse effects on their immune status and performance of broiler when receiving LE in drinking water for 42 days [59].

Experiments were performed to determine the effect of diet supplementation with thyme, peppermint, green tea, and licorice in 245 one-day-old broiler chickens on enhancing their growth performance, serum lipid profile, immune response, and carcass characteristics. An aqueous blend of 400 g thyme extract, 300 g peppermint extract, 200 g green tea extract, and 100 g LE, which finally provided 1.4% essential oils (0.4% thyme oil/0.9% peppermint oil), 25% polyphenols, 15% catechins, and 0.5% glycyrrhizinic acid as active principles, was used. A total of seven experimental groups were set up: five groups supplied with 100, 200, 500, 1000, and 2000 ppm of the aqueous blend and one negative (no supplementation) and one positive (antibiotic: oxytetracycline) control group. The results depicted an overall increase in the performance of chicks at 200 and 1000 ppm levels as compared to the negative control and a significant increase in humoral immunity as compared to the positive control. These findings recommended the inclusion of the plant extract blend (at 200 ppm concentration) in poultry diet to support the broiler performance and immune status, in addition to its use as a growth promoter and an alternative to antibiotics [60].

A study performed on hundred 40-week-old laying hens showed that the diets supplemented with LE improved the production of functional eggs and modulated the productive performance of laying hens by lowering the egg cholesterol level, some plasma parameters (reduction in LDL and egg-yolk cholesterol level with an increase in the HDL level and total antioxidant capacity of plasma [61]. Scientific literature has witnessed the global impact of this herb on the performance, carcass traits, and meat quality of poultry, when their diet is supplemented with licorice (*G. glabra*) either in feed and/or in drinking water [6]. In broiler chickens, probiotic and licorice extract (500 ppm) increased body weight gain of broilers exposed to high stocking density [62]. Drinking water supplementation of LE has been suggested to be an alternative to in-feed antibiotic growth promoter in broiler chickens [45]. Body weight gain (BWG) was improved in birds reared at high stocking density by licorice extract (500 ppm) but not the feed conversion ratio (FCR) [62]. This was further improved by the addition of probiotics (200 ppm).

An overview of the beneficial effects and modes of action of *Glycyrrhiza glabra* in poultry health and production is depicted in Figure 2.

4. Conclusions and Future Prospects

The extract of *G. glabra* might play an important role in the preparation of several pharmaceutical compounds for further use in the poultry industry. Licorice contains bioactive components, such as flavonoids and glycyrrhizin, which have pharmacological properties and medicinal applications. The licorice extract has been found to show immunogenic and antioxidant activities, which might improve the growth performance, feed efficiency, carcass traits, and blood biochemical indices of the poultry birds, and act as a potential solution for solving respiratory, digestive, and immune problems in poultry. The use of LE up to 0.4 g/L in the drinking water of poultry increased the feed intake, and improved the immune response and antioxidant parameters as well as lipid profile. The addition of LE at 50 μg/mL in the diet of laying hens has been found to produce some beneficial effects on their cellular immunity. A dose of 300 μg/mL of *G. glabra* extract showed potent antiviral action against NDV. Further studies need to be conducted to evaluate the beneficial effects of using the licorice herb as poultry feed additive, as well as to explore other properties of this medicinal plant that might enhance productivity and health in poultry. Efforts need to be made to enhance the delivery of this important

herb in poultry by exploring the nanodelivery and in ovo delivery techniques, thereby efficiently enhancing production and safeguarding the health of birds in a better way.

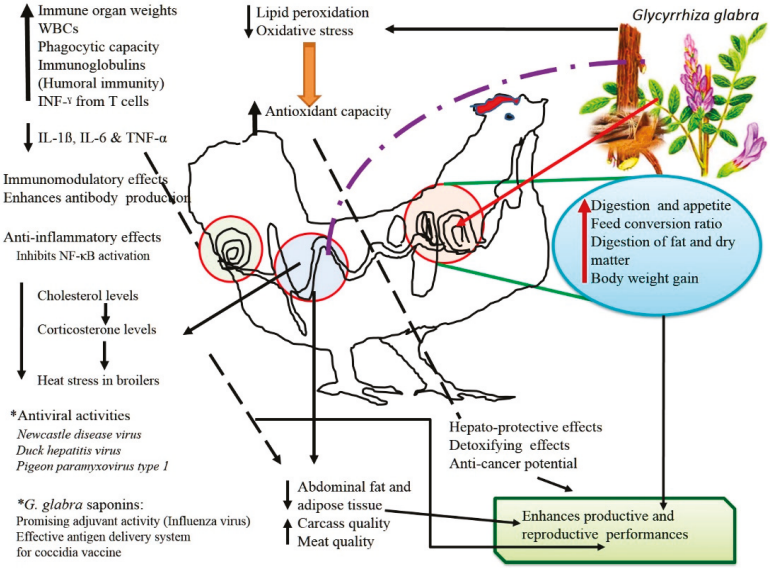

Figure 2. Beneficial effects and modes of action of *Glycyrrhiza glabra* in poultry health and production.

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Abbreviations

LE	licorice extract
LFO	Licorice flavonoid oil
GA	glycyrrhetinic acid
GL	glycyrrhizin
SNMC	Stronger Neo-Minophagen C
CHX	chlorhexidine
TNF-α	tumor necrosis factor alpha
IL10	interleukin 10

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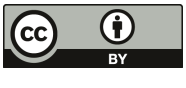

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Review

Omega-3 and Omega-6 Fatty Acids in Poultry Nutrition: Effect on Production Performance and Health

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Simple Summary: In this review, we discuss previous studies, state-of-the-art technology, and the potential implications of utilizing omega-3 and omega-6 fatty acids in poultry diets, as well as the application of these fatty acids in the poultry industry for improving poultry production and health. Essential roles are played by these fatty acids in development and metabolism, growth and productive performance, immune response and anti-oxidative properties, improving meat quality, bone growth and development, and improving fertility rates and semen quality.

Abstract: Omega-3 (ω-3) and omega-6 (ω-6) fatty acids are important components of cell membranes. They are essential for health and normal physiological functioning of humans. Not all fatty acids can be produced endogenously owing to the absence of certain desaturases; however, they are required in a ratio that is not naturally achieved by the standard diet of industrialized nations. Poultry products have become the primary source of long-chain polyunsaturated fatty acids (LC-PUFA), with one of the most effective solutions being to increase the accretion of PUFAs in chicken products via the adjustment of fatty acids in poultry diets. Several studies have reported the favorable effects of ω-3 PUFA on bone strength, bone mineral content and density, and semen quality. However, other studies concluded negative effects of LC-PUFA on meat quality and palatability, and acceptability by consumers. The present review discussed the practical application of ω-3 and ω-6 fatty acids in poultry diets, and studied the critical effects of these fatty acids on productive performance, blood biochemistry, immunity, carcass traits, bone traits, egg and meat quality, and semen quality in poultry. Future studies are required to determine how poultry products can be produced with higher contents of PUFAs and favorable fatty acid composition, at low cost and without negative effects on palatability and quality.

Keywords: omega-3; omega-6; fatty acid; nutrition; performance; antioxidant; egg and meat quality; fertility; immunity; health

1. Introduction

Fatty acids, especially essential fatty acids, are gaining importance in poultry feeding systems not only for improving the health and productivity of birds, but also because of our health-conscious society that prefers properly balanced diets to minimize adverse health issues [1–3]. Among various fatty acids, omega-6 (ω-6) and omega-3 (ω-3) fatty acids are proving indispensable in a properly maintained ratio for numerous biological [4,5], physiological [2], developmental [6], reproductive [7], and beneficial health functions [3,8,9]. Adequate supplementation of poultry diets with novel and beneficial feed additives or supplements is gaining importance as it significantly improves overall poultry production and performance as well as safeguards the health of birds [10–13]. In poultry production, the advantages of using oils in diets involves a reduction of feed dust and improvement in hydrolysis and absorption of the lipoproteins that supply fatty acids [14]. In addition, oils are the main source of energy for the birds and have the highest caloric value among all dietary nutrients. They can also enhance the absorption of fat-soluble vitamins, increase diet palatability, and improve the utilization of the consumed energy. Moreover, the rate of food passage through the gastrointestinal tract can be reduced, with subsequent better absorption of all dietary nutrients [15].

In the diets of humans, ω-6 and ω-3 are essential fatty acids. However, considerable modification in dietary patterns has resulted in alterations of the consumption of such fatty acids, with subsequent elevation in the consumption of ω-6fatty acids and a marked decrease in the consumption of ω-3 fatty acids. This modification has led to an imbalance in the ω-6/ω-3 ratio, which at 20:1 now differs considerably from the original ratio (1:1). Therefore, dietary supplements of foodstuffs such as eggs and meat are a clear alternative to increase the daily consumption of ω-3 to meet the recommended doses [16]. Foods that provide ω-6 fatty acids include soybean, palm, sunflower, and rapeseed oils, whereas foods that provide ω-3 fatty acids include certain nuts, and plant and fish oils [17–20]. Omega-9 fatty acids are not essential and are found in olive oil and animal fat [21]. Fish oil (FO) includes two types of ω-3 fatty acids: docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Certain vegetables, nuts, and seed oils include alpha-linolenic acid (ALA), which can be converted to DHA and EPA in the body, with linseed oil containing more than 50% ALA [22].

High doses of omega sources in the diet may have deleterious effects on humans, such as increased bleeding risk and higher levels of low density lipoprotein (LDL) cholesterol. Omega-3 fatty acids may influence heart rates. The consumption of high rates of fatty acids (e.g., ω-6) has been linked with a higher occurrence of health problems, such as type 2 diabetes, obesity, and coronary artery diseases [23]. Maintaining a proper ratio of ω-3 and ω-6 fatty acids not only improves performance, but also prevents these health risks. Devising the correct ratios requires the addition of oils having appropriate ω-3 and ω-6 fatty acid levels [24]. Omega-3 fatty acids EPA and DHA have shown many health benefits; they are helpful in fetal development and cardiovascular function, and prevent Alzheimer's disease [25]. In addition, they play a role in modulating immunity [26,27]. The ratio of n-6:ω-3 fatty acids also plays an important role in the immune response, production performance of broilers and designing meat enriched with ω-3 polyunsaturated fatty acids (PUFAs) [26,27]. The addition of FO to the poultry diet may yield poultry products (such as eggs and meat) that are enriched with ω-3, such as EPA and DHA. Additionally, FO is more effective than other vegetable oils [28]. However, many aspects of essential fatty acids are still unknown and their diverse functions and importance in health and production should be explored. The objective of the present review is to assess the influence of dietary ω-6 and ω-3 PUFAs on the productive performance, antioxidant properties, immunity, carcass traits, bones, egg and meat quality, and semen quality of poultry, as well as their limitations in the poultry industry.

2. Beneficial Applications of ω-3 and ω-6 Oils in Poultry

Adding ω-3 and ω-6 fatty acids to the diet has become more important recently [3,29]. For at least the past three decades, studies on the beneficial activities of long-chain PUFAs (LC-PUFAs) in biological processes have been conducted. Dietary intervention with ω-3 may influence chicken immunity and lead to the production of poultry products with health benefits for the consumer [30]. Therefore, research on broiler chickens has focused on the functional action of different LC-PUFA forms and their dietary levels on the metabolism of lipids in birds. Other than the LC-PUFA source, high levels of fatty acids, particularly of the ω-3 family, lead to accelerated lipid oxidation when broiler chickens are under oxidative stress because of genetic selection [31].

Using PUFAs in poultry diets significantly reduces the cholesterol and total lipid content in the blood and egg yolk. Several studies have been conducted to minimize the harmful effects of triglycerides and total cholesterol in poultry products (edible parts). Ahmad et al. [32] reported that the cholesterol content of eggs was decreased when birds were fed a diet supplemented with ω-3 fatty acids. Moreover, increasing dietary levels of FO and milled flaxseed improved the concentration of linoleic acid (LA), EPA, and DHA in the yolk, and the fatty acid deposition from FO was found to be two times greater than that from milled flaxseed when fed at the same dietary levels [33].

Designer eggs offer balanced ratios of PUFA: SFA (1:1) or ω-6/ω-3 PUFA (1:1). Omega-3 UFAs are important nutritional factors that modulate immune functions and are of great importance for nervous system development and for lowering blood platelet aggregation and the incidence of thrombosis, hypertension, and atherosclerosis, and have anti-tumor, anti-inflammatory, and cardioprotective effects [34]. The content of ω-3 fatty acids in eggs can be increased by supplementing the diets of laying hens with certain dietary supplements, such as flaxseed, fish oil, safflower oil, linseed, fish meal, or algae. Omega-3 fatty acids can be introduced to the human consumer's body through these designer eggs; they play an important role in the maintenance of the normal functioning of the body in that they protect the body from cardiovascular problems such as heart attacks. In addition, they can replace fish products in consumer diets [35]. Ebeid et al. [36] stated that hens fed a diet containing different concentrations of ω-3 PUFA showed a linear decrease and increase ($p < 0.05$) in egg yolk content of ω-6 PUFA and ω-3 PUFA, respectively, compared to the control hen group. The levels of ALA, EPA, DHA, and docosapentaenoic acid (DPA) were higher in the egg yolks of laying hens fed linseed meal and fish oil as dietary supplements than in the un-supplemented hen group [37]. Similarly, ALA was higher in the egg yolk of hens fed diets that contained ω-3 fatty acid dietary supplements than in the control bird group [32]. It has been previously concluded that laying hens can synthesize EPA and DHA from ALA during metabolic processes if ALA is present in adequate quantities [38,39]. However, in mammals, the synthesis rates of DHA from ALA are low compared to the dietary intake and tissue demand, with the estimation of percent conversion of ALA to DHA differing widely, ranging from 0% to 9.2%, supporting the conclusion that DHA synthesis from ingested ALA is not an efficient process in humans [40]. Moreover, metabolization of ALA in vivo is not adequate to improve meat quality in ω-3 LC-PUFA, and direct supplementation of the diet with ω-3 LC-PUFA is a better alternative to modulate an increase in beneficial fatty acids of broiler meat [41]. The efficiency of ALA conversion to ω-3 LC-PUFA derivatives and deposition in peripheral tissues might not be sufficiently high to improve the nutritional value of muscle. Because there is competition among the enzymes involved in the elongation and desaturation of both LA and ALA, high amounts of LA suppress the conversion of ALA to EPA or DHA; therefore, an optimal intake of LA relative to ALA is crucial for normal metabolism [42].

Regarding egg production performances, Buitendach et al. [43] investigated the effects of dietary fatty acid saturation on the production performance of laying hens at end-of-lay (58–74 weeks of age). These authors reported no significant differences in hen-day egg production, egg weight, egg output, feed efficiency, and body weights at end-of-lay. Similar results were reported by Cachaldora et al. [44,45] who concluded that dietary fatty acid saturation had no significant effects on the egg production performance of layer hens. In contrast, Shang et al. [46] stated that body weight gain, rate of egg production, egg

weight, and feed efficiency decreased linearly with an increase in dietary fatty acid unsaturation levels during the 8-week experimental period between 40 and 48 weeks of age. Yin et al. [47] reported a decrease in egg and body weights with an increase in dietary UFAs at 50–58 weeks of age. This decrease in the performance parameters of hens as recorded by Shang et al. [46] and Yin et al. [47] is mostly attributed to the fact that these authors used conjugated linoleic acid (CLA) at higher inclusion levels (up to 7.8%) to enhance the UFA profile of their experimental diets. CLA causes weight loss in humans [47]; therefore, it appears that this specific type of unsaturated fatty acid has a similar negative effect on the body weights of laying hens and consequently on egg production and egg size.

3. Improved Growth and Productive Performance

Growth and production performance of poultry are improved by supplementation of fatty acids or their sources. The supplementation of fats and oils (as an omega source) in limited amounts leads to better utilization of feed and energy, with subsequent improvement in growth and performance [48]. The body mass and percentage body mass gain of quails was improved via dietary supplementation of sunflower and soyabean oil for a 12-week period [49]. The feed conversion ratio (FCR) and growth performance of broilers were improved via dietary supplementation with sunflower and canola oil [14]. Smith et al. [50] stated that the supplementation of animal fat, corn oil, fish oil, and a blend of vegetable and animal oils did not affect the feed intake, but positively influenced the body gain and FCR in heat-stressed broilers compared to heat-stressed un-supplemented broilers. Jalali et al. [51] found that the addition of soybean oil (high in ω-3 PUFA) significantly ($p < 0.05$) improved the FCR and body weight gain of broilers during the total and growth rearing periods. Abdulla et al. [52] found that the supplementation of soybean oil in broiler diets increased the body weight and weight gain at 6 weeks of age compared with the supplementation of LO ($p < 0.05$).

Fébel et al. [53] noticed that the difference in diets supplemented with sunflower oil (SO) or lard was not significant for the growth performance of broilers. Supplementation of fish oil in poultry diets had no influence on feed consumption, live weight, or weight gain [48] compared with the control diet (without fat). Ebeid et al. [54] reported that dietary ω-3 PUFA in Japanese quail had no adverse effects on the growth performance, such as the final body weight, feed intake, or FCR. Raj Manohar and Edwin [55] declared that dietary ω-3 PUFA in quails had a significant ($p < 0.01$) influence on body weight gain and non-significant differences in feed intake and feed efficiency. In addition, Qi et al. [56] demonstrated that decreasing dietary ω-6/ω-3 improved FCR, with the best result obtained from the diet with 5:1 ω-6/ω-3. Puthpongsiriporn and Scheideler [57] reported that four dietary ratios (17:1, 8:1, 4:1, and 2:1) of LA to ALA had no significant effect on the body weight of chickens over 16 weeks. However, Ayerza et al. [58] observed a marked reduction in the body weight and FCR of broilers fed on chia and flaxseed (rich in ALA), which may be attributed to one or more of the anti-nutritional factors present in flaxseed. Crespo and Esteve Garcia [59], Newman et al. [60], and Ferrini et al. [61] reported that the digestibility of fat increased with increasing unsaturation; therefore, the effect of the type of fat on feed efficiency could reflect the degree of unsaturation. This is consistent with the improvement of growth performance noted by Zollitsch et al. [62], Huo et al. [63], and Lopez-Ferrer et al. [48] with increasing content of UFA.

Generally, a dietary supplementation of a PUFA-enriching ingredient has an improvement effect on live weight, weight gain, and FCR of poultry. However, no adverse effects on feed intake have been reported in almost all designed studies to date.

4. Improved Immune Response and Anti-Oxidative Properties

Fatty acid supplementation affects both immune and oxidative status in poultry. It can modulate immune system through both cellular and humoral immune responses. Proliferation, maturation, function and cytokine production of lymphocytes, heterophils and splenocytes are influenced by omega fatty acids, besides antibody production like IgM and IgG (Table 1). Similarly, neutralization of oxidants and an increase of antioxidants level directly or indirectly by fatty acids minimizes the risk

of oxidative stress. The immune response and oxidative mechanisms are interlinked and affect one another, hence modulation of one can impact the other. The supplementation of natural antioxidants has become a pressing research topic [64–66]. Different studies have confirmed the many favorable effects of dietary ω-3 PUFA, including anti-oxidative properties and lipid peroxidation, as well as immune response effects [36,67]. For instance, dietary ω-3 PUFA can modulate the immune response in poultry [68]. Ebeid et al. [36] found that dietary FO supplementation below a level of 35 g/kg in the diet induced antibody titers in hens. The levels of antibodies were higher in laying hens fed oils (FO or LO) rich in ω-3 PUFAs than those in laying hens fed oil rich in ω-6 PUFAs (maize oil) [69]. Ebeid et al. [54] stated that dietary ω-3 PUFA (FO or LO) had a positive influence on humoral immunity ($p \leq 0.05$) at 42 days of age as measured by antibody titers against Newcastle disease virus (NDV) compared to the control diet. Al-Khalifa et al. [70] declared that SO replaced by fish oil at low levels showed no evidence of adverse effects on the immune function of broilers. Jameel et al. [71] showed that chicks fed a diet supplemented with FO had significantly higher ($p \leq 0.05$) antibody titers and percentages of spleen and bursa than that of the control group.

One of the earliest studies on the effects of fatty acid supplementation on immune tissues was by Fritsche et al. [72]. They noted that the supplementation of diets rich in ω-3 fatty acids to chicks decreased levels of arachidonic acid (AA) (C20:4n-6) in the serum and immune tissues by 50–75%. However, the levels of EPA (C20:5n-3) and DHA (C20:6n-3) increased [72], suggesting an influence on the immune system. During the early stages of life, ω-3 and ω-6 fatty acids are more important for immunity in chicks as they play a role in cellular and humoral immunity, and are regulators of inflammation [1,73]. They determine the immunoglobulin G (IgG) content of chicks produced by maternal hens, which are essential for passive immunity [74]. An inflammatory role for these fatty acids in delayed-type hypersensitivity has also been reported [75]. Further, they help in maintaining membrane integrity, thus preventing pathogen entry or infection.

Wang et al. [68] noted that the LA to ALA ratio may influence IgG-receptor activity in yolk sac membranes and thereby influence the maternal-embryo transfer of yolk IgG. Adding fish oil to broiler diets significantly induced antibody titers for the LaSota vaccine at 35 days of age after vaccination against Newcastle disease because of ω-3, which plays a role in the production of immunomodulators (leukotriene and prostaglandin). In addition, fish oil has the capacity to modulate the production of cytokines via signal transduction and lymphocytes in a population of immune cells [76]. Al-Mayah [76] showed that chickens fed a diet supplemented with fish oil at a level of 50 g/kg showed a higher production of antibodies (IgM and IgG) and globulins in the serum and maintained immune function after vaccination compared to the control group.

A moderate intake of ω-3 PUFAs enhances anti-oxidative properties, such as glutathione peroxidase (GSH-Px) activity, in laying hens [36] and decreases lipid peroxidation in abdominal fat and serum [36,67]. Ebeid et al. [54] reported that adding FO and LO at a level of 20 g/kg to Japanese quail diets significantly increased both the total antioxidant capacity and GSH-Px activity, and decreased the thiobarbituric acid reactive substances in the serum compared with the negative control. SO-enriched diets led to a reduction in the deposition of abdominal fat [77]. Additionally, adding SO and LO to the diet of birds led to a greater decrease in abdominal fat deposition than that observed after adding olive oil and tallow [67]. The addition of SO in broiler diets significantly increased ($p < 0.05$) the relative weight of the abdominal fat pad [51], whereas the abdominal fat of broilers was decreased with fish oil [64,78]. Diets high in ω-3 fatty acids increased the incorporation of these fatty acids into tissue lipids, leading to oxidative stress in cells [30]. A diet enriched in ω-3 PUFA improved the gene expression of lipin-1, which regulates triglyceride synthesis, in chicken abdominal fat [79]. Ibrahim et al. [26] stated that FO and LO supplementation had a low but significant effect ($p \leq 0.05$) on the malondialdehyde (MDA) concentration in broilers. Reducing the ratios of ω-6:ω-3 PUFAs were found to be linked to a significant ($p \leq 0.05$) induction of glutathione S transferase (GSH-ST). Moreover, superoxide dismutase, GSH-ST, and cardiac GSH-Px activities were augmented in the ω-3 PUFA-rich treatment, and MDA was reduced [79]. Omega-3 PUFAs have shown beneficial immune responses in infectious bursal disease

challenged broilers [80]. However, despite the noted improvements, these fatty acids must be evaluated and properly monitored for ratios to prevent adverse effects on immune status [70].

5. Improving Egg Quality and Nutritional Value of Eggs

The nutraceutical value and health benefits of eggs can be enhanced by adapting appropriate feeding strategies in poultry as well as by developing designer eggs [10,12,20]. These improve the quality and quantity of eggs [33]. Eggs are not naturally rich in ω-3 PUFA; therefore, ω-3 PUFA supplementation in poultry rations is required to obtain enriched ω-3 PUFA eggs [81,82]. Designer eggs are enriched in ω-3 fatty acids for beneficial health effects in human nutrition [12,83]. Designer eggs offer balanced ratios of PUFA: SFA (1:1) or ω-6/ω-3 PUFA (1:1) and provide more than 600 mg of ω-3 PUFA [34]. The content of ω-3 fatty acids in eggs can be increased by supplementing the diets of laying hens with certain dietary supplements, such as groundnut oil, fish oil, safflower oil, linseed, fish meal, or algae [15–18,58]. Omega-3 fatty acids include EPA, DPA, DHA, and linolenic acid (LNA), whereas AA and LA are examples of ω-6 fatty acids. Omega-3 fatty acids can be introduced to the body through designer eggs [35]. Omega 3-PUFAs serve as good fats for human health, therefore increasing PUFA contents in the egg yolk helps to decrease the bad cholesterol content [84]. The stability of ω-3 PUFAs can be improved by vitamin E and/or organic selenium, which reduces oxidation in raw eggs; thus, these confer protective effects during the marketing, storage, and cooking of ω-3 enriched eggs [85,86].

Meluzzi et al. [87] reported that the key ω-3 and ω-6 PUFAs are LNA and LA, respectively. LNA is metabolized inside the body to EPA, DHA, and DPA, whereas LA is metabolized to AA. LNA was higher in the egg yolk of hens after feeding them diets that contained ω-3 fatty acid dietary supplements than those of the control birds [88].

Ceylan et al. [89] evaluated the effect of dietary supplementation on two levels (15 g/kg and30 g/kg diet) of SO, rapeseed oil, and LO for 12 weeks in laying hens. They concluded that egg production, egg weight, feed intake, FCR, and live weight were not significantly affected by the treatments. However, hens receiving SO produced less intensively colored egg yolks than those receiving other oils in their diet ($p < 0.01$). Moreover, the composition of fatty acid in egg yolks was significantly ($p < 0.01$) affected by the treatment, whereas the cholesterol content was not influenced. There was a significant ($p < 0.05$) interaction between fat source and the level of inclusion in the diet, and LNA content was increased when hens were fed diets with linseed and rapeseed oil (30 g/kg diet). In contrast, da Silva Filardi et al. [90] studied the effects of the dietary inclusion (for 12 weeks) of different fat sources (cottonseed oil, soybean oil, lard, SO, or canola oil) on egg quality, and egg yolk lipid profiles. The different fat sources did not affect eggshell quality; however, the lipid profile of the egg yolk changed based on dietary fat sources. Optimal changes were considered to be lower levels of SFA and LA, and higher levels of ALA and DHA. Such changes were promoted by the addition of different fat sources, particularly canola oil; however, it did not enhance the egg content of PUFAs.

6. Improving Meat Quality

In human diets, there is a marked reduction in ω-3PUFA and an imbalance in the ratio of ω-6/ω-3 PUFA. Currently, the ratio of ω-6 to ω-3 fatty acids is approximately 10 to 20:1 rather than the recommended ratio (1 to 4:1). The decrease in ω-3PUFA consumption is due to the low intake of sea fish, which are the major source of ω-3 PUFA. An accepted solution for this situation could be based on the production of suitable functional foods with adjusted PUFA content, which is generally accepted to confer nutritional effects and beneficial physiological properties. The enrichment of poultry meat with ω-3 PUFA may provide an excellent alternative source for such acids in the human diet because of their relative availability [87]. Schiavone et al. [91] illustrated that the content of lipids, protein, and moisture in breast meat was not significantly affected by the addition of fish oil to the diet of the Muscovy duck. Additionally, Ebeid et al. [54] reported that adding n-3 PUFA to Japanese quail diets had no significant influence on the content of crude protein, ash, and dry matter in the meat, whereas the addition of ether extract significantly influenced these parameters. Additionally,

the physical traits of the meat except for the water-holding capacity were not significantly influenced when ω-3PUFA was added to the diet. Though the fatty acid composition of meat is influenced by ω-3 PUFA supplementation or their sources in diet, meat quality parameters like meat pH, tenderness, grilling loss, toughness, and juiciness are not affected [48,54,87,91]. This can be exploited for designing functional foods with adjusted PUFA and having no differences in palatability.

In broiler diets, replacing soybean oil with LO along with the addition of pomegranate peel extract enriched muscle meat with antioxidants and ω-3 and improved broiler immunity and their serum lipid profile [92]. Also, natural antioxidants, especially those extracted from herbal plants, have greater potential for increasing the stability, palatability, and shelf-life of meat products [93,94]. The meat quality of broilers improved with fish oil supplementation in the diet [78]. Inclusion of fish oil (FO) and different fat sources [linseed oil (LO), rapeseed oil (RO), sunflower oil (SO)] for providing different PUFA (ω-3 and ω-6 PUFA) in diets and their deposition into the eggs' fat revealed that smaller proportions of FO resulted in lower values of saturated and higher values of ω-6 FA contents. Replacing FO with LO showed the lowest turn down of its derivatives by elongation and desaturation and an increase in the total ω-3 FA in the form of linolenic acid [95]. The use of LO as ground or whole flaxseed before slaughter is recommended to broiler breeders and producers as a feeding strategy to optimize ω-3 enrichment, without compromising poultry performance [96]. The fat and cholesterol content in poultry meat may decrease because of dietary supplements, such as ω-3 PUFA, and high PUFA concentrations in the diet (addition of vegetable oils) decrease the storage stability of meat [97].

A proper ratio of ω-6:ω-3 fatty acids is essential for maintaining health, oxidative balance and quality of meat. Recently, Konieczka et al. [8] found that feeding birds with a diet containing a PUFA ω-6:ω-3 ratio exceeding the recommended levels resulted in damage to the intestinal epithelial cells. Further, low PUFA ω-6:ω-3 ratio diets increased MDA in tissues including the meat. This can affect meat quality owing to peroxidative changes [8]. Hence, these authors recommended balanced supplementation to prevent oxidative damage and loss of meat quality. Similarly, Kalakuntla et al. [6] noted that the supplementation of ω-3 PUFA-rich oil sources in the broiler diet during starter and finisher phases can affect fatty acid composition, quality, and organoleptic characters of broiler chicken meat. At 2% and 3% addition levels, mustard oil, fish oil, and LO improved ω-3 PUFA levels and sensory attributes such as the appearance, flavor, juiciness, tenderness, and overall acceptability of meat; however, due to an increase of thiobarbituric acid-reacting substances, the meat quality might be compromised as it might cause oxidative damage to meat.

The content of ω-3 fatty acids in poultry meat, especially as EPA and DHA, can be readily improved by increasing the levels of ω-3 PUFA in poultry diets via the inclusion of oily fish by-products [98]. Qi et al. [56] concluded that substituting ω-3 for ω-6 in the diets of chickens resulted in a significant effect on the subcutaneous and intramuscular fat content and on meat quality (color and tenderness).

Unfortunately, although poultry meat is considered one of the main potential sources of ω-3 LC-PUFA for humans, particularly in developed countries [99,100], there are some disadvantages related to meat oxidative stability. LC-PUFAs are very susceptible to oxidation, producing off-flavors and odors in meat that are often associated with a fishy flavor [101]. This oxidative instability can influence meat quality and, consequently, reduce acceptability to consumers [41,102,103]. Oken et al. [104] concluded that the supplementation of chicken diets with fish-derived products led to unacceptable odors in the product, which has restricted the adoption of this strategy [105]. Vegetable sources such as LO may clearly increase the ω-3 PUFA content in the form of ALA, which is the precursor of the entire ω-3 family [42].

Conclusively, dietary supplementation of ω-3 fatty acids in poultry diet, particularly in the form of EPA and DHA, can improve various parameters of meat quality. However, LC-PUFAs are extremely susceptible to oxidative deterioration, resulting in off-flavors and odors, which adversely affect acceptability to consumers, especially when fish-derived products are used.

7. Effects of Dietary ω-3 and ω-6 Fatty Acids on Bones

The ω-3 and ω-6 fatty acids or their sources like fish oil, linseed oil, soybean oil and palm oil have bearing effects on mineral metabolism and hence promote bone formation, growth and development. Fat supplementation in the diet influences mineral metabolism, especially calcium, zinc, and magnesium [106], because of insoluble soap formation between these minerals and fatty acids during digestion, which makes them unavailable [107]. This can affect mineral retention, and influence bone and eggshell quality in birds. Dietary lipids play a remarkable role in the growth, development, and formation of bones [106–108]. Sun et al. [108] reported that dietary fish oil supplementation led to significantly higher bone mineral density in the proximal tibia and distal femur than supplementation with maize oil. Some studies have reported non-significant correlations between fatty acid supplementation and bone characteristics. Baird et al. [109] reported that feeding laying chickens a diet high in ω-3 PUFA did not have a significant effect on bone morphological characteristics, bone mineral content, or bone mineral density. However, there are many studies that can prove interrelation of fatty acid supplementation and bone growth and development. Ebeid et al. [36] declared that using ω-3 PUFA in Japanese quail diets improved the tibia bone and morphological characteristics and, in quails fed diets supplemented with FO and LO at a level of 20 g/kg diet, there was increased tibia bone wall thickness, tibia diameter, and the percentage of tibia ash and tibia bone breaking strength compared with that in quails fed the control diet. Abdulla et al. [52] clarified that chicks fed a diet supplemented with LO had non-significantly higher ash percentage, tibia weight, and bone-breaking strength than those fed diets supplemented with SO and palm oil. In addition, ω-3 PUFA may improve bone health by inducing calcium absorption in the gut and inducing osteoblast activity and differentiation, decreasing osteoclast activity, and stimulating the deposition of minerals in developing bones [110]. Reproducible and consistent beneficial effects of ω-3 fatty acids have been observed for bone/joint diseases and bone metabolism [111]. Recently, the importance of yolk as a mineral source for chicks and possible alterations via interventions strategies for future usage has been analyzed [112]. The amount of minerals in yolk reflects that the uptake content and enrichment can have beneficial effects [113]. In-ovo supplementation of minerals has improved bone properties and development in hatchlings and mature broilers [113–115].

8. Improved Fertility Rates and Semen Quality

Fatty acid supplementation, especially of ω-3 and ω-6, helps in improving fertility, semen quality and quantity. Kelso et al. [114] found that dietary fish oil or corn oil supplementation to chickens at a level of 50 g/kg in their diet led to significantly higher (96%) fertility rates than the rates prior to supplementation (89%). Kelso et al. [115] noticed that the supplementation of ALA in male diets led to higher fertility at 39 weeks of age because of the increased ω-3 fatty acid proportion in the phospholipids of sperm. Cerolini et al. [116] reported that dietary FA supplementation can influence spermatozoa traits. Hudson and Wilson [117] stated that the supplementation of menhaden oil at 30 g/kg in the diets of male broiler breeders improved the quality of semen and increased fertility and hatchability. Bongalhardo et al. [118] reported that supplementing cockerel diets with fish oil improved fertility, which was attributed to the lower fatty acid ratio (ω-6:ω-3) in the membrane of spermatozoa that may change the membrane resistance to peroxidative damage or its physical characteristics [119].

Fertility and quality of sperms both have been found to be affected during cryopreservation, and fatty acids act as protectants for sperms. Cryopreservation of semen affects survivability, which to a greater extent is dependent on lipid content in spermatozoa [119]. Blesbois et al. [119] noted a decrease in cholesterol/phospholipid ratio in poultry sperms following cryopreservation and a relation with fluidity, hence affecting survivability. Fatty acids can prevent damage to sperms, whether it be physical (cryopreservation) or chemical (oxidative). Zaniboni and Cerolini [120] stated that the dietary ω-3 LC-PUFA treatment of turkey prevented the negative influence of sperm storage on sperm sensitivity and quality and promoted in-vitro peroxidation and sperm death. Additionally, dietary maize oil supplementation decreased the spermatozoa number per ejaculate by 50% between 26 and 60 weeks of age. Al-Daraji et al. [121] noticed that dietary fish oil supplementation produced the best results

for sperm concentration ($p < 0.05$) based on the ejaculate volume, live sperm, total sperm count, and sperm quality factors, followed by flax oil; however, the worst results for these traits were found with treatments of corn oil and SO. Al-Daraji [122] determined that the correlation between the spermatozoa number and glucose concentration in the seminal plasma was highly significant and negative, indicating that the spermatozoa utilized glucose. Additionally, Al-Daraji [123] noticed that spermatozoa used glucose in the seminal plasma for metabolism. Al-Daraji et al. [121] also clarified that dietary SO or corn oil supplementation had a significant effect ($p < 0.05$) on the semen glucose content, alanine aminotransferase activity, and semen protein content, followed by the results for flax oil and fish oil.

A diet supplemented with a moderate ratio of ω-3:ω-6 fatty acids increased DHA and ω-3PUFAs and decreased docosatetraenoic acid and AA in rooster sperm [124]. Sperm motility, progressive motility, membrane functionality, and viability were significantly improved; the testosterone concentration increased; and a higher fertility rate was noted [125]. Feng et al. [7] reported no significant effect on the testis index; however, the spermatogonial development and germ cell layers and gonadotropin-releasing hormone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone hormone levels increased. Further, they reported that PUFAs regulate the expression of hormone receptors and steroid acute regulator protein (StAR). PUFAs significantly increased the mRNA levels of all hormone-related genes (GnRHR, FSHR, LHR, and StAR mRNA levels).

An overview of different dietary manipulations for improving nutritional quality of poultry products (egg and meat) is presented in Table 1.

9. Conclusions and Future Perspectives

The present review revealed that ω-3 and ω-6 fatty acids could be successfully utilized in poultry feeds to promote immune responses and improve the nutritional value of eggs, meat quality and growth in poultry. Omega-3 fatty acids have anti-inflammatory or inflammation-reducing properties because they can reduce the liberation of cytokines. Omega-6 fatty acids at high levels are associated with an increased prevalence of severe conditions, such as depression and heart disease. However, these fatty acids have a tremendous range of health benefits, including improved cholesterol levels and a reduced occurrence of coronary heart disease. Numerous studies have reported the favorable effects of ω-3 PUFA on bone strength, bone mineral content, and bone mineral density. Furthermore, the content of ω-3 fatty acids in eggs can be increased by supplementing the diets of laying hens with certain dietary supplements, such as groundnut oil, fish oil, safflower oil, linseed, fish meal, or algae. Dietary supplementation with different sources of ω-3 or ω-6 to cockerels improved the semen quality and increased fertility and hatchability. In the present review, we proposed that supplementing poultry diets with different sources of ω-3 and ω-6 fatty acids represented a potential strategy for poultry produced for human consumption. However, some disadvantages were related to meat oxidative stability, where LC-PUFAs were very susceptible to oxidation, resulting in off-flavors and odors in poultry meat, which negatively influence meat quality and acceptability by consumers. Therefore, future studies should investigate how we can produce poultry products with higher contents of PUFAs and favorable fatty acid composition, with low cost and without negative effects on palatability and quality, and subsequently on acceptability by consumers.

Table 1. Studies showing the different dietary manipulation to improve nutritional quality in poultry products (egg and meat).

Author/s	Supplements	Results
[88]	Linoleic acid to α-linolenic acid ratio from 20:1 to 1:2	Increased ALA from 0.95 to 5.09% of fatty acid, EPA from 0.07 to 0.31% of fatty acids, DHA from 0.44 to 3.54% of fatty acids, and n6/n3 from 9.37 to 1.31.
[124]	Calcium in diet (3.2 and 3.7%), sodium butyrate, probiotic, herbal blend or chitosan	Chitosan increased eggshell thickness, strength, and decreased cholesterol in yolk; herbal extract increased eggshell thickness and had no effect on performance, egg shell quality, fatty acid, and lipid profile.
[125]	* Hemp seed (Cannabis sativa) (100 to 200 g/kg diet)	Increased EPA (0.2 versus 0.9 to 1.2% of fatty acids) and DHA (17.1 versus 39.2 to 47.4% of fatty acids)—decreased n6 to n3 fatty acid ratio (44.9 versus 4.92 to 11.7).
[125]	* Hemp oil (40 and 120 g/kg diet)	Increased EPA (0.3 versus 1.2 to 3.2% of fatty acids), DHA (17.1 versus 40.9 to 48.1% of fatty acids) and ALA (15.8 versus 58.7 to 192% of fatty acids).
[126]	* Hemp seed (Cannabis sativa) (150–250 g/kg diet)	Decreased cholesterol linearly with maximum reduction of 32% (281 versus 191 mg/egg) Increased EPA + DHA from 1.33 mg to 5.76 mg/g of unsaturated fatty acids.
[127]	* Hemp seed (Cannabis sativa) (100 to 300 g/kg diet)	Increased EPA (0 versus 1.12 to 2.66% of fatty acids) and DHA (16.2 versus 41 to 41.3% of fatty acids)—decreased n6 to n3 fatty acid ratio (44.9 versus 4.92 to 11.7).
[127]	* Hemp oil (45 and 90 g/kg diet)	Increased EPA (0 versus 1.35 to 2.13% of fatty acids) and DHA (15.8 versus 39.3 to 43.6% of fatty acids).
[127]	** Medicago sativa (Alfalfa sprouts) 40 g/d	Decreased cholesterol by 9.5% in the egg. Improved isolariciresinol by 220% and daidzein by 173%. Improved EPA by 109%, DHA by 22% and LNA by 22% and also increased many other antioxidant significantly.
[128]	Flax sprouts (Linum usitatissimum) 40 g/d	Decreased cholesterol by 8.7%. Increased isolariciresinol by 142% and daidzein by 327%. Enhanced EPA by 64%, DHA by 91% and LNA by 55%. Increased many other antioxidants significantly.
[129]	*** Fermented buckwheat extract (Fagopyrum esculentum) 16 g/kg diet for 4 weeks	Enriched L-carnitine (13.6%) and GABA (8.4%) in the egg yolk.
[130]	Gynura procumbens (Lour) plant (2.5 to 7.5 g/kg)	Lowered total cholesterol by 12% in the egg yolk.
[131]	Stearidonic acid-enriched soybean oil (50 g/kg diet)	Improved EPA (1 versus 10 mg), DHA (46 versus 84 mg) and total ω-3fatty acids (94 versus 244 mg) per egg yolk.
[131]	**** Fish oil (50 g/kg diet)	Increased EPA (1 versus 56 mg), DHA (46 versus 211 mg) and total n-3 fatty acids (94 versus 340 mg) per egg yolk.
[131]	***** Flaxseed oil (50 g/kg diet)	Improved EPA (1 versus 6 mg), DHA (46 versus 72 mg) and total ω-3fatty acids (94 versus 376 mg) per egg yolk.
[132]	***** Flaxseed oil (10 to 40/kg diet)	Increased EPA (0 versus 0.01 to 0.7% of fatty acid) and DHA (0.74 versus 1.25 to 1.72% of fatty acid) content. Decreased n6/n3 fatty acid (13.3 versus 6.8 to 2.3).
[133]	Feeding of Lacobacillus reuteri (10(6) CFU/mL of bacteria to 1-d-old broiler chickens weekly for 6 weeks)	Enhanced conjugated linoleic acid concentration in eggs (0.16 to 1.1 mg/g fat at 4–5 week of supplementation).
[134]	Pomegranate seed oil, used as a source of punicic acid (5–15 g/kg diet)	Pomegranate seed oil, used as a source of punicic acid (0.5 to 1.5% level. Improved EPA and DHA content in eggs).
[135]	Microalgae (Schizochytrium) powder (5 and 10 g/kg diet)	Increased DHA, but not EPA.

Table 1. *Cont.*

Author/s	Supplements	Results
[136]	Addition of PUFA at a ratio of ω-3: ω-6(1:5)	Reduced the cholesterol level of breast meat.
[137]	Increasing doses (0. 3 to 4 g/kg n3-PUFA from microalgae Isochrysis galbana)	Increased n3 long-chain PUFA in egg yolk linearly from 14.7 to 129 mg Transfer efficiency was maximum (53%) at 0.12% level of supplementation with lowest efficiency (28%) at 0.4% level.
[138]	Different n3-PUFA supplementation (0.56% extruded flaxseed, 2.03% Isochrysis galbana, 0.68% fish oil, and 0.44% DHA Gold)	The lowest enrichment efficiency (6%) was observed with flaxseed (α-linolenic acid source). Fish oil, microalgae and DHA Gold had enrichment efficiencies of about 55%, 30% and 45%, respectively.
[139]	Microalgae, Phaeodactylum tricornutum, Nannochloropsis oculata, Isochrysis galbana and Chlorella fusca (25 mg and 250 mg extra n-3 PUFA per 100 g feed)	The highest efficiency of ω-3long-PUFA enrichment was obtained by supplementation of Phaeodactylum and Isochrysis. Yolk color shifted from yellow to a more intense red color with supplementation of Phaeodactylum, Nannochloropsis and Isochrysis.
[140]	PUFA in diet	Meat fat content and composition, meat quality and shelf life, nutritive value.
[141]	Basal diet + 100 mg l-theanine/kg diet; basal diet + 200 mg l-theanine/kg diet; and basal diet + 300 mg l-theanine/kg diet.	Intermediate level of l-theanine (200 mg/kg diet) showed better results in terms of body weight gain (BWG), feed consumption (FC), and feed conversion ratio (FCR). Visceral weight and meat color improved, cholesterol decreased HDL increased, and antioxidant status improved. Higher levels have l-theanine deleterious effects.
[142]	Feeding laying hens with alpha-linolenic acid (ALA) resources [flax (10%), perilla (10%), and Eucommia ulmoides (10%) and eicosapentaenoic acid/docosahexaenoic acid (EPA/DHA) resources (Schizochytrium sp.) (1.5%)	Combination of microalgae and perilla seeds increased ALA from 19.7 to 202.5 mg/egg and EPA + DHA from 27.5 to 159.7 mg/egg. n-3 PUFA enrichment was 379.6 mg/yolk. Combination feeding increased ALA, EPA, and DHA content.
[143]	Feeding linseed (4.5%) + tomato-red pepper mix (1 + 1%)	Linseed decreased palmitic acid (25.41% to 23.43%) and stearic acid (14.75% to 12.52%), no effect on α-Linolenic acid, and increased eicosapentaenoic acid (EPA) (0.011% to 0.047%) and docosahexaenoic acid (DHA) (1.94% to 2.73%). Linseed combined with tomato-red pepper mix did not affect these parameters.
[144]	Feeding wheat-soybean meal basal diet along with sunflower oil (SO), animal oil (AO), linseed oil (LO), or menhaden fish oil (FO)@ 5% (wt/wt)	Significantly lower splenocyte proliferative response to ConA has been noted in chicks fed LO or FO than the chicks fed SO or AO. Significantly lower splenocyte response to PWM has been noted in chicks fed AO, LO, and FO than fed with SO. Significantly lower thymus lymphocyte proliferation in response to ConA in chicks fed AO, LO, and FO than in the chicks fed SO. Proportion of IgM + lymphocytes in spleen increased in both chicks fed LO and FO, however serum IgG concentration increased in FO-fed chicks only. Significant increase in CD8 + T-lymphocytes percentage has been noted in LO-fed chicks.

* One ounce of hulled hemp seeds providing 2.5 g of omega-3. ** Total Omega-3 fatty acids in Medicago sativa equal to 1522 mg (levels per 200-Calorie serving). *** Fermented buckwheat extract providing Omega-3: 0.08 g and Omega-6: 0.96 g (in each100 gm). **** Fish oil contain the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). ***** One tablespoon of flaxseed oil is providing about 700 milligrams (mg) of EPA and DHA.

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Threonine Requirements in Dietary Low Crude Protein for Laying Hens under High-Temperature Environmental Climate

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Simple Summary: The threonine (Thr) requirement of laying hens in a high-temperature climate is scarcely referred in the review of literature. Therefore, our aim was to estimate the dietary Thr requirement in low CP diets in a high-temperature environmental climate. Based on our findings, the optimal dietary Thr requirements to optimize egg production, serum uric acid, and serum CuZn-SOD were 0.58%, 0.59%, and 0.56%, respectively, by regression analysis.

Abstract: Lohmann Brown hens (n = 420), at 28 weeks of age, were divided into five dietary treatments, and each treatment included six replicates of 14 laying hens. Dietary crude protein (14%) was presented as the control diet. Dietary L-Thr was added to the control diet for 12 weeks. Dietary Thr levels are 0.43%, 0.49%, 0.57%, 0.66%, and 0.74%, based on digestible base. From 28 to 40 weeks, hen-day egg production presented a quadratic trend to supplementing dietary Thr (R^2 = 0.96, p = 0.02), and reached a maximum level at 0.58%. Serum uric acid demonstrated a quadratic trend (R^2 = 0.62, p = 0.02) at 0.59%. Both serum total cholesterol and 3-hydroxy-3-methylglutaryl (HMG-CoA) reductase showed lower levels ($p < 0.05$) at 0.66% Thr. Serum CuZn-SOD elevated ($p < 0.05$) at 0.49%, 0.57%, and 0.66% Thr, as compared to the control group, and showed a quadratic trend (R^2 = 0.87, p = 0.003) at 0.56%. Supplemental L-Thr decreased ($p < 0.05$) the expression of ileal HSP70 at 0.66% Thr. In summary, the optimal dietary Thr requirements to optimize egg production, serum uric acid, and serum CuZn-SOD were 0.58%, 0.59%, and 0.56%, respectively, by regression analysis.

Keywords: cholesterol; CuZn-SOD; HMG-CoA; HSP70; laying hens; L-Thr

1. Introduction

High temperatures negatively affect protein utilization efficiency [1]. In addition, diets that contain a high content of dietary crude protein (CP) will increase internal heat production by the elevated heat increment in a high-temperature climate [2]. Therefore, low levels of CP with supplementing limiting amino acids can overcome the bad effects of heat stress [3,4] and enhance protein utilization [5,6]. Recently, it has been reported that laying performance was equal among 14%, 15%, and 16% dietary CP [7]. Since synthetic dietary L-Thr became commercially available, it is possible to decrease CP.

Thr affects protein synthesis and is the third limiting amino acid [8,9]. Recently, it has been reported that Thr is a limiting amino acid in diets containing 14% CP [10].

It has been showed that heat stress provoked lipid accumulation by elevated de novo lipogenesis, decreased lipolysis, and enhanced amino acid catabolism [11]. In addition, during stress times, the bird's body begins freeing heat shock proteins to secure itself from the harmful cellular effects of reactive oxygen species [12]. Heat stress is usually accompanied by increasing levels of 70 kilodalton heat shock proteins (HSP70) [13,14]. In addition, high temperatures disturb oxidative status [15] and increase serum total cholesterol, triglyceride, and zinc [16–20].

The present research aimed to estimate the dietary Thr requirement in low CP diets for laying hens in a high-temperature environmental climate. In addition, the effects of increasing Thr on lipid peroxidation, antioxidants enzymes activities, mineral levels, and HSP70 were investigated.

2. Material and Methods

2.1. Management

All procedures in this study were conducted according to the guide for the care and use of agricultural animals in research and teaching (American society of animal science and poultry science association, 2010), through research group (No. RG-1440-146).

Lohmann Brown hens (n = 420), at 28 weeks of age, and with almost similar live body weights (1800 g), were divided into five dietary treatments. Each treatment included six replicates of 14 laying hens (4 birds/cage; 471.5 cm^2/hen). They were exposed to 16-h light. The study began during the middle of May and ended in August; it lasted 13 weeks, including one week for acclimation. The mean daily temperature and humidity are both presented in Figure 1.

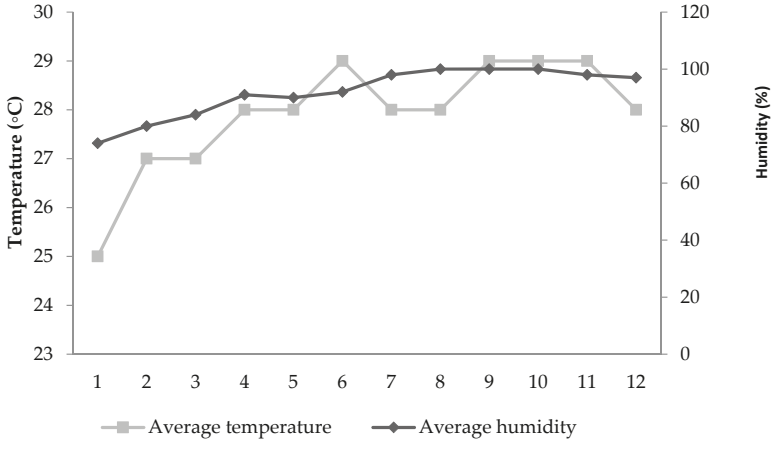

Figure 1. Average temperature (°C) and humidity (%) inside the experimental farm by week.

2.2. Experimental Diets

Hens were fed *adlibitum* (mash form), and water was available through nipples. Dietary CP (14%) was presented as the control diet (Table 1). Dietary Thr levels are 0.43%, 0.49%, 0.57%, 0.66%, and 0.74%, based on digestible base. Ingredient and analyzed CP and total amino acids are presented in Tables 1 and 2, respectively. L-Thr (98.5% purity) was supplied at the expense of kaolin (inert filler). Samples from each diet were analyzed for CP and amino acids according to [21]. Total amino acids in diets were analyzed using HPLC, as described by the authors of [10].

Table 1. The ingredients and nutrient level of the control diet.

Ingredients	**%**
Yellow Corn	65.6
Soybean meal	11.0
Peanut meal (47.8%)	8.5
Soybean oil	3.0
Limestone.38% Ca	8.1
CaHPO4	1.8
L-Lys. HCL	0.23
DL-Meth	0.15
L-Thr	0.0
L-Isoleucine	0.22
L-Trp	0.04
L-Val	0.16
Premix [1]	0.5
NaCl	0.3
Filler (Kaolin)	0.4
Total	100
Nutrient Level [2]	**%**
Analyzed CP	14.0
Digestible Lys	0.69
Digestible Meth + Cysteine	0.54
Digestible Thr	0.43
Ca	3.60
P	0.43
Metabolizable Energy, Kcal/kg	2850

[1] Premix per kilogram of diet: Vitamins (A, 12,000 IU; E, 20 IU; D3, 2,500 IU; K3, 1.8 mg; B1, 2.0 mg; B2, 6.0 mg; B6, 3.0 mg; B12, 0.020 mg; niacin, 25 mg; pantothenic acid, 10 mg; folic acid, 1.0 mg; biotin, 50 mg). Minerals per mg: Fe, 50; Zn, 65; Mn, 65; Co, 0.250. [2] Values of digestible amino acids were calculated according to (Rostagno et al., 2011).

Table 2. Amino acids (g/kg) in experimental diets.

Amino Acids (g/kg)	**Dietary Treatments (L-Thr, g/kg)**				
	0.0	**1.0**	**2.0**	**3.0**	**4.0**
Digestible Thr [1]	4.3	4.9	5.7	6.6	7.4
Arginine	8.7	8.5	8.4	8.7	8.6
Isoleucine	7.1	6.9	6.9	6.8	6.9
Lysine	7.4	7.2	7.4	7.4	7.3
Methionine	3.4	3.3	3.5	3.4	3.4
Threonine	4.5	5.2	6.0	6.9	7.8
Valine	7.7	7.5	7.4	7.6	7.5
Glycine	6.2	6.0	6.0	6.2	6.0
Serine	6.6	6.8	6.8	6.6	6.7

[1] Values of digestible Thr were calculated according to (Rostagno et al., 2011).

2.3. Laying Performance

Mortalities were recorded daily. Egg numbers and egg weight were recorded daily. However, feed consumption was recorded weekly. Egg mass was calculated according to this formula (egg weight × egg production), while feed conversion ratio (FCR) was calculated according to grams of feed consumption/grams of egg mass produced.

2.4. Blood, Liver, and Ileum Sampling and Laboratory Analyses

At the end of the trial (40 weeks), 6 hens per treatment were slaughtered. The blood was collected and was centrifuged (3000× *g*) for 10 min. It was aspirated by pipette and stored in Eppendorf tubes

at −70 °C. Serum concentrations of zinc (Zn), copper (Cu), triglyceride (TG), total cholesterol (CHO), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) were determined by kits from (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The levels of serum uric acid, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were analyzed by commercial kits from the previous company. The HDL-C levels in serum supernatant were determined after precipitation of lipoprotein-B using phosphotungstic acid/Mg^{2+} (PTA/Mg^{2+}).

Serum 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was determined following the manufacturer's protocols from Sigma (St. Louis, MO, USA).

After slaughter, the liver of each hen was collected immediately, snap-frozen with liquid nitrogen, and stored at −80 °C until analysis. About 0.5 g of liver of every bird was homogenized and analyzed for CHO, TG, GOT, and GPT, as described above in the serum.

2.5. Oxidant and Antioxidant Status

Serum levels malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidative capability (T-AOC), and copper zinc superoxide dismutase (CuZn-SOD) were analyzed as described by [22,23]. Liver tissues were homogenized in ice-cold isotonic physiological saline to form homogenates at the concentration of 0.1 g liver/mL. The samples of liver tissues were homogenized and centrifuged, and the supernatants were collected to analyze MDA, T-AOC, and SOD.

2.6. HSP70 mRNA Expression Assay

Total RNA was isolated from 50 mg of ileum, according to the instructions (TRIzol; Invitrogen, Carlsbad, CA, USA). The quality of RNA was examined by both native RNA electrophoresis on 1.0% agarose gel and the UV absorbance at 260 nm and 280 nm. The cDNA was synthesized from 2 lg of total RNA by a reverse transcriptase at 42 °C for 60 min with oligo dT-adaptor primer, using the protocol of the manufacturer (M-MLV; Takara, Dalian, China). The abundance of mRNA was determined based on a Step-One-Plus Real-Time PCR (ABI 7500; Applied Biosystems, Foster, CA, USA). The PCR used a kit (SYBR Premix PCR kit; Takara, Dalian, China) as described by [10]. Average gene expression relative to the endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method [24]. The calibrator for each studied gene was the average $^{\Delta}$Ct value of the control group (Table 3).

Table 3. Gene and primer sequence.

Gene Name	(5′–3′) Primer Sequence (5′–3′)	Reference
18s rRNA	R: ATTCCGATAACGAACGAGACT F: ATTCCGATAACGAACGAGACT	[25]
GAPDH	R: TCCTAGGATACACAGAGGACCA F: CGGTTGCTATATCCAAACTCA	[26]
HSP70	R: GTCAGTGCTGTGGACAAGAGTA F: CCTATCTCTGTTGGCTTCATCCT	[27]

HSP70 means the 70 kilodalton heat shock proteins.

2.7. Statistical Analyses

Data (Replicate; n = 6) were statistically analyzed by one-way ANOVA (SPSS Inc., Chicago, IL, USA). Polynomial comparisons were applied to test for linear and quadratic responses of dependent variables to dietary Thr. Inflection points in response curves at increasing dietary Thr levels were calculated following [28]. To estimate the optimal Thr requirement, a quadratic regression equation based on 95% of the maximum or minimum response was used [29,30].

3. Results

3.1. Laying Performance and Optimal Dietary Thr

The results showed that both egg mass and hen-day egg production increased quadratically ($p < 0.05$) (Table 4).

From 28 to 34 weeks, hen-day egg production presented a quadratic trend to increasing dietary Thr ($R^2 = 0.97$, $p = 0.03$) at 0.58%. In addition, hen-day egg production presented a quadratic trend ($R^2 = 0.96$, $p = 0.02$), at 0.58% from 28–40 weeks (Table 5).

Table 4. Effect of graded levels of dietary Thr on laying performance of laying hens [1,2].

Items	Thr Levels (%)					SEM	*p*-Value		
	0.43	0.49	0.57	0.66	0.74		Thr	Linear	Quadratic
28–34 weeks									
Egg production, %	91.57	94.66	95.93	95.73	94.26	1.76			0.03
Egg weight, g	62.49	63.02	62.39	62.73	62.31	0.83			
Egg mass, g/hen/day	57.22	59.65	59.84	60.06	58.72	1.43			0.04
ADFI, g/hen/day	123.15	116.3	135.22	122.51	130.2	10.35			
FCR	2.16	1.95	2.26	2.04	2.22	0.18			
35–40 weeks									
Egg production, %	90.14	93.39	94.89	94.36	93.42	1.95			0.05
Egg weight, g	63.55	64.08	63.34	63.71	63.33	0.89			
Egg mass, g/hen/day	57.28	59.83	60.11	60.16	59.16	1.63			
ADFI, g/hen/day	117.27	115.77	122.27	115.09	121.58	9.34			
FCR	2.05	1.94	2.04	1.92	2.05	0.16			
28–40 weeks									
Egg production, %	90.86	94.02	95.41	95.04	93.84	1.7			0.03
Egg weight, g	63.02	63.55	62.86	63.22	62.81	0.85			
Egg mass, g/hen/day	57.25	59.74	59.98	60.12	58.94	1.43			
ADFI, g/hen/day	120.21	116.04	128.74	118.8	125.89	10.35			
FCR	2.1	1.94	2.15	1.98	2.13	0.18			

[1] Data are means of 6 replications with 14 hens/replicate; [2] Throughout the entire experimental period, [2] birds died.

Table 5. Estimations of the dietary Thr requirements based on quadratic regressions.

Variables	Equations	Requirements	*p*-Value	R^2
Hen-day egg production (28–34 week)	$Y = -132.406X^2 + 162.416X + 46.446$	0.58	0.03	0.97
Hen-day egg production (28–40 week)	$Y = -131.368X^2 + 161.869X + 45.809$	0.58	0.02	0.96
Serum uric acid	$Y = 3207.394X^2 - 3995.371X + 1470.705$	0.59	0.02	0.62
Serum CuZn-SOD	$Y = -1130.848X^2 + 1336.98X - 1130.848$	0.56	0.003	0.87

Y = Dependent variables; X = The dietary Thr level (%).

3.2. Serum Biochemical Parameters

Serum uric acid declined ($p < 0.05$) at 0.57% Thr (Figure 2) and showed a quadratic trend ($R^2 = 0.62$, $p = 0.02$) at 0.59% (Table 5).

Serum total cholesterol decreased ($p < 0.05$) at 0.66% dietary Thr. Serum HMG-CoA reductase activity decreased ($p < 0.05$) at 0.49% and 0.66% dietary Thr (Table 6). No effects were observed in the liver for total CHO, HDL-C, HDL-C, and triglyceride.

Table 6. Effect of graded levels of dietary Thr on the levels of lipoproteins and activities and HMG-CoA reductase of laying hens [1,2].

Items	Thr Levels (%)					SEM	*p*-Value		
	0.43	0.49	0.57	0.66	0.74		Thr	Linear	Quadratic
Serum, mmol/L									
Triglyceride	15.94	14.09	16.17	14.05	13.04	1.62			
Total cholesterol	4.67 [a]	3.35 [a,b]	4.26 [a]	2.61 [b]	3.14 [a,b]	0.46	0.008	0.005	
High-density lipoprotein	0.32	0.25	0.25	0.27	0.18	0.06			
Low-density lipoprotein	1.01	0.88	0.9	1.0	0.84	0.14			
HMG-CoA reductase activity, ng/L	189.80 [a]	104.41 [b]	171.88 [a]	103.29 [b]	172.77 [a]	14.97	0.0001		0.00
Liver, μmol/gprot									
Triglyceride	154.6	123.49	146.15	159.66	149.99	12.91			
Total cholesterol	31.51	19.68	30.1	27.64	27.59	4.63			
High-density lipoprotein	17.55	18.64	17.87	17.24	18.09	1.79			
Low-density lipoprotein	25.33	20.63	23.75	14.2	21.08	4.34			

[1] n = 6 hens/treatment; [2] means with different superscripts; [a,b] differ ($p < 0.05$).

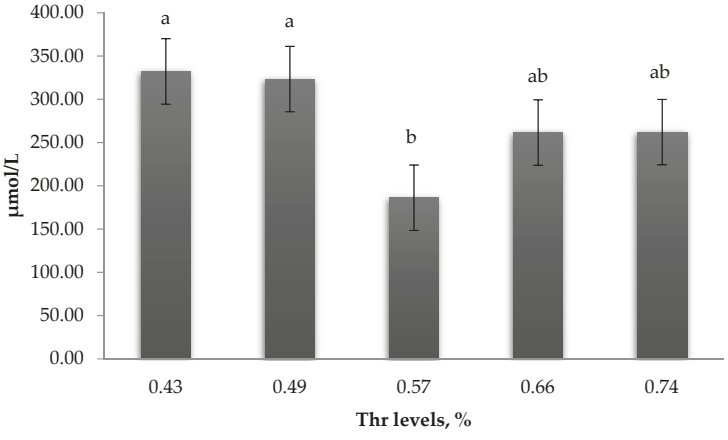

Figure 2. Effect of Thr levels on the levels of serum uric acid. Values are means ± standard SEM. Means on each bar with no common letter differ ($p < 0.05$).

Serum T-SOD increased ($p < 0.05$) at 0.49% dietary Thr. In addition, serum level of CuZn-SOD elevated ($p < 0.05$) from 0.49% to 0.66% dietary Thr (Table 7) and showed a quadratic trend ($R^2 = 0.87$, $p = 0.003$) at 0.56% (Table 5).

Graded levels of dietary Thr did not affect serum or liver concentration of T-AOC, MDA, Zn, Cu, GOT, and GPT (Table 7).

Table 7. Effect of graded levels of dietary Thr on the levels of antioxidants in the liver and serum of laying hens [1,2].

Items	Thr Levels (%)					SEM	*p*-Value		
	0.43	0.49	0.57	0.66	0.74		Thr	Linear	Quadratic
Serum									
T-AOC, U/mL	6.319	7.353	7.283	6.882	5.149	1.15			
T-SOD, U/ mL	131.566 [b]	184.003 [a]	162.439 [a,b]	161.869 [a,b]	178.778 [a,b]	16.34	0.03		
CuZn-SOD, U/ mL	75.03 [b]	103.85 [a]	102.80 [a]	102.34 [a]	83.60 [a,b]	9.03	0.008		0.001
MDA, nmol/ mL	8.071	8.93	10.043	8.945	8.795	1.01			
Zn, µmol/L	51.81	62.9	66.92	56.44	60.5	8.56			
Cu, µmol/L	18.05	17.42	18.07	17.44	16.86	0.95			
GOT, IU/L	9.45	9.84	10.22	8.6	8.92	0.65			
GPT, IU/L	3.15	2.83	3.87	3.3	3.41	0.66			
Liver									
T-AOC, U/mgprot	1.78	1.62	1.8	1.87	1.6	0.31			
T-SOD, U/mgprot	93.73	105.69	91.01	111.94	102.72	13.66			
MDA, nmol/mgprot	0.68	0.66	0.88	0.81	0.78	0.14			
GOT, U/gprot	40.01	41.59	42.06	38.39	39.5	3.04			
GPT, U/gprot	13.49	13.14	15.14	14.38	15.3	1.19			

[1] n = 6 hens/treatment; [2] means with different superscripts; [a,b] differ ($p < 0.05$).

3.3. Ileal HSP70 mRNA Expression

The expression of ileal HSP70 decreased ($p < 0.05$) at 0.66% Thr (Figure 3).

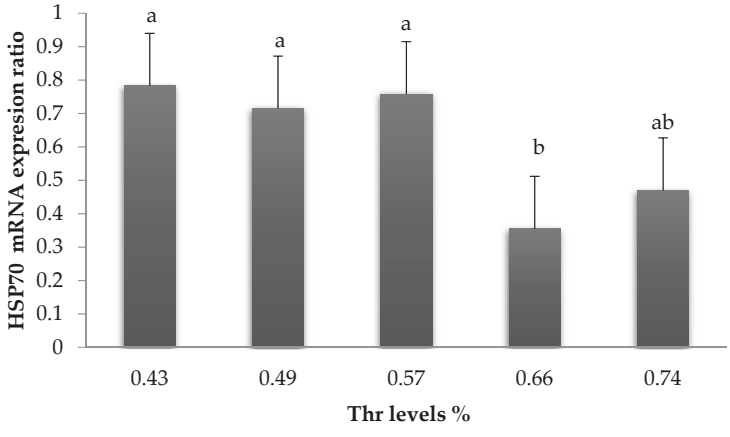

Figure 3. Effect of graded levels of dietary Thr on mRNA expression of ileal HSP70. Values are means ± SEM. means with different superscripts; [a,b] differ ($p < 0.05$).

4. Discussion

It is important to formulate accurate diets to meet the requirements of laying hens because feed ingredients are expensive. In addition, laying hens have been selected for massive egg production, resulting in greater metabolic activity and reduced thermo-tolerance [31,32]. Heat stress has adverse effects on laying hens [33–35]. In addition, high temperatures increase the hens' discomfort and lead to behavioral and endocrinological changes.

In the present study, rapid panting was noticed. In addition, the expression of ileal HSP70 protein decreased ($p < 0.05$) at 0.66% Thr. It has been reported that heat shock protein protects birds from high temperatures by preventing unwanted protein aggregation and channelizing their degradation [36]. The expression of mRNA HSP70 was measured in the gut [14,37], liver [38], hypothalamus [27,39], and blood and feather [40]. Here, we focused on detecting HSP70 in ileum because it plays a vital role

in digestion and absorption, as well as immunity. In addition, the effects of Thr on intestinal function were known [41,42], and the effects of heat stress on gut function become obviously clear.

Both serum CHO and serum 3-HMG-CoA reductase decreased significantly ($p < 0.05$) at 0.66% dietary Thr. It has been reported that 3-hydroxy-3-methylglutaric acid (HMG) is a potent agent for reducing serum triglyceride and cholesterol concentrations [43,44]. It has been reported that HMG causes a 40% to 50% reduction of [1–^{14}C] acetate incorporation into cholesterol in male rats [45]. In addition, HMG inhibited fatty acid synthesis in vivo [46]. In vitro, HMG inhibited 3-HMG-CoA reductase [mevalonate: NADP oxidoreductase (CoA-acylating), EC 1.1.1.34] and interfered with the enzymatic steps involved in the conversion of acetate to HMG-CoA [46]. Taken together, the data suggest that dietary Thr level affect CHO, especially biochemical pathways in which HMG-CoA reductase is involved. Our findings are in agreement with the results of the previous study in broiler chickens [47]. They found that plasma CHO levels decreased significantly ($p < 0.05$) when dietary Thr was sufficient [47]. Here, we did not find a decrease in total CHO levels in the liver. Recently, it has been reported that Thr supplementation did not have an effect ($p > 0.05$) on hepatic cholesterol in Pekin ducks [48]. They suggested that dietary Thr supplementation enhanced hepatic lipid metabolism by regulating lipid synthesis, transport, and oxidation. It has been reported that there is no relationship between the plasma CHO level and the level of yolk cholesterol [49–51], and, consequently, liver CHO.

The levels of GOT and GPT did not change due to treatments. The enzymatic activity of GOT and GPT are indicators of liver health. These enzymes are elevated in acute hepatotoxicity, but they are decreased with prolonged intoxication [52].

In the present study, dietary Thr at 0.49% increased serum levels of T-SOD ($p < 0.05$). In addition, dietary Thr at 0.49%, 0.57%, and 0.66% increased the levels of CuZn-superoxide dismutase (Cu-ZnSOD). The present result suggests that Thr may promote the antioxidative ability of laying hens. Previous studies [22,53] also found that supplemental amino acids (L-Thr and L-Trp) increased T-SOD in serum and the liver.

Uric acid is the metabolic product of protein metabolism and has been suggested as a dominant scavenger of free radicals [54]. We found that level of serum uric acid was declined at 0.57% Thr, which confirms that sufficient Thr increases amino acid utilization. Thr is considered a limiting amino acid in low CP diets [55,56], affecting utilization of TSAA and Lys [57]. It has been reported that the levels of plasma uric acid and excreta were higher from increasing CP than from lowering CP in the diet [58]. In addition, a decrease in the level of uric acid excretion was reported with supplementing limiting amino acids, which indicated better N utilization [59,60]. The plasma urea nitrogen and uric acid have been used to estimate amino acids requirements in swine and broilers [61–63].

It has been reported that laying performance decreased by feeding low CP diets [64]. Here, reduction CP (14%) in the control group reduced egg production quadratically. The effect of the low crude protein diet was pronounced clearly during the late cycle of laying production (43–63 weeks of age) [65]. This study was conducted during the first cycle of egg production (28–40 weeks).

Increasing dietary Thr to 0.57% improved egg production quadratically. It has been found that egg mass and hen-day egg production were reduced ($p < 0.05$) by feeding hens a Thr deficient diet [66]. This means that increasing recognition of Thr as a critical amino acid in the diet of laying hens fed a low CP diet under high-temperature environmental climate. The current results showed that 0.58% dietary Thr based on quadratic regressions guaranteed the best egg production. The present results are in agreement with [67]. They estimated that Thr requirement was 0.57% of dietary Thr from 24 to 40 weeks in Hy-Line W36.

Egg weight, feed consumption, and FCR were similar among dietary Thr levels. Previous studies reported no effect of Thr levels on the egg weight and FCR in laying hens [68–71]. It has been indicated that total Thr deficiency beyond 0.42% decreased feed intake [72].

5. Conclusions

From 28 to 40 weeks of age, the optimal dietary Thr requirements to optimize egg production, serum uric acid, and serum CuZn-SOD were 0.58%, 0.59%, and 0.56%, respectively, by regression analysis. In addition, serum total cholesterol, serum HMG-CoA reductase, and expression of ileal HSP70 decreased at 0.66% Thr.

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Effect of Supplemental Cyanocobalamin on the Growth Performance and Hematological Indicators of the White Pekin Ducks from Hatch to Day 21

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Simple Summary: Vitamin B12 plays a key role in the normal functioning of the brain and nervous system as well as creation and regulation of nucleic acids (DNA and RNA). Furthermore, vitamin B12 plays a significant role in fatty acid metabolism and energy generation. Deficiency of vitamin B12 in animals may lead to weakness and anemia because it is involved in the formation of hemoglobin, which transports oxygen to body cells and red blood cells. Similarly, deficiency of vitamin B12 may also lead to hyperhomocysteinemia (increased level of homocysteine in the blood) which may depress immunity and cause cardiovascular diseases. There is no literature available regarding cyanocobalamin requirement for Pekin ducks. Therefore, the aim of our study is to determine its requirement. However, we find that cyanocobalamin has no influence on growth performance (weight gain), but it has more effect on hematological indicators (blood). On the basis of growth performance and hematological indicators we suggest that 0.02 mg cyanocobalamin/kg of feed is the dietary requirement of male Pekin ducks from hatch to day 21.

Abstract: The experiment was conducted to evaluate the requirement of cyanocobalamin of male Pekin ducks from hatch to 21 days of age. A total of three-hundred-eighty-four, one-day-old meat-type male Pekin ducks were randomly allocated to six treatments, i.e., dietary cyanocobalamin (vitamin B12) concentrations of 0.00, 0.02, 0.04, 0.06, 0.08 and 1.00 mg/kg, respectively in their feed. Each treatment had eight replicated pens with eight ducks for each pen. Feed and water were provided ad libitum. The experiment was conducted for 21 days. Different growth parameters including average daily weight gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR), and hematological indicators were evaluated because, on the basis of hematological indicators, the health and nutritional status of an animal can be accessed. It is observed that supplemental cyanocobalamin has no significant effect on ADG, ADFI, and FCR but it improves hematological parameters such as white blood cells, red blood cells, and its indices and platelet counts compared to the control group ($p < 0.05$). On the basis of growth performance and hematological indicators it is concluded that 0.02 mg cyanocobalamin/kg of feed is the dietary requirement of male Pekin ducks from hatch to day 21 of age.

Keywords: vitamin B12; pekin ducks; weight gain; feed intake; feed conversion ratio; hematological indicator

1. Introduction

Growth of ducks is faster than all other poultry species [1]. To bias the increased growth rate, these have been genetically selected like all the other poultry species [2]. Numerous studies have suggested that vitamin B12 is necessary for chick growth and egg hatchability [3–7]. Deficiency of vitamin B12 has a critical impact on growth and existence of rats. It is also reported that the addition of high levels of protein in a diet with a deficiency of vitamin B12 causes high mortality [8]. In another

study, Dryden and Hartman (1971) observed a steady increase in growth rate with every increased unit of protein content that might not be associated with the breakdown of surplus nitrogen but with complications in the disposal of the carbon skeleton of certain or total amino acids of added protein contents in vitamin B12 deficient diets [9].

The natural vitamin contents of diets depend upon ingredients and compositions that can vary considerably between particular feedstuffs based on the season, region, and processing conditions which can induce natural variations in vitamin contents. Natural dietary contents rarely provide the intakes thought to fulfill the needs of birds to meet their normal requirements and to provide a margin of safety to meet extra metabolic demands imposed by stress and other factors [10]. Whitehead (2002) [11] demonstrated that diets supplemented with vitamins play a vital role for disease prevention and treatment. He also illustrated that biological functions are carried out by the involvement of vitamins that allow an animal to use energy and proteins for growth, health, maintenance, reproduction, and feed conversion [11]. Poultry intake B12 either by feed supplementation or ingesting feces. This vitamin plays a vital role in the nervous system and proper brain functioning, homocysteine metabolism, energy metabolism, normal blood functions, cell division, and in the immune system [12]. Similarly it is also anti-anemic in function [13]. Vitamin B12 acts as a co-factor for L-methylmalonyl-CoA mutase and methionine synthase. Methionine synthase increases the rate of homocysteine conversion to methionine which is further required for DNA and RNA synthesis while L-methylmalonyl-CoA mutase converts L-methylmalonyl-CoA to Succinyl-CoA which is a crucial biochemical reaction in fat and protein metabolism. Succinyl-CoA is also required for hemoglobin synthesis [14].

Ceca of the birds synthesize vitamin B12 by using cobalt, but its production is lower than daily requirements, and suggestions were given to supplement diets with vitamin B12 [15]. In another study, researchers reported that for an increased growth rate and maximum feed utilization, a growing ration fortified with vitamin B12 had appreciable effects but it may not be optimum for achieving maximum feed efficiency [16]. Folic acid, vitamin B12, and dietary total non-structural carbohydrates have no effect on milk production and milk total solid yield. There was also a non-significant difference observed on average daily gains among the calves supplemented with these vitamins [17]. Vitamin B12 deficiency also caused health problems in many ways, from hematological manifestations to neurological disorders, varied as leucoenia, numbness in fingers, unsteadiness of gait, fasciculation, and macrocytic anemia [18]. Erythrocytes depend on vitamin B12 for their maturation and proliferation. Therefore vitamin B12 deficient erythrocytes cannot mature, resulting in hemolysis and hyperbilirubinemia [19].

Blood parameters are indices of the internal environment of the living body and they also indicate the health status of ducks [20]. To improve animals' productivity, it is important to understand their physiology and hematological characteristics. For the establishment of a diagnostic baseline for blood characteristics, hematological studies are usually undertaken on farm animals as routine management practices [21,22]. Hematological constituents serve as authentic tools for monitoring animal health because they usually reflect the physiological reactions of an animal to its external and internal environment [23,24]. Hematological indicators are very important as they designate an animal's health and nutritional status [25]. These parameters in poultry are also sensitive to stress reactions [26]. Hematological indicators are considered to be biomarkers for the immune system [27]. There is a limited literature available for hemogram parameters in vitamin B12 deficiency [28]. That is why we investigate these hematological indicators on different levels of vitamin B12 in Pekin ducks. The National Research Council (NRC) (1994) also has no data regarding the vitamin B12 requirement of meat-type Pekin ducks for growth and hematological indicators. Therefore, one of the main goals of this study is to estimate the requirement of cyanocobalamin as a supplement for the growth performance and hematology of male Pekin ducks from hatch to 21 days of age.

2. Materials and Methods Estimate

2.1. Study Design

All procedures of this study were permitted by the Animal Care and Welfare Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (No. IASCAAS-AE-03, No. 20180416). A dose-response experiment with six supplemental vitamin B12 levels (0, 0.02, 0.04, 0.06, 0.08 and 1.00 mg/kg) was designed. Vitamin B12 deficient basal diet was prepared (Table 1) and it was comprised of 0.00 mg/kg total vitamin B12, according to the data of feed ingredients of the NRC (1994).

Table 1. Composition of basal diet (% as fed).

Items	Content (%)
Ingredients	
Corn	63.6
Soyabean meal	32.4
Calcium hydrophashate	1.6
Lime stone	0.8
Premix [1]	1
NACL	0.3
Methionine	0.2
Lysine	0.1
Calculated Composition	
Metabolizable energy (Mcal/kg) [2]	2.9
Crude protein	20.03
Calcium	0.96
Nonphytic acid phosphor	0.42
Lysine	1.11
Methionine	0.51
Methionine + Cystine	0.85
Threonine	0.81
Tryptophan	0.25
Arganine	1.41
Valine	0.93
Isoleucine	0.81
Phosphorus	0.62
Vitamin B12	0

[1] Supplied per kilogram of total diet: Cu ($CuSO_4 \cdot 5H_2O$), 8 mg; Fe ($FeSO_4 \cdot 7H_2O$), 60 mg; Zn (ZnO), 60 mg; Mn ($MnSO_4 \cdot H_2O$), 100 mg; Se ($NaSeO_3$), 0.3 mg; I (KI), 0.4 mg; Mg(MgO), 200 mg; K(K_2CO_3), 1500; choline chloride, 1000 mg; vitamin A (retinyl acetate), 4000 IU; vitamin D_3 (Cholcalciferol), 2000 IU; vitamin E (DL-α-tocopheryl acetate), 20 IU; vitamin K_3 (menadione sodium bisulfate), 2 mg; thiamin (thiamin mononitrate), 2 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 4mg; calcium-D-pantothenate, 20 mg; nicotinic acid, 50 mg; folic acid, 1 mg; and biotin, 0.20 mg.[2] The values were calculated according to the apparent metabolizable energy of chickens.

2.2. Chicks and Diets

The basal diet was formulated first and then six experimental diets were added with various supplemental levels of crystalline vitamin B12. A total of 384 one-day-old male White Pekin ducks with average body weights around 54 g were divided into 6 experimental groups and each group was replicated 8 times with 8 birds per pen. These ducklings were reared in steel cages with plastic floors (200 × 100 × 40 cm), from hatch to 21 days of age. They were raised and allocated randomly to an environmental control shed. The birds were offered feed and water ad libitum. Provision of water was done by using a drip-nipple, and feed was offered in pellet form. Birds were provided 24-h lighting, and temperature was maintained at 33 °C with relative humidity at 20% from 1 to 3 days of age, then temperature was decreased steadily to room temperature while relative humidity gradually increased to 65% until birds were 21 days of age.

2.3. Growth Performance and Hematological Parameters

On the 21st day, the average daily weight gain, average daily feed intake, and feed conversion ratio (FCR) of ducks from every pen were calculated using the following formula.

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed intake}}{\text{Weight gain}}$$

Feed intake and FCR were all corrected for mortality.

2.4. Sampling and Analysis

After fasting 12 h, two ducks from each pen were selected randomly for blood collection on the basis of average body weight gain of the corresponding pen. Three to four milliliters of blood were collected in an Ethylenediaminetetraacetic Acid (EDTA)-containing tube from the jugular vein of each selected bird. These blood samples were analyzed by using an automated hematological analyzer (ABX PENTRA DX 120, Munich, Germany).

2.5. Statistical Analysis

Data were analyzed using SPSS Statistics 22.0 (Statistical Packages for the Social Sciences, released December 2013, Armonk, New York, USA). The mean comparison test was applied by using Duncan's new multiple-range test to find out the significant differences among the applied treatments at 5% the level of significance ($p < 0.05$).

3. Results

3.1. Growth Performance

The effect of dietary cobalamin on average daily weight gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) of the experiment is described in Table 2. ADG, ADFI, and FCR showed non-significant results among the vitamin B12 supplemented groups and the control group ($p > 0.05$), as presented in Table 2.

Table 2. Effect of dietary vitamin B12 on average daily weight gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) in meat-type Pekin ducks (mean ± SD).

Parameters	Vitamin B-12 (mg/Kg) [1]						*p*-Value
	C	0.02	0.04	0.06	0.08	1	
ADG (g/bird/day)	55.73 ± 1.6	56.30 ± 0.9	56.72 ± 1.1	56.25 ± 1.5	56.35 ± 1.5	56.29 ± 1.6	0.853
ADFI (g/bird/day)	89.95 ± 4.7	92.10 ± 2.2	93.6 ± 2.8	92.4 ± 2.5	94.0 ± 3.4	94.1 ± 4.7	0.174
FCR	1.61 ± 0.10	1.63 ± 0.02	1.65 ± 0.02	1.64 ± 0.03	1.66 ± 0.04	1.67 ± 0.03	0.301

[1] Dietary treatments where C = control (without vitamin B12), all others are vitamin B12 supplemented groups. Each mean represents values from 8 replicates (8 ducks/ replicate).

3.2. Hematology

3.2.1. White Blood Cells

On day 21, ducks from the control group had significantly lower ($p < 0.05$) values for the white blood cell count (WBC) and had significantly higher ($p < 0.05$) values for the percentage of intermediate cells (MID %) than the supplemented groups. However, there were no further significant differences among the vitamin B12 supplemented groups. The absolute value of granulocytes (GRA) and percentage of granulocytes (GRA %) were lower for the control (C) group as compared to the vitamin B12 supplemented groups, although these values were not statistically significant ($p > 0.05$). There were non-significant results observed for the absolute value of lymphocytes (LYM), percentage of lymphocytes (LYM %), and absolute value of intermediate cells (MID), as presented in Table 3.

Table 3. Effect of dietary vitamin B12 on white blood cell count and its indices in meat-type Pekin ducks (mean ± SD).

Parameters	Vitamin B-12 (mg/Kg) [1]						*p*-Value
	C	0.02	0.04	0.06	0.08	1	
WBC (10^9/L)	126.7 ± 22.5 [b]	139.2 ± 7.2 [a]	141.8 ± 4.3 [a]	137.3 ± 14.9 [a]	142.5 ± 3.0 [a]	133.8 ± 9.5 [ab]	0.004
LYM (10^9/L)	60.2 ± 13.4	62 ± 11.2	62.9 ± 7.2	64.1 ± 9.4	61.5 ± 8.3	60.9 ± 13.1	0.474
MID (10^9/L)	20.4 ± 2.9	21.1 ± 3.2	20.9 ± 1	20.9 ± 1.8	20.7 ± 1.7 [b]	20.5 ± 3.0	0.061
GRA (10^9/L)	46.1 ± 33.9	61.1 ± 16.9	57.9 ± 8.7	54.2 ± 21.9	62.3 ± 9.9	54.5 ± 14.3	0.211
LYM (%)	51.7 ± 17.2	41.2 ± 9.1	44.6 ± 5.8	47.7 ± 10.4	41.9 ± 6	44.9 ± 9.6	0.139
MID (%)	16.5 ± 3.8 [a]	15.2 ± 2.8 [ab]	14.7 ± 0.7 [b]	13.9 ± 1.2 [b]	14.5 ± 1.1 [b]	14.9 ± 2.4 [b]	0.028
GRA (%)	33.3 ± 20.5	43.6 ± 11.4	40.7 ± 5.5	38.4 ± 11.3	43.6 ± 6.4	40.8 ± 10.9	0.161

[a,b] Superscript letters show significant differences ($p \leq 0.05$). [1] Dietary treatments where C = control (without vitamin B12), all other are vitamin B12 supplemented groups. Each mean represents values from 8 replicates (2 ducks/ replicate). WBC: White Blood Cells; LYM: Absolute value of lymphocytes; MID: The absolute value of the intermediate cell; GRA: Absolute value of granulocytes; LYM (%): Percentage of lymphocytes; MID (%): Percentage of intermediate cells; GRA (%): Percentage of granulocytes.

3.2.2. Red Blood Cells

At the end of the experiment, ducks with different levels of vitamin B12 had higher significant ($p < 0.05$) values for red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and standard deviation of RBC distribution width (RDW-SD) compared to the control group (Table 4). By the end of the experiment, mean corpuscular hemoglobin concentration (MCHC) and coefficient of variation in RBC width (RDW-CV) values of the control ducks had increased more significantly ($p < 0.05$) than the treated ducks. The values of RBC, Hb, HCT, MCV, MCH, and RDW-SD of the control ducks were significantly lower (1.4 to 1.8 10^{12}/L, 82.4 to 116.9 g/L, 0.17 to 0.26 L/L, 107.5 to 148.9 fL, 54.3 to 66.6 pg, and 43.2 to 63.9%) than supplemented ducks respectively. Results also concluded that vitamin B12 plays a vital role in the synthesis of blood necessary for the different proper and optimal types of functions for the ducks and their well-being (Table 4).

Table 4. Effect of dietary vitamin B12 on red blood cell count and its indices in meat-type Pekin ducks (mean ± SD).

Parameters	Vitamin B-12 (mg/Kg) [1]						*p*-Value
	C	0.02	0.04	0.06	0.08	1	
RBC (10^{12}/L)	1.4 ± 0.5 [b]	1.7 ± 0.2 [a]	1.6 ± 0.4 [ab]	1.5 ± 0.4 [b]	1.8 ± 0.1 [a]	1.6 ± 0.3 [a]	0.005
HGB (g/L)	82.4 ± 42.6 [b]	111.9 ± 11.5 [a]	103.1 ± 22.8 [a]	109.1 ± 37.5 [a]	111.8 ± 7.1 [a]	116.9 ± 17.6 [a]	0.001
HCT (L/L)	0.17 ± 0.11 [b]	0.25 ± 0.03 [a]	0.23 ± 0.05 [a]	0.20 ± 0.09 [b]	0.26 ± 0.02 [a]	0.26 ± 0.04 [a]	0.001
MCV (fL)	107.5 ± 38.6 [b]	145.7 ± 3.5 [a]	141.8 ± 5.3 [a]	120.3 ± 28.6 [b]	142.5 ± 3.5 [a]	148.9 ± 2.9 [a]	0.001
MCH (pg)	54.3 ± 12.15 [b]	65.1 ± 4.6 [a]	64.1 ± 6.67 [a]	59.8 ± 12.12 [b]	61.7 ± 4.05 [a]	66.6 ± 4.3 [a]	0.001
MCHC (g/L)	505.6 ± 82.3 [a]	424.2 ± 22.1 [b]	428.5 ± 33.9 [a]	398.1 ± 57.6 [b]	410.6 ± 20.9 [b]	424.5 ± 21.6 [b]	0.001
RDW-SD (fL)	43.2 ± 18.9 [c]	64.4 ± 4.2 [a]	63.4 ± 3.9 [a]	52.6 ± 16 [b]	61.4 ± 2.3 [a]	63.9 ± 13.6 [a]	0.001
RDW-CV (%)	32.1 ± 18.8 [a]	13.7 ± 1.6 [b]	15.1 ± 2.2 [b]	27.9 ± 16.8 [b]	15.2 ± 1.3 [b]	13.1 ± 1.1 [b]	0.001

[a,b,c] Superscript letters show significant difference ($p \leq 0.05$). [1] Dietary treatments where C = control (without vitamin B12), all others are vitamin B12 supplemented groups. Each mean represents values from 8 replicates (2 ducks/replicate).

3.2.3. Platelet Count

The values of the platelets indices by day 21 are shown in Table 5. The values of the platelet count, thrombocytocrit, and mean platelet volume of the control group had significantly lower values than the supplemented groups, (41.3 to 84.6 10^9/L, 0.05 to 0.11 L/L and 11.4 to 13.7 fL) respectively, whereas the values of platelet distribution width of the control group were significantly higher (51.9 to 22.9%) than treated groups (Table 5).

Table 5. Effect of dietary vitamin B12 on platelet count and its indices in meat-type Pekin ducks (mean ± SD).

Parameters	Vitamin B-12 (mg/Kg) [1]						*p*-Value
	C	0.02	0.04	0.06	0.08	1	
PLT (10^9/L)	41.3 ± 23.4 ^{c}	55.8 ± 33.9 ^{ab}	76.7 ± 38.9 ^{bc}	84.6 ± 33.7 ^{a}	73.4 ± 23.1 ^{bc}	43.6 ± 19.7 ^{b}	0.001
PCT (L/L)	0.05 ± 0.03 ^{b}	0.08 ± 0.05 ^{ab}	0.11 ± 0.05 ^{a}	0.11 ± 0.05 ^{a}	0.09 ± 0.03 ^{a}	0.06 ± 0.03 ^{b}	0.001
MPV (fL)	11.4 ± 2.1 ^{c}	13.4 ± 0.41 ^{a}	13.7 ± 0.3 ^{a}	12.4 ± 1.6 ^{b}	13.5 ± 0.26 ^{a}	13.5 ± 0.37 ^{a}	0.001
PDW (%)	51.9 ± 24.4 ^{a}	37.6 ± 23.7 ^{b}	27.5 ± 21.5 ^{ab}	22.9 ± 8.4 ^{c}	24.7 ±10.03 ^{ab}	34.4 ±17.01 ^{ab}	0.001

^{a,b,c} Superscript letters show significant difference ($p \leq 0.05$). [1] Dietary treatments where C = control (without vitamin B12), all other are vitamin B12 supplemented groups. Each mean represents values from 8 replicates (2 ducks/replicate).

4. Discussion

The influence of different levels of vitamin B12 on growth performance and hematological analysis was studied in Pekin ducks during the starter phase (0–21 days). There is no data available on the requirement of vitamin B12 in the Pekin duck, also the literature regarding vitamin B12 and Pekin ducks is limited.

In our study there were no significant differences found for the average daily feed intake and average daily weight gain in the group without vitamin B12 supplementation and groups supplemented with vitamin B12 in the Pekin ducks, respectively. The mean ADG, ADFI, and FCR in the present study is similar to that described in ducks earlier with vitamin B12 [12]. Our results also match the findings of other researchers who found that increasing levels of vitamin E in the diet had no significant effect on live body weight gain, body weight gain, feed intake, and FCR [29].

Without supplementation of vitamin B12 in the feed, the ducks did not show any clinical signs related to vitamin deficiency because the liver can preserve vitamin B12 for long periods of time, even feeding a vitamin B12 deficient diet. They further illustrated that about 2–5 months may be required for the elimination of B12 preserved by hens to such an extent that progeny will hatch with a low reserve of vitamin B12 [30].

Vitamin B12 had no effect (Table 2) on growth performance ($p > 0.05$). This might be due to the short period of the experiment (21 days) and its very low requirement for proper growth. In the present study, ducklings hatched from parent flocks which were feeding according to the nutrient requirements of breeder ducks. Therefore, it can be assumed that the storage of vitamin B12 in the egg yolk was stabilized, and the freshly hatched ducklings had an optimum B12 depot in the liver and in the last part of the yolk sac, and that the liver maintained the level of vitamin B12 for 21 days in the ducklings even without its supplementation in the diet [12].

Supplementation of vitamin B12 had no significant effect on the production and fertility of eggs. It was also revealed that vitamin B12 deficiency in the breeder diet had adverse effect on growth of chicks and when the chicks were collected from breeders during hatching they were supplemented with vitamin B12 but their deficiency was fulfilled up to some extent. It is therefore concluded that the supplementation of vitamin B12 in the diet of hens was more effective than the chick diet for the growth in their early stage [7].

However, limited research work has been done on hematological profiles and limited information is available on these parameters in ducks. Vitamin B12 is essential for proper red blood cell production, neurological function, and DNA formation [31–34]. The effects of vitamin B12 on the hematological indicators are shown in (Tables 3–5). The hematological indicators weremeasured in WBCs, RBCs, and platelets after both the addition and subtraction of vitamin B12. Vitamin B12 supplemented groups showed significantly higher values than the control group ($p < 0.05$).

Vitamin B12 works as a co-factor for methionine synthase and L-methylmalonyl-CoA mutase. Methionine synthase speeds up the transformation of homocysteine to methionine. Methionine is mandatory for the creation of S-adenosylmethionine, which is a universal methyl donor for nearly 100 different substrates, including DNA, RNA, hormones, proteins, and lipids. L-methylmalonyl-CoA mutase converts L-methylmalonyl-CoA to Succinyl-CoA in the degradation of propionate, which is

a critical biochemical reaction in fat and protein uptake. Succinyl-CoA is necessary for synthesis of hemoglobin [35].

Dietary supplementation with a combination of probiotics and prebiotics markedly increased the PCV, RBC, and Hb of guinea fowls [36]. Molasses also had significant effect on MCV and platelets in broiler chickens during hot dry seasons. Presence of high MCV may indicate an active erythropoiesis, as MCV is thought to be the average size of an individual erythrocyte [30]. Younger erythrocytes are larger than older ones [37,38]. Betaine had highly significant effects on hematological parameters in the meat-type ducks under stress conditions; specially values of RBC, HCT, HGB, MCV, MCHC, RDW, platelets PLT, PCT, and MPV were significantly higher ($p < 0.05$) in betaine added groups as compared to the control group but no further significant differences were found among the betaine supplemented groups [39]. Similarly the control group had significantly lower ($p < 0.05$) values for RBC, Hb, PCV, MCV, and MCH in different breeds of Omani goats from treated groups which were receiving dietary cobalt and cutaneous injections of hydroxycobalamin [40]. Hemorrhagic anemia and hemolytic anemia are caused by the decreased value of RBCs, whereas the decrease of hemoglobin causes microcythemia. It is also reported that hematocrit and packed red cells volume have an immense correlation with each other. Consequently, when the mean hematocrit decreases, the levels of hematoglobin and hemoglobin becomes lower [39]. In a study, MPV was found to be lower in patients with vitamin B12 deficiency than in the patients without vitamin B12 deficiency as a result of production of smaller platelets [18,41].

5. Conclusions

To the best of our knowledge, this is the first time that someone determined the dietary requirement of cyanocobalamin for male Pekin ducks during the starter phase on the basis of growth performance and hematological indicators. However, we found that cyanocobalamin had a non-significant effect on growth performance, but it had more significant improvements on hematological indicators. On the basis of our findings we suggest that 0.02 mg cyanocobalamin/kg of the feed may be supplemented to improve hematological variables of Pekin ducks from hatch to day 21.

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animals

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Article

Effect of Chestnut Tannins and Short Chain Fatty Acids as Anti-Microbials and as Feeding Supplements in Broilers Rearing and Meat Quality

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Simple Summary: The poultry industry needs to replace antibiotics with natural or synthetic compounds able to overcome problems linked to the development of bacterial resistance. Tannins and short chain fatty acids are valid alternatives to contrast the growth of pathogens. However, tannins may induce detrimental effects on animal performances, especially in monogastrics, causing damage on gut villi. In contrast, short chain fatty acids are very efficient in influencing positively the morphology of small intestine wall. Hence, the aim of this trial was to develop a feeding strategy for broiler rearing, based on the use of chestnut tannins and short chain fatty acids administered as blends. No differences in animal performances or in meat quality were found among feeding groups. The results suggested that the mix of these supplements did not have negative effects on the productive performances, representing a promising alternative to antibiotics. However, further investigation is needed to better understand the effects of these supplements on animals in stress conditions.

Abstract: Chestnut tannins (CT) and saturated short medium chain fatty acids (SMCFA) are valid alternatives to contrast the growth of pathogens in poultry rearing, representing a valid alternative to antibiotics. However, the effect of their blends has never been tested. Two blends of CT extract and Sn1-monoglycerides of SMCFA (SN1) were tested in vitro against the proliferation of *Clostridium perfringens*, *Salmonella typhymurium*, *Escherichia coli*, *Campylobacter jejuni*. The tested concentrations were: 3.0 g/kg of CT; 3.0 g/kg of SN1; 2.0 g/kg of CT and 1.0 g/kg of SN1; 1.0 g/kg of CT and 2.0 g/kg of SN1. Furthermore, their effect on broiler performances and meat quality was evaluated in vivo: one-hundred Ross 308 male birds were fed a basal diet with no supplement (control group) or supplemented with CT or SN1 or their blends at the same concentration used in the in vitro trial. The in vitro assay confirmed the effectiveness of the CT and SN1 mixtures in reducing the growth of the tested bacteria while the in vivo trial showed that broiler performances, animal welfare and meat quality were not negatively affected by the blends, which could be a promising alternative in replacing antibiotics in poultry production.

Keywords: antibiotic; hydrolysable polyphenol; monoglyceride; pathogen; poultry feeding

1. Introduction

Conventional antimicrobial agents are commonly used in the poultry industry to control diseases and to prevent the mortality of birds. However, this approach conflicts with the worldwide aim to eliminate antibiotics in animal feeding. Indeed, the use of pharmaceuticals as preventing tools against pathogens has contributed to the acquisition of bacterial resistance. Moreover, problems for dejection disposal occur, due to the presence of residual antibiotics. Therefore, the poultry industry needs alternatives able to replace drugs with natural compounds or with synthetic compounds able to simulate natural molecules [1].

Polyphenols from plant kingdom are efficient antimicrobials, even if major differences can be noted. Indeed, their efficacy is affected by the solubility, which is strongly linked to the molecular structure. Among the others, chestnut tannins (CT) are hydrolysable and water-soluble compounds. Their antimicrobial activity has been previously demonstrated in poultry by Tosi et al. [2], while Redondo et al. [3] reported that *Clostridium perfringens* is unable to develop resistance against hydrolysable tannins, compared to antibiotics as avilamycin or bacitracin. However, the use of tannins in animal feeding, with particular reference to monogastrics, is discouraged for their potential anti-nutritional effects [4]. The reason is their ability in binding proteins, lowering feed intake and digestibility [4–6]. Hence, to evaluate the inclusion level of polyphenols is extremely important to avoid detrimental effects on animal welfare and performances [7,8].

Furthermore, literature shows that free saturated short-medium chain fatty acids (SMCFA; from C4:0 to C12:0) can protect the gut against several pathogenic bacteria [9–11], but their employment is limited because they are quickly absorbed in the jejunum [12–14]. Similarly, Sn2-monoglycerides are easily carried from the gut into the blood stream [6,15]. A hypothetical alternative could be represented by synthetic monoglycerides, because the industrial synthesis occurs under kinetic control of the reaction and the end-products are Sn1-substituted monoglycerides. These molecules, according to a not-natural structure, are little absorbed at the gut level, where they can exert antimicrobial effects against pathogens [16].

Several studies have been published on the efficiency of CT and synthetic monoglycerides in exercising antimicrobial activities and in ameliorating animal performances when used separately [12–14,17]. Nevertheless, no information is available in literature on the effect of blends of CT and Sn1-monoglycerides (SN1) of SMCFA as antimicrobials against the proliferation of *C. perfringens*, *Salmonella typhymurium*, *Escherichia coli*, *Campylobacter jejuni* and as dietary supplement in poultry diets. Hence, the aims of this trial were: (i) to test a possible synergic antimicrobial activity of two blends obtained from a mixture of two commercial supplements (i.e., CT extract from *Castanea sativa* Mill. and SN1) by an in vitro study, then (ii) to evaluate the effect of the same mixtures on broiler performance and meat quality.

2. Materials and Methods

2.1. Chestnut Hydrolysable Tannins and Sn1-Monoglycerides Composition

Chestnut tannins were extracted from the wood of *C. sativa* Mill. by distillation with water flow (Saviotanfeed®®, Gruppo Mauro Saviola srl Radicofani, Siena, Italy) and contained 750 g/kg (on dry matter basis, DM) of equivalent tannic acid. The chromatographic characterization of this lot of CT extract is reported in Bargiacchi et al. [18]. Sn1-monoglicerydes contained a mix of SMCFA from C4:0 to C12:0 (Silohealth®®, Silo SpA, Firenze, Italy). The glycerides and fatty acid (FA) profile of SN1 was determined according to Christie [19] and it is shown in Table 1. These supplements were the same used in the microbiological assay and during the in vivo trial.

Table 1. Lipid profile of Sn1-monoglycerides.

Glycerides Composition g/100 g of Product	
monoglycerides	95.0
diglycerides	4.9
triglycerides	0.1
Fatty Acid Composition g/100 g of Total Lipids	
C4:0	50.0
C6:0	12.5
C8:0	12.5
C10:0	12.5
C12:0	12.5

2.2. Microbiological Assay

The microbiological assay was carried out according to Elizondo et al. [20] modified as described below. The microorganisms used in this study were the following: *C. perfringens* NetB positive (strain number 191999/2014), isolated from broiler chickens affected by necrotic enteritis; *S. typhimurium* (strain number 198306/2014), isolated from viscera of egg-table layers; *E. coli* serotype O45 (strain number 184049/2014), isolated from broiler chickens affected by avian colibacillosis; *C. jejuni* (strain number 18818/2015), isolated from the skin of broiler chickens. The bacterial strains were isolated and identified using the standard procedures adopted by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (section located in Forlì, Italy) and maintained on slants with heart infusion agar (HIA; Becton Dickinson GmbH, Germany) at +4 °C. To ensure culture purity, before the assays, a sample of *C. perfringens* culture was streaked on blood agar base (Oxoid Ltd., Basingstoke, UK) with 5 g/100 g of sheep blood and incubated overnight at 37 °C under anaerobic conditions (GENbag anaer, bioMérieux S.A., Marcy l'Etoile, France). As similar, the samples of *E. coli* and *S. typhimurium* cultures were streaked on Hektoen Enteric Agar (Becton Dickinson GmbH, Germany) and incubated overnight at 37 °C. The samples of *C. jejuni* cultures were streaked on modified charcoal cefoperazone deoxycholate (mCCD) agar (Oxoid Ltd., Basingstoke, UK) and incubated at 44 °C for 48 h in a microaerobic atmosphere (GENbag microaer, bioMérieux S.A., Marcy l'Etoile, France). Then, one colony of each strain was grown in Brain Heart Infusion (BHI) broth (Becton Dickinson GmbH, Germany) and incubated overnight at 37 °C (under anaerobic conditions for *C. perfringens*; microaerobic atmosphere at 44 °C for *C. jejuni*) and then titrated. For this purpose, 10-fold serial dilutions of each suspension were carried out in Buffered Peptone Water (Oxoid Ltd., Basingstoke, UK); each dilution was streaked on specific media and incubated overnight at 37 °C (under anaerobic conditions for *C. perfringens*; microaerobic atmosphere at 44 °C for *C. jejuni*). Based on the titration results, each bacterial suspension was diluted in BHI broth to obtain a concentration of 2×10^3 CFU/mL and then used as inoculum in the antibacterial test described below.

The antimicrobial activity in vitro assay was carried out according to Basri and Khairon [21] modified as follow. Five milliliters of each bacterial suspension (described above) was mixed with 5.0 mL of the following concentrations (in BHI broth, expressed as *w/v*) of the tested compounds: 6.0 g/kg of CT (T group), 6.0 g/kg of SN1 (S group), 4.0 g/kg of CT + 2.0 g/kg of SN1 (TS group) and 4.0 g/kg of SN1 + 2 g/kg of CT (ST group). Untreated tubes containing 5.0 mL of each bacterial suspension and 5.0 mL of BHI broth served as control (C group). This procedure resulted in a final concentration of the bacterial inoculum of 1×10^3 CFU/mL and in the following final concentrations (*w/v*) of the tested compounds: 3.0 g/kg of CT (T group), 3.0 g/kg of SN1 (S group), 2.0 g/kg of CT + 1.0 g/kg of SN1 (TS group) and 2.0 g/kg of SN1 + 1.0 g/kg of CT (ST group). The concentrations of supplements in T and in S groups were chosen according to the producers' guidelines. The ratio among CT and SN1 in the two blends was decided referring to preliminary studies [2,22]. The mixtures were incubated at 37 °C (under anaerobic conditions for *C. perfringens*; microaerobic atmosphere at 44 °C for *C. jejuni*). Each suspension was assayed at 0.5 h, 3 h and 24 h of incubation by making 10-fold

dilutions in Buffered Peptone Water (Oxoid Ltd., Basingstoke, UK), streaking 100 µL of each dilution on specific media and incubated as described above (adapted from Elizondo et al. [20]). The viable bacterial counts (expressed as CFU/mL) of the tested compounds for each concentration were compared with the bacterial counts obtained in the C group. The assays were repeated three times and results were expressed as the average values.

The microbial growth rate was calculated as ratio: Δ_{Conc} (CFU/mL)/Δ_{Time} (h), where Δ_{Conc} is calculated as difference among microbial concentrations at the ranges of 0.5–3 h or 3–24 h and Δ_{Time} is the related interval between the sampling times (0.5–3 h; 3–24 h).

2.3. In Vivo Trial

2.3.1. Animals

Animal handling was in accordance with Italian Government guideline (D.lgs 26/2014, protocol number 232/2016PR). One hundred one-day-old Ross 308 male chicks were provided by a local hatchery (Incubatoio Settecrociari, Forlì-Cesena, Italy), where they were vaccinated against Marek's disease, infectious bronchitis and Newcastle disease. Birds were allotted in 20 pens (5 animals per pen singularly identified by ring) and randomly assigned to one of the 5 experimental diets (4 pens each diet). The feeding groups, summarized in Table 2, were: control group (C group), fed with a basal diet containing tannins free and SN1-monoglycerides of SMCFA free ingredients (Table 3); T group, fed with the basal diet supplemented with 3.0 g/kg on DM of CT; S group, fed with the basal diet supplemented with 3.0 g/kg on DM of SN1; TS group, fed with the basal diet supplemented with 2.0 g/kg on DM of CT and 1 g/kg on DM of SN1; ST group, fed with the basal diet supplemented with 1.0 g/kg on DM of CT and 2.0 g/kg on DM of SN1. The diets were formulated according to animal requirements (NRC, 1994) with 3 periods of growth: starter (0–12 days), grower (13–21 days) and finisher (22–35 days). The dosage of CT, SN1 and of their blends was the same used in the microbiological assay. Animals were fed *ad libitum* and had free access to water for all the 35 days of the trial. Every week, the animals from each pen were individually weighted. The individual feed intake was registered weekly for each pen and calculated dividing the total amount consumed by the number of animals present in the pen (the approved protocol did not allow the use of individual pens). Feed efficiency was calculated as estimated ratio of the individual feed intake/registered individual weight gain for each group.

Table 2. Experimental design.

Group	Number of Animals Per Pen	Number of Pens	Diet
C	5	4	basal diet
T	5	4	basal det + 3 g/kg DM of chestnut tannin extract
S	5	4	basal det + 3 g/kg DM of SN1 monoglicerides
TS	5	4	basal det + 2 g/kg DM of chestnut tannin + 1 g/kg DM of SN1 monoglicerides
ST	5	4	basal det + 1 g/kg DM of chestnut tannin + 2 g/kg DM of SN1 monoglicerides

Table 3. Ingredient composition (g/kg of DM) of basal diets formulated according to growing periods.

Ingredients	Starter (0–12 d)	Grower (13–21 d)	Finisher (22–42 d)
Maize	330.0	360.0	380.0
Wheat	240.0	240.0	230.0
Soy bean meal	220.0	220.0	200.0
Animal fat	39.0	43.0	60.0
Maize gluten feed	30.0	22.0	15.0
Hydrolysed protein	33.0	10.0	10.0
Sunflower meal	50.0	50.0	50.0
Pea	10.0	10.0	10.0
Dicalcium phosphate	19.0	19.0	19.0

Table 3. *Cont.*

Ingredients	Starter (0–12 d)	Grower (13–21 d)	Finisher (22–42 d)
Calcium carbonate	15.0	12.0	12.0
Sodium bicarbonate	2.5	2.5	2.5
Sodium chloride	2.5	2.5	2.5
DL Methionine	2.5	2.5	2.5
Lysine HCl	1.5	1.5	1.5
Vitamin mineral premix	5.0	5.0	5.0

The litters of each group have been checked weekly for faeces compactness using an arbitrary but comparative score: 0, dry litter; 1, medium wet; 2, wet. At the 36th day, the animals were sacrificed at a slaughterhouse.

2.3.2. Diet Proximate Analysis

Diets were analyzed for proximate profile as follows: crude protein (CP), ether extract (EE), crude fiber (CF) and ash were determined according to the AOAC methods 976.06, 920.39, 962.09 and 942.05, respectively (AOAC 1995). Neutral detergent fiber (NDF) was determined according to van Soest et al. [23], using heat stable amylase and sodium sulphite, and expressed inclusive of residual ash. Metabolizable Energy (ME) was estimated from feed tables according to Sauvant et al. [24]. The chemical and nutritional profile of the basal diets are reported in Table 4.

Table 4. Nutritional traits of basal diets according to growing periods.

Item	Starter (0–12 d)	Grower (13–21 d)	Finisher (22–35 d)
Dry matter, g/kg	884	866	869
Crude protein, g/kg on DM	225	200	188
Ether extract, g/kg on DM	60	65	82
NDF, g/kg on DM	28	28	32
Ash, g/kg on DM	63	54	49
Calcium, g/kg on DM	8	7	6
Phosphorus, g/kg on DM	6	5	4
Lysine, g/kg on DM	13	12	11
Methionine, g/kg on DM	6	4	3
Metabolizable Energy, kcal/kg [1] on DM	2950	3010	3090

[1] Estimated from feed tables according to Sauvant et al. (2004).

2.3.3. Physical and Chemical Analysis

All carcasses were evaluated for dressing out and major traits. Breast meat from three animals of each pen was sampled for color analysis, antioxidant capacity, and oxidative status as follows.

- Color analysis. The samples of breast were poured into a clean glass petri dish to be evaluated for color using the portable spectrophotometer (Minolta CR 200 Chroma Meter4, Konica Minolta Chiyoda, Tokyo, Japan, calibrated using a standard yellow calibration tile, model CRA471). The top of the Chroma Meter measuring head was placed flat against the surface of the meat and the reflective color was determined from the average of three consecutive pulses from the optical chamber of the spectrophotometer. Data are reported in the L* a* b* color notation system [25] with L* axis representing lightness, the a* axis representing the red-green color axis (redness) and the b* axis representing the blue-yellow (yellowness) color axis. The numerical total color difference (ΔE2000) among samples was calculated by the formula proposed by Mokrzycki and Tatol [26].
- Antioxidant capacity. Meat samples (5.0 g) were extracted with ethanol as reported by Mancini et al. [27]. The filtrate was used to measure ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) reducing activity, DPPH (1,1-diphenyl-2-pircydrazyl) radical scavenging activity and ferric reducing ability (FRAP), as reported by Mancini et al. [27] and modified from Blois [28], Re et al. [29] and Descalzo et al. [30].

Results were expressed as mmol of Trolox equivalent per kg of fresh meat for ABTS and DPPH methods and as mmol of Fe^{++} equivalent per kg of fresh meat for FRAP determination.

- Oxidative status of meat. Meat samples (5.0 g) were considered for TBARS (thiobarbituric acid-reactive substances) determination. TBARS were measured to determinate malondialdehyde (MDA) levels, according to the method described by Ke et al. [31] and modified by Dal Bosco et al. [32]. Briefly, the meat samples were homogenized with a water solution of trichloroacetic acid (7.5% *w*/*v*) and diethylenetriaminepentaacetic acid (0.1% *w*/*v*). After centrifugation and filtration, the solutions were reacted with a water solution of 2-thiobarbituric acid (0.288% *w*/*v*) and heated in a water bath at 95 °C for 45 min. The absorbance of the samples was determined at 532 nm (V-530 Jasco International, Milan, Italy) and a calibration curve was plotted with TEP (1,1,3,3-tetraethoxypropane; 0–15 μM, final concentrations) to obtain the MDA concentration. Results were expressed as mg of MDA-equivalents per kg of fresh meat.

2.3.4. Statistical Analysis

Data related to bacterial counts were expressed as log10 (CFU/mL) and normalized according to Snedecor and Cochran [33]. Data related to microbial growth rate were processed as completely randomized design with repeated measures using the MIXED procedure of SAS [34]:

$$Y_{ijkl} = \mu + T_i + D_j + I_k(D) + (T \times D)_{ij} + e_{ijkl}, \quad (1)$$

where y_{ijkl} is the observation; μ is the overall mean; D_j is the fixed effect of treatment (j = 1 to 5); T_i is the fixed effect of assaying time (i = 1 to 3); I_k is the random effect of the replicate nested within the treatment (k = 1 to 3); $(T \times D)_{ij}$ is the interaction between treatment and assaying time and e_{ijkl} is the residual error. The covariance structure was compound symmetry, which was selected based on Akaike's information criterion of the mixed model of SAS [34]. The statistical significance of the treatment effect was tested against variance of bacterial cultures nested within treatment, according to repeated measures design theory [35]. Multiple comparisons among means were performed using the Tukey's test.

One-hundred animals divided in 5 groups is the minimum number of animals in order to obtain significant differences among treatments according to the power analysis based on alpha 0.05 beta 0. 08 [33]. Data related to the feed intake, weight gain, feed efficiency of each period, were processed as completely randomized design with repeated measures using the MIXED procedure of SAS [34]:

$$Y_{ijkl} = \mu + T_i + D_j + I_k(D) + (T \times D)_{ij} + e_{ijkl}, \quad (2)$$

where y_{ijkl} is the observation; μ is the overall mean; D_j is the fixed effect of treatment (i = 1 to 5); T_i is the fixed effect of assaying time (j = 1 to 5); I_k is the random effect of the replicate nested within the treatment (k = 1 to 5); $(T \times D)_{ij}$ is the interaction between treatment and assaying time and e_{ijkl} is the residual error. The covariance structure and the statistical significance were tested as described above.

The data related to feed intake, weight gain, feed efficiency of the whole period, physical and chemical parameters of meat, dressing out and the major carcass traits of slaughtered birds were analysed by one-way ANOVA, keeping the factor "diet" as the fixed one [34]:

$$y_{ij} = \mu + D_i + e_{ij}, \quad (3)$$

where y_{ij} is the observation; μ is the overall mean; D_i is the diet (i = 1 to 5) and e_{ij} is the residual error. Multiple comparisons among means were performed using the Tukey's test. Probability of significant effect due to experimental factors was fixed for $p < 0.05$.

3. Results and Discussion

3.1. Microbiological Assay

All the treatments were efficient in decreasing the bacterial growth of each species compared to the control (Figure 1). The T resulted the most effective treatment in controlling the growth of each bacterial species at 3 h and 24 h. For the other treatments, the behavior of the tested bacteria was different. *C. perfringens* and *S. typhymurium* resulted more sensitive to the TS than to the ST and S at 3 h and 24 h. At 3 h no significant differences were found for the S, TS and ST for *E. coli* but at 24 h the TS was more efficient in limiting the growth compared to the other two treatments (Figure 1C). No significant different growth was observed for *C. jejuni* with S and TS at 3 h. However, at the same sampling time, the growth with ST was higher than the growth with S and TS. At 24 h the growth of *C. jejuni* was lower with the TS than with the S and ST (Figure 1D).

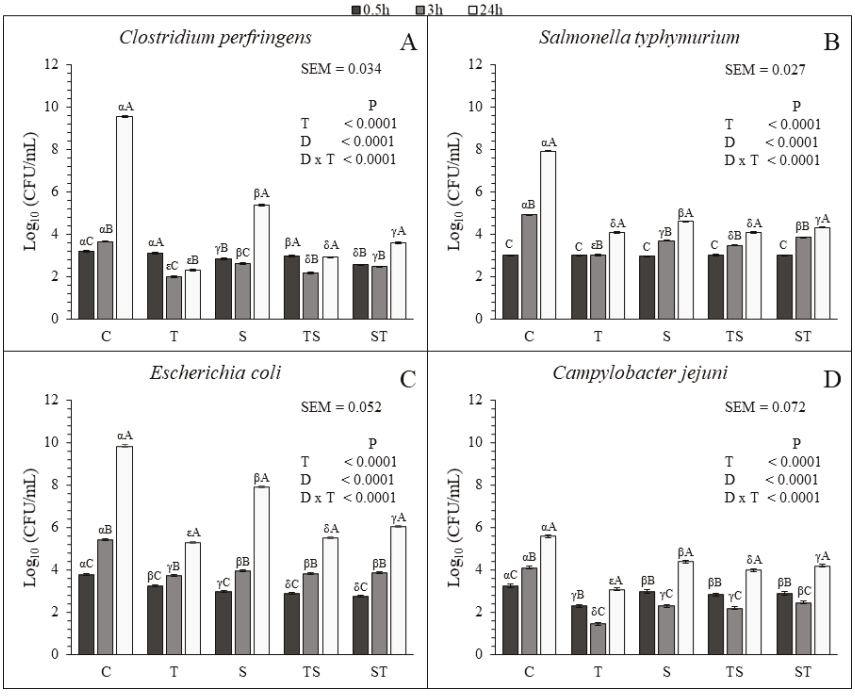

Figure 1. Results of microbial in vitro assay for *Clostridium perfringens* (**A**), *Salmonella typhymurium* (**B**), *Escherichia coli* (**C**), *Campylobacter jejuni* (**D**) (data are reported as Log10 (CFU/mL)). C, control; T, 3.0 g/kg of chestnut tannin; S, 3.0 g/kg of Sn1-monoglycerides; TS, 2.0 g/kg of chestnut tannin added to 1.0 g/kg of Sn1-monoglycerides; ST, 1.0 g/kg of chestnut tannin added to 2.0 g/kg of Sn1-monoglycerides. Concentration are expressed as *w*/*v*. SEM, Standard Error Mean. The probability of significant effect due to experimental factors is reported as: α, β, γ, δ, ε for the treatments (means with different Greek superscripts are significantly different ($p < 0.05$)); A, B, C for the sampling time (means with different Latin superscripts are significantly different ($p < 0.05$)).

All the treatments lowered the growth rate of each microbial species, except for *C. jejuni* that did not show significant decreasing with the tested compounds between 3 h and 24 h, compared to the control. Furthermore, both *C. perfringens* and *C. jejuni* decreased in all the treatments between 0.5 h and 3 h, and increased between 3 h and 24 h (Table 5 and Figure 1). This observation suggested that the bactericidal effect of CT and SN1, supplied alone or in combination, is stronger at the beginning of

the treatment. For *E. coli* and *S. typhymurium* only a bacteriostatic effect was observed in the treated cultures (Table 5 and Figure 1).

Table 5. Rate of microbial growth in the in vitro assay (data are reported as Log10 (CFU/mL)/h).

Pathogen	Tested Compounds [1]						SEM [2]	p [3]
	Δ_{time} [4]	C	T	S	TS	ST		
C. perfringens	0.5–3 h	0.186 ^{a}	−0.446 ^{d}	−0.096 ^{b}	−0.323 ^{c}	−0.032 ^{b}	0.025	<0.0001
	3–24 h	0.281 ^{a}	0.015 ^{e}	0.131 ^{b}	0.035 ^{d}	0.054 ^{c}	0.001	<0.0001
S. typhymurium	0.5–3 h	0.764 ^{a}	0.039 ^{e}	0.298 ^{c}	0.186 ^{d}	0.338 ^{b}	0.011	<0.0001
	3–24 h	0.143 ^{a}	0.022 ^{c}	0.043 ^{b}	0.029 ^{d}	0.022 ^{c}	0.001	<0.0001
E. coli	0.5–3 h	0.665 ^{a}	0.192 ^{d}	0.382 ^{b}	0.383 ^{b}	0.449 ^{b}	0.028	<0.0001
	3–24 h	0.210 ^{a}	0.074 ^{d}	0.188 ^{b}	0.080 ^{d}	0.104 ^{c}	0.003	<0.0001
C. jejuni	0.5–3 h	0.344 ^{a}	−0.336 ^{c}	−0.275 ^{cb}	−0.254 ^{cb}	−0.175 ^{b}	0.048	<0.0001
	3–24 h	0.071 ^{b}	0.077 ^{b}	0.100 ^{a}	0.086 ^{ab}	0.083 ^{b}	0.005	0.0219

[1] C, control; T, 3.0 g/kg chestnut tannin; S, 3.0 g/kg of Sn1-monoglycerides; TS, 2.0 g/kg of chestnut tannin added to 1.0 g/kg of Sn1-monoglycerides; ST, 1.0 g/kg of chestnut tannin added to 2.0 g/kg of Sn1-monoglycerides. [2] SEM, Standard Error Mean. [3] Probability of significant effect due to experimental factors; ^{a,b,c,d,e} within a row, means with different Latin letters are significantly different ($p < 0.05$). [4] Time range considered for growth rate calculation.

Our results are in accordance with Tosi et al. [2] and Redondo et al. [3] who demonstrated that the CT can inhibit the growth of *C. perfringens*. The antimicrobial activity of tannins seems to be due to their ability to bind microbial enzymes and proteins, in ion deprivation and in inhibiting the topoisomerase, fundamental for the DNA replication [36–38]. Moreover, Ramìrez et al. [6] and Timbermont et al. [10] showed that the SN1 of SMCFA were efficient in controlling the growth of *S. typhimurium* and *C. perfringens*, respectively, consistent with our study. Their antimicrobial effect was explained with their ability to penetrate through the bacterial wall, because of their affinity with lipoteichoic acid, present in microbial membrane. Their ability to destroy the inner the membrane is probably due to their compatibility with hydrophilic and hydrophobic moieties [6,39–41]. These results suggest that the CT and SN1 alone or in combination could be useful to control the proliferation of pathogenic bacteria tested in this trial. Hence, these molecules could represent a valid alternative to antibiotics both used alone or in mixture.

3.2. In Vivo Trial

No differences among the groups were found for feed intake (Table 6), both in each single growth period and in the whole period of bird life, showing that the supplementation with CT and SN1 blends did not affect the palatability of the diets. No differences were found for weight gain and feed efficiency among groups, suggesting that the blends of CT and SN1, at the inclusion level adopted in this study, did not interfere with nutrient absorption, with respect to the single supplementation or to the control diet (Table 6). Hence, no synergic effect was found when the CT and SN1 mixtures were included in the diets. The results showed a higher feed/gain ratio during the first two weeks, compared to the growing and finisher periods. Usually, young chicks have higher feed efficiency than old birds. This trend could be due to an adaptation period of birds to the rearing condition because also the control group, fed with only the basal diet, did not show significant differences with the other feeding groups. The literature reports information on the effect of CT and SN1 when they are included alone in the diets, but few data are available on the effect of blends composed by a mixture of tannin extracts and monoglycerides. The results of this trial are in accordance with Jamroz et al. [42] and Antongiovanni et al. [13,14,43] who studied respectively the effect of CT (inclusion level of 0, 250, 500 and 1000 mg/kg on DM) and of several monoglycerides (inclusion level of 200, 350, 500 mg/kg of DM) separately, as dietary supplementation on the performance and histological characteristics of the intestine wall in chickens. No impairment of the growth performance emerged, despite a slight modification on the small intestine wall, due to the introduction in the diet of chestnut tannins

and monobutyrin (CT degrades enterocytes while monobutyrin modifies positively villi, microvilli and crypts), was observed. Moreover, previous results, reported by Schiavone et al. [17], showed that the inclusion of a natural extract of chestnut wood did not affect the apparent digestibility of CP and that this supplement had a positive effect on average daily gain and feed intake in the first two weeks of addition. For monoglycerides, especially with butyric acid, the literature confirms that they can ameliorate growth performances and health in broilers [12–14]. In contrast, for the CT, several studies reported that polyphenols reduce protein digestibility in monogastrics, decreasing the productive performances in accordance with a lower availability of this nutrient for the animal nutritional requirements [5,44–46]. In particular, tannins stimulate hypersecretion of endogenous enzymes leading to losses of sulphur aminoacids in poultry species [47–49]. The inconsistence of the results reported in many papers, including those shown here, is probably due to the kind of tannin used as dietary supplement, the animal species and the dietary dose formulation. In this study, by an accurate observation, an astringent effect of tannin has been noted in T, TS and ST groups, whose litters resulted drier than the litters of S group (at 35th day: C = 1; T = 0; TS = 0; ST = 0 and S = 1).

Table 6. Live performance of birds.

Item	Diets [1]					SEM [2]	*p* [3]
	C	T	S	TS	ST		
0–14 Days							
Weight gain, g	299.85	325.90	303.15	320.60	318.70	34.69	0.7850
Feed intake, g	526.00	571.50	502.25	509.25	538.25	68.34	0.8535
Feed/gain ratio	1.82	1.77	1.66	1.65	1.72	0.08	0.9248
15–21 Days							
Weight gain, g	440.50	453.75	483.50	467.25	416.25	34.69	0.7850
Feed intake, g	498.25	555.25	558.00	537.50	585.75	68.34	0.8535
Feed/gain ratio	1.32	1.27	1.20	1.20	1.24	0.08	0.9248
22–35 Days							
Weight gain, g	1487.50	1491.50	1497.00	1418.50	1479.75	34.69	0.7850
Feed intake, g	2179.50	2169.00	2145.00	2095.00	2,307.50	68.34	0.8535
Feed/gain ratio	1.52	1.47	1.46	1.56	1.60	0.08	0.9248
Whole Period							
Weight gain, g	2227.85	2271.15	2283.65	2206.35	2214.70	48.60	0.7304
Feed intake, g	3203.75	3295.75	3205.25	3141.75	3331.50	68.34	0.8535
Feed/gain ratio	1.45	1.46	1.41	1.44	1.52	0.04	0.4105

[1] C, control; T, 3.0 g/kg chestnut tannin; S, 3.0 g/kg of Sn1-monoglycerides; TS, 2.0 g/kg of chestnut tannin added to 1.0 g/kg of Sn1-monoglycerides; ST, 1.0 g/kg of chestnut tannin added to 2.0 g/kg of Sn1-monoglycerides. [2] SEM, Standard Error Mean. [3] Probability of significant effect due to experimental factors.

No significant differences in carcass quality were found among groups (Table 7), except for the liver that was smaller in the animals fed the T and S diets than the other feeding groups. This result is consistent with the findings reported by Jamroz et al. [42] and Antongiovanni et al. [13] who noted that the supplementation with polyphenols or the monoglycerides of butyric acid did not affect carcass quality, even though monoglycerides represented an energy source for animal growth and tannins are considered to be antinutritive. Unfortunately, in literature, no information is available on blends of CT and SN1, which were not able to affect carcass traits at the tested levels in our study.

Table 7. Major carcass traits of slaughtered birds.

Carcass Trait	Diet [1]					SEM [2]	p [3]
	C	T	S	TS	ST		
Dressing out, %	86.27	84.68	84.65	85.35	86.27	0.79	0.4687
Live weight, g	2868	2624	2627	2560	2760	143	0.5996
Carcass weight, g	2800	2503	2504	2452	2659	151	0.5250
Eviscerate weight, g	2465	2223	2224	2185	2384	139	0.5919
Breast, g	581	527	504	516	615	67	0.7474
Tights, g	551	513	502	497	514	31	0.7655
Liver, g	78 [a]	65 [b]	59 [b]	72 [a]	82 [a]	2	0.0023

[1] C, control; T, 3.0 g/kg chestnut tannin; S, 3.0 g/kg of Sn1-monoglycerides; TS, 2.0 g/kg of chestnut tannin added to 1.0 g/kg of Sn1-monoglycerides; ST, 1.0 g/kg of chestnut tannin added to 2.0 g/kg of Sn1-monoglycerides. Concentration are expressed as *w*/*v*. [2] SEM, Standard Error Mean. [3] Probability of significant effect due to experimental factors; [a,b] within a row, means with different letters are significantly different ($p < 0.05$).

Changes in the a* and b* values are related to changes in meat color because these parameters are markers of browning [50]. Where the SN1 was present in the diet alone an increase of the L* and b* values occurred (Table 8). Specifically, the S group showed the highest L* and b* values and it significantly differed compared to the other groups. Despite the statistical differences, ΔE calculation for the samples of chicken breast can be useful to understand how the values of color can be perceived by human eyes (Table 9). As suggested by Mokrzycki and Tatol [26], a standard observer is able to see the difference in color as follows: $0 < \Delta E < 1$ observer does not notice the difference, $1 < \Delta E < 2$ only experienced observers can notice the difference, $2 < \Delta E < 3.5$ unexperienced observer also notices the difference, $3.5 < \Delta E < 5$ clear difference in color is noticed, $\Delta E > 5$ observer notices two different colors. Regarding the ΔE, from Table 9 it emerged that the T and TS were the most similar groups ($1 < \Delta E < 2$), while the ST assumed a $2 < \Delta E < 3.5$ when compared with C, T, and TS. Interestingly, the S group was found to be the only one with a $\Delta E > 5$ when compared to the other experimental groups, thus underlining that Sn1 monoglycerides strongly impacted meat coloration. Whether this modification could be accepted or not by consumers should be further investigated.

Table 8. Colour traits of breast meat.

Item [2]	Diet [1]					SEM [3]	p [4]
	C	T	S	TS	ST		
L*	53.01 [c]	57.32 [bc]	63.80 [a]	58.36 [b]	55.91 [bc]	2.21	0.0435
A*	5.37	3.58	5.60	4.46	6.04	1.14	0.3689
B*	3.47 [b]	3.32 [b]	8.99 [a]	3.56 [b]	2.74 [b]	1.04	0.0041

[1] C, control; T, 3.0 g/kg chestnut tannin; S, 3.0 g/kg of Sn1-monoglycerides; TS, 2.0 g/kg of chestnut tannin added to 1.0 g/kg of Sn1-monoglycerides; ST, 1.0 g/kg of chestnut tannin added to 2.0 g/kg of Sn1-monoglycerides. Concentration are expressed as *w*/*v*. [2] Data are reported in the L* a* b* color notation system with L* axis representing lightness, the a* axis representing the red-green color axis (redness) and the b* axis representing the blue-yellow (yellowness) color axis. [3] SEM, Standard Error Mean. [4] Probability of significant effect due to experimental factors; [a, b] within a row, means with different letters are significantly different ($p < 0.05$).

Table 9. Calculated ΔE2000 values for breast.

	C	T	S	TS	ST
C	-	4.5565	10.5648	5.1233	2.9569
T	4.5565	-	7.2859	1.4070	3.1987
S	10.5648	7.2859	-	6.3161	8.5273
ST	5.1233	1.4070	6.3161	-	2.9534
TS	2.9569	3.1987	8.5273	2.9534	-

C, control; T, 3.0 g/kg chestnut tannin; S, 3.0 g/kg of Sn1-monoglycerides; TS, 2.0 g/kg of chestnut tannin added to 1.0 g/kg of Sn1-monoglycerides; ST, 1.0 g/kg of chestnut tannin added to 2.0 g/kg of Sn1-monoglycerides.

Data reported in this study showed that both the tested blends of CT and SN1 could be utilized as dietary supplements without impairing animal welfare, growing performances and meat quality, thus representing valid alternatives to antibiotics in poultry rearing. Contrariwise, the S diet deeply modified the color of breast meat which could result in a modification of consumers' acceptance.

In this trial, data related to the antioxidant status of the breast meat did not show significant differences among the groups (Table 10). Several studies demonstrated the antioxidant power of tannins and of polyunsaturated fatty acids but not for SMCFA, because of the lack of double bounds on carbon chain [51–53]. Indeed, several authors reported that ellagic tannins in humans and rats are gradually metabolized by the intestinal microbiota to produce different metabolites with antioxidant effects [54–57]. Luciano et al. [53] found that the inclusion at 8.96% (DM basis) of quebracho tannins in lamb diet produced an improvement in the antioxidant status of *Longissimus dorsi* muscle, measured as both its ferric reducing ability and its radical scavenging ability. Other authors reported similar results in beef meat [30,58,59]. In contrast, Gladine et al. [60] found no effect of polyphenols in rat muscle for the radical scavenging activity. The inability of many polyphenols to be metabolized by the gastrointestinal tract of animals is strongly linked to their molecular structure and solubility, the dose of inclusion and the animal species.

Table 10. Oxidative status of meat.

Item [2]	Diet [1]					SEM [3]	p [4]
	C	T	S	TS	ST		
ABTS	0.140	1.194	1.239	0.715	0.804	0.1330	0.1127
DPPH	0.188	0.228	0.224	0.158	0.183	0.0157	0.0983
FRAP	0.264	0.280	0.282	0.261	0.246	0.0141	0.4160
TBARS	0.134	0.134	0.130	0.137	0.138	0.0017	0.1469

[1] C, control; T, 3.0 g/kg chestnut tannin; S, 3.0 g/kg of Sn1-monoglycerides; TS, 2.0 g/kg of chestnut tannin added to 1.0 g/kg of Sn1-monoglycerides; ST, 1.0 g/kg of chestnut tannin added to 2.0 g/kg of Sn1-monoglycerides. [2] ABTS and DPPH are expressed as mmol of Trolox equivalent per kg of meat; FRAP as mmol of Fe^{++} equivalent per kg of meat; TBARS as mg of MDA-Eq per kg of meat. [3] SEM, Standard Error Mean. [4] Probability of significant effect due to experimental factors; a, b within a row, means with different letters are significantly different ($p < 0.05$).

Nowadays, in conventional and intensive poultry production, antibiotics are used to control diseases and to prevent the mortality of birds, responsible for a huge economical loss. This approach conflicts with the sustainability of animal productions because several issues about the development of bacterial resistance, dejection disposal, food safety and human health occur. Therefore, the poultry industry needs alternatives able to replace antibiotics with natural or synthetic compounds able to simulate natural molecules. Chestnut tannins are a by-product of wood industry because they are obtained by distillation of wood used in the building industry. SN1 is obtained by recycling glycerol derived from biodiesel production. Hence, CT and SN1 are part of the concept of bio-economy. Moreover, FAO reported that livestock support the livelihoods and food supply of almost 1.3 billion people, being one of the fastest growing areas of the agricultural economy in the world. In developing countries, poultry production plays an important role in food, and pathogen proliferation represents an important public health problem that cannot be underestimated. At the same time, environmental sustainability must be ensured [61]. Data reported in this study showed that CT, SN1 or their blends could represent a valid alternative to antibiotics in poultry rearing. Although literature shows several studies in which tannins exert antinutritional effects in monogastric, in the present in vivo trial, no detrimental effects were observed on animal welfare, performance or meat quality. However, it is well known that the kind of polyphenols and the dietary inclusion level are fundamental to explain the biological and nutritional effects of dietary tannins. Besides, the literature shows unequivocal positive effects of SN1 monoglycerides in protecting gut from pathogens, by providing energy to enterocytes and by favoring the development of gut villi [12,13,16]. Hence, the blends of CT and SN1

could represent a good compromise among antimicrobial activities, animal gut protection, meat quality and production sustainability.

4. Conclusions

The in vitro study suggested that blends of CT and SN1 could be efficient against the proliferation of *C. perfringens*, *S. typhymurium*, *E. coli* and *C. jejuni*. Additionally, the in vivo trial suggested that the mixture of these supplements did not have negative effects on animal productive performances, representing a promising alternative to antibiotics. Considering all the recorded information about in vitro antimicrobial effectiveness and the broilers growing performances, meat color and the overall antioxidant capacity of meat, both the TS and ST tested blends might be good alternatives to antibiotics in the poultry sector. However, further investigation is needed to better understand the effects of these supplements on animals in stress conditions.

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Effect of Bamboo Leaf Extract on Antioxidant Status and Cholesterol Metabolism in Broiler Chickens

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Simple Summary: Cholesterol is an important lipid substance in organisms. As the precursor of bile acid, steroid hormones and vitamin D3, cholesterol plays important roles in lipid metabolism. Chicken is among the most consumed meat products worldwide; however, its cholesterol level is higher than that of other meat products. High cholesterol in a human diet will increase the risk of atherosclerosis. In addition, low-density lipoprotein cholesterol is susceptible to be oxidized, which will cause the death of broilers. Therefore, it is of great significance to enhance the antioxidant capacity and improve cholesterol metabolism in broiler chickens. Bamboo leaf extract (BLE) contains active ingredients such as flavonoids, polyphenols, and active polysaccharides, which possess anti-inflammatory, antioxidant and lipid-lowering effects. Our results show that supplementation of BLE in the basal diet improved growth and slaughter performance, antioxidant status and cholesterol metabolism in broilers. Therefore, the application of BLE as a feed additive has a certain economic value.

Abstract: The objective of this study was to investigate the effects of dietary bamboo leaf extract (BLE) on antioxidant status and cholesterol metabolism in broilers. One-day-old male Arbor Acres (576) broilers were randomly divided into six groups. A control group was fed a basal diet, while five experimental groups were supplemented with 1.0, 2.0, 3.0, 4.0, and 5.0g BLE per kg feed in their basal diets. The result indicated that BLE supplementation linearly improved eviscerated yield and decreased abdominal fat ($p < 0.05$). A significant decrease of serum triglyceride (TG) and low-density lipoprotein cholesterol (LDL-c) content was observed with BLE supplementation ($p < 0.05$). BLE supplementation linearly improved the total antioxidant capacity and catalase activity in both serum and liver ($p < 0.05$). Glutathione peroxidase was quadratically increased in serum and linearly increased in the liver with BLE supplementation ($p < 0.05$). The malonaldehyde content in liver showed a linear and quadratic decrease with BLE supplementation ($p < 0.05$). BLE supplementation up-regulated the mRNA expression of cholesterol 7- alpha hydroxylase and low-density lipoprotein receptor and downregulated 3-hydroxy3-methyl glutamates coenzyme A reductase mRNA expression in the liver. The antioxidant enzyme mRNA expressions were all up-regulated by BLE supplementation in the liver. In conclusion, supplemental BLE improved antioxidant status and cholesterol metabolism in broilers, which eventually led to a decrease of serum TG, LDL-c content, and abdominal fat deposition.

Keywords: antioxidant status; bamboo leaf extract; broiler; cholesterol metabolism

1. Introduction

In the past few decades, the proportion of animal products in the human diet has increased considerably, and the high cholesterol content in animal products has attracted great interest of researchers [1,2]. If the human diet contains high concentrations of cholesterol from the animal products, the blood cholesterol concentration will raise, and the risk of hypercholesterolemia will increase [3,4]. As a result, atherosclerosis and coronary heart disease will happen because hypercholesterolemia is one of the major factors leading to these diseases [4]. As reported previously, every 1% reduction in serum cholesterol could reduce the incidence of coronary heart disease by 2% [5]. However, chicken products are widely consumed worldwide and are also the most cholesterol-containing meat [6]. Therefore, reducing the cholesterol content in broilers is of great importance for human health. In addition, there are many oxidative stresses in poultry production, such as heat stress, immune stress and transport stress; all these stresses will increase oxidizing substances in broilers [7]. If there is a large amount of low-density lipoprotein cholesterol (LDL-c) in chicken serum, LDL-c will react with oxidizing substance, and the oxidized LDL-c will be produced [8]. While the oxidized LDL-c is highly poisonous to the cells, it will cause damage to endothelial cells and accelerate the platelet adhesion and aggregation, release growth factors, cause hyperplasia of fibroblasts and organization, and eventually speed up the development of atherosclerosis [9,10]. As a result, it will cause sudden death of broilers and lead to economic losses. For all these reasons, it becomes a hot research topic and solutions need to be further explored for enhancing antioxidant status and improving cholesterol metabolism in poultry production.

Bamboo is widely distributed around the world, and its leaves have been used for medicinal and culinary purposes in China. It is reported that bamboo leaves contain active ingredients such as flavonoids, polyphenols, and active polysaccharides [11]. Research showed that bamboo leaf extract (BLE) has multiple biological effects, especially on cardiovascular and cerebrovascular protection. As mentioned before, BLE was able to reduce the cholesterol concentration of hyperlipidemia mice and improve liver function [12]. In addition to the effect of lowering serum cholesterol, BLE is capable to increase antioxidant capacity in hyperlipidemia mice [13]. Meanwhile, the research of Sunga et al. has shown that BLE reduced the adhesion of vascular epithelial factors, regulated endothelial cells to increase vascular mobility, and reduced the risk of atherosclerosis [14]. BLE has super antioxidant ability for scavenging free radicals in vitro [15,16], and improves antioxidant enzyme activity in vivo [17]. BLE is confirmed as an ideal choice for the body to supplement exogenous antioxidants and prevent hyperlipidemia, and some Chinese functional food or medicine made with BLE, like bamboo rice, bamboo beer, bamboo toothpaste, is very popular among Chinese consumers. The existing research about BLE in poultry production mainly focuses on the growth performance, meat quality and immune organ index [18]; little research has been conducted on the antioxidant effect. Furthermore, although multiple studies showed that flavonoids are able to affect fat metabolism in animals, and BLE holds the function to improve lipid metabolism in rat or mice, the effect of BLE on cholesterol metabolism in broiler chickens is still not clear. BLE supplementation in the basal diet could enhance antioxidant status and improve cholesterol metabolism in broiler chickens, which will be beneficial for poultry production and the human diet. Therefore, this study was conducted to investigate the effect of BLE on antioxidant status and cholesterol metabolism in broiler chickens.

2. Materials and Methods

2.1. Ethical Statement

Animal feeding experiments were carried out at Jiangpu farm of Zhujiang campus of Nanjing Agricultural University, and experimental analyses were conducted at the College of Animal Science and Technology of Weigang campus of Nanjing Agricultural University. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University

(GB14925, NJAU-CAST-2011-093), and the serial number of the laboratory animal use certificate issued by Science and Technology department of Jiangsu province is SYXK (Su) 2017–0007.

2.2. Animals, Diets and Experimental Design

Bamboo leaf extract (BLE) was obtained from Zhejiang XinHuang Biotechnology Co., Ltd. (Zhejiang, China), and its main components include flavonoids, polyphenols (the bamboo leaf flavonoid concentration is 70 mg per gram of BLE, and the polyphenol concentration is 50.42 mg per gram of BLE). A total of 576 one-day-old male Arbor Acres broiler chicks were obtained from a local commercial hatchery (Hewei Company, Anhui Province, China) and were randomly allotted into 6 groups with 6 replicates containing 16 birds each. Basal diets were designed for the starter phase (1–21 d) and growth phase (22–42 d) (Table 1). Chickens were supplied according to NRC (1994) recommendations for nutrition requirements. The control group (CON) was fed with a basal diet, while five experimental groups BLE1, BLE2, BLE3, BLE4, and BLE5 were fed the basal diet supplemented with 1.0, 2.0, 3.0, 4.0 and 5.0 g BLE per kg feed for 42 days. All birds were kept in three-layer pens; each replicate was divided into two pens. Temperature was maintained at 32–35 °C for the first five days, then gradually decreased to 22 °C and kept stable until the end of the experiment. During the trial period, birds had free access to feed and water.

Table 1. Composition and nutrient level of basal diets.

Item	Starter Phase (1–21 d)	Growth Phase (22–42 d)
Ingredient (%)		
Corn	57.02	61.36
Soybean	31.3	28.3
Corn gluten meal	3.7	1.7
Soya oil	3	4
Dicalcium phosphate	2	1.6
Limestone	1.2	1.3
L-Lysine	0.33	0.31
DL-Methionine	0.15	0.13
Sodium chloride	0.3	0.3
Premix [1]	1	1
Nutrient levels [2]		
ME (MJ/kg)	12.57	12.91
CP (%)	21.42	19.23
Lys (%)	1.20	1.10
Met (%)	0.50	0.44
Calcium (%)	1	0.93
Available Phosphorus (%)	0.46	0.39

[1] Premix provided per kilogram of diet: VA 10 000 IU, VD_3 3 000 IU, VE 30 IU, VK_3 1.3 mg, thiamine 2.2 mg, riboflavin 8 mg, niacin 40 mg, choline chloride 600 mg, calcium pantothenate 10 mg, pyridoxine 4 mg, biotin 0.04 mg, folic acid 1 mg, VB_{12} 0.013 mg, zinc 65 mg, iron 80 mg, copper 8 mg, manganese 110 mg, iodine 1.1 mg, selenium 0.3 mg; [2] Calculated value.

2.3. Slaughter Performance

Two birds from each replicate were weighted before slaughter and after sufficient exsanguination at 42 d. Then, the feathers, head, feet, abdominal fat and viscera were removed from the bird (except for kidney) and the carcass was reweighed. In addition, liver and abdominal fat weight were recorded separately. The right–side breast and thigh meat were weighed after removing skin and bone. The percentage weight of eviscerated yield, breast, thigh, abdominal fat and liver, compared with the live-weight was used to evaluate slaughter performance.

2.4. Sample Collection

At the end of the experiment, two birds (near the average body weight of each replicate) from each replicate were selected, and non-anticoagulant sterile blood vessels were used to collect blood samples from the jugular vein. The blood was left for 2 hours at 4 °C, then centrifuged at 3500 rpm for 10 min, and the supernatant was stored at −20 °C for analysis. A sample was cut from liver tissue from the middle of the left lobule in anatomical location and stored at −80 °C for antioxidant enzyme and mRNA expression analysis.

2.5. Cholesterol Metabolism Parameter Analysis

The total cholesterol (TC, kit number: A111-1-1), total triglyceride (TG, kit number: A110-1-1), high density lipoprotein cholesterol (HDL-c, kit number: A112-1-1), low density lipoprotein cholesterol (LDL-c, kit number: A113-1-1) and blood glucose (GLU, kit number: F006-1-1) in serum were measured by different commercial kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China).

2.6. Serum and Liver Homogenate Antioxidant Enzyme Analysis

One gram of liver tissue from a sample preserved at -80°C was homogenized with 4.5 mL of 0.9% sodium chloride buffer with tube embed in ice by using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH, USA), and the homogenates were centrifuged at 3500 rpm for 10 min. The supernatant was used to measure superoxide dismutase (SOD, kit number: A001-1-1), glutathione peroxidase (GSH-Px, kit number: A005-1-1), catalase (CAT, kit number: A007-1-1), total antioxidant capacity (T-AOC, kit number: A015-1-1) activities, and Malondialdehyde (MDA, kit number: A003-1-1) content by different commercial kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China) according to its instruction.

2.7. RNA Extraction and Quantitative Real-Time PCR

Trizol Reagent (Vazyme, NanJing, China) was used to extract total RNA from liver tissue, which was then treated by deoxyribonuclease I to remove the contaminant DNA. RNA was quantified based on the absorption of light by a Nanodrop ND-2000c spectrophotometer (Thermo Scientific, Camden, UK) at 260 nm (A260) and 280 nm. From each sample, 1 μg of RNA was used to synthesize cDNA in a 20 μL reaction mixture using the Primer-ScriptTM reagent kit (TaKaRa, Dalian, China) according to the manufacturers' instructions. The real-time quantitative polymerase chain reaction was carried out by using the SYBR Premix Ex Taq II kit (TaKaRa) in an ABI 7300 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). The 20 μL reaction system included 10 μL of SYBR Premix Ex Taq buffer, 0.4 μL each of forward and reverse primers and dye, 2 μL of cDNA template, and 6.8 μL of distilled water. The real-time PCR cycling conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The relative mRNA expression was determined using β-actin as an internal reference gene. The significance and correlation of quantitative results were analyzed by using 2-ΔΔct as per Livak and Schmittgen [19]. Primer sequences are shown in Table 2.

Table 2. Primer sequences used for Real-time PCR.

Gene Name [1]		Primers Sequence (5′–3′)	Gene Bank Number
β-Actin	Forward	TGCTGTGTTCCCATCTATCG	NM_205518.1
	Reverse	TTGGTGACAATACCGTGTTCA	
CYP7A1	Forward	CACCATGGATCTGGGGAACA	NM_001001753.1
	Reverse	AGGCACATCCCAGGTATGGA	
LDLR	Forward	CTTCTGGTCTGACTGCGGTT	NM_204452.1
	Reverse	CAGAACACGGAGTCCTCGAA	
HMGCR	Forward	TTCTCGGCCGGGCGATTT	NM_204485.2
	Reverse	GGCACTCATAGTTCCAGCCAC	
SREBP-2	Forward	GTTCCTGGAGGTGTCAAGCA	AJ414379.1
	Reverse	CAGACTTGTGCATCTTGGCG	
SOD	Forward	CCGGCTTGTCTGATGGAGAT	NM_205064.1
	Reverse	TGCATCTTTTGGTCCACCGT	
CAT	Forward	GGTTCGGTGGGGTTGTCTTT	NM_001031215.2
	Reverse	CACCAGTGGTCAAGGCATCT	
GSH-Px	Forward	GACCAACCCGCAGTACATCA	NM_001277853.2
	Reverse	GAGGTGCGGGCTTTCCTTTA	

[1] CYP7A1: cholesterol 7- alpha hydroxylase; LDLR: low-density lipoprotein receptor; HMGCR: 3-hydroxy 3-methyl glutamates coenzyme A reductase; SREBP-2: sterol regulatory element binding transcription factor 2; SOD: Superoxide dismutase; CAT: Catalase; GSH-Px: Glutathione peroxidase.

2.8. Statistical Analysis

All data were preliminarily processed by using Excel 2016 and analyzed through one-way analysis of variance (ANOVA) using SPSS statistical software (Ver. 20.0 for Windows, SPSS, Inc., Chicago, IL, USA). The data were analyzed as a completely randomized design with a replicate as an experimental unit. Duncan's multiple range test was performed to determine differences between treatments. The effect of BLE supplementation at various levels was evaluated using an orthogonal polynomial contrast test for linear and quadratic effects. Differences were regarded as significant at $p < 0.05$.

3. Results

3.1. Growth Performance

In the starter phase, compared to the CON group, average daily feed intake was significantly higher in the BLE2 and BLE5 groups ($p < 0.05$), and average body weight in the BLE5 group increased significantly ($p < 0.05$). In the growth phase, compared with the CON group, average daily feed intake and average daily gain in the BLE2 and BLE5 groups were significantly higher than that of the CON group ($p < 0.05$). However, the BLE1 and BLE2 groups showed a significant decrease in feed: gain ratio ($p < 0.05$). Average daily gain and feed: gain ratio showed a quadratic improvement with increasing BLE dosage, and there was a linear and quadratic enhancement on average body weight when the BLE levels increased. During the whole rearing period, average daily gain and feed: gain ratio improved significantly in the BLE2 group over the CON group ($p < 0.05$).

3.2. Slaughter Performance

As shown in Table 3, compared with the CON group, the percentage of eviscerated yield in BLE supplementation groups was significantly ($p < 0.05$) increased (except for birds in BLE3 group), the percentage of abdominal fat was decreased significantly in BLE supplementation groups ($p < 0.05$). In addition, the percentage of eviscerated yield showed a linear improvement ($p = 0.007$), and abdominal fat percentage showed a linear ($p = 0.027$) decrease with increasing level of BLE supplementation. Moreover, there was a quadratic decrease in the percentage of liver weight as BLE supplementation increased. No difference was observed in breast and thigh meat percentage among BLE groups ($p > 0.05$).

Table 3. Effect of dietary BLE on slaughter performance of broilers.

Item: Percentage of (%)	Diet Treatment [3]						SEM [1]	*p* Value	
	CON	BLE1	BLE2	BLE3	BLE4	BLE5		Linear [2]	Quadratic [2]
Eviscerated yield	75.368 [c]	78.233 [a b]	77.862 [a b]	76.665 [b c]	77.618 [a b]	78.475 [a]	0.247	0.007	0.341
Breast meat	20.213	22.232	22.344	23.428	22.631	23.380	0.375	0.608	0.229
Thigh meat	18.763	18.154	18.119	18.206	18.332	18.088	0.208	0.533	0.610
Abdominal fat	1.278 [a]	0.847 [b]	0.900 [b]	0.963 [b]	0.902 [b]	0.869 [b]	0.042	0.027	0.085
Liver weight	2.536 [a]	2.163 [b]	2.113 [b]	2.103 [b]	2.145 [b]	2.296 [a b]	0.047	0.178	0.005

Note: [a.b.c] means within the same row with no common superscript differ significantly ($p < 0.05$); [1] standard error of the means; [2] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of BLE treatment; [3] CON: basal diet, BLE1, BLE2, BLE3, BLE4 and BLE 5 group, basal diet adding 1.0, 2.0, 3.0, 4.0 and 5.0g/kg BLE, respectively.

3.3. Serum Cholesterol Metabolism Parameters

Compared with the CON group (Table 4), the serum content of TG in BLE2, BLE3 and BLE4 groups were significantly decreased ($p < 0.05$), except for BLE1, the LDL-c content in serum was significantly decreased with increasing levels of BLE supplementation ($p < 0.05$). In addition, there was a quadratic ($p = 0.002$) decrease in the TG content and a linear ($p < 0.001$) decrease in the LDL-c content with the increasing inclusion of BLE in the diet. No significant difference was observed in TC, HDL-C and GLU contents among groups ($p > 0.05$).

Table 4. Effect of dietary BLE on serum cholesterol metabolism parameters of broilers.

Item	Diet Treatment [3]						SEM [1]	*p* Value	
	CON	BLE1	BLE2	BLE3	BLE4	BLE5		Linear [2]	Quadratic [2]
TC (mmol/L)	4.478	4.417	4.104	4.244	4.293	4.287	0.226	0.367	0.235
TG (mmol/L)	0.404 [a]	0.369 [a b]	0.305 [b]	0.327 [b]	0.322 [b]	0.373 [a b]	0.010	0.148	0.002
HDL-c (mmol/L)	1.617	1.786	1.841	1.839	1.681	1.791	0.030	0.355	0.083
LDL-c (mmol/L)	2.958 [a]	2.917 [a]	2.488 [b]	2.356 [b]	2.401 [b]	1.843 [c]	0.068	<0.001	0.565
GLU (mmol/L)	12.523	13.080	12.444	12.358	11.931	12.713	0.124	0.299	0.473

Note: [a.b.c] means within the same row with no common superscript differ significantly ($p < 0.05$). TC: total cholesterol; TG: triglyceride; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; GLU: glucose; [1] standard error of the means; [2] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of BLE treatment; [3] CON: basal diet, BLE1, BLE2, BLE3, BLE4 and BLE 5 group, basal diet adding 1.0, 2.0, 3.0, 4.0 and 5.0g/kg BLE, respectively.

3.4. Antioxidant Index of Serum

Birds in BLE4 and BLE5 groups showed higher T-AOC activity in serum (Table 5) than other groups ($p < 0.05$). The CAT activity in serum of BLE2, BLE3 and BLE5 groups was significantly higher than that in the CON group ($p < 0.05$). Supplementation with BLE significantly increased GSH-Px activity in the serum of broilers ($p < 0.05$). In addition, BLE linearly increased T-AOC and CAT activity ($p < 0.001$, and $p = 0.003$), and quadratically increased GSH-Px activity in the serum as the addition level increased ($p < 0.001$), and GSH-Px activity in BLE2 and BLE3 groups was significantly higher than that in other BLE supplementation groups ($p < 0.05$). SOD activity and MDA concentration were not affected by BLE supplementation ($p > 0.05$). Birds in the BLE2 group showed a numerical minimum serum MDA concentration among groups.

Table 5. Effect of dietary BLE on serum antioxidant index of broilers.

Item	Diet Treatment [3]						SEM [1]	*p* Value	
	CON	BLE1	BLE2	BLE3	BLE4	BLE5		Linear [2]	Quadratic [2]
T-AOC (U/ml)	5.848 [c]	5.946 [c]	6.804 [b c]	6.778 [b c]	8.099 [a]	7.688 [a b]	0.162	<0.001	0.818
CAT(U/ml)	5.405 [b]	6.504 [a b]	7.437 [a]	7.919 [a]	6.730 [a b]	8.551 [a]	0.288	0.003	0.302
SOD(U/ml)	162.928	167.121	163.238	165.775	168.467	164.067	1.027	0.565	0.476
GSH-Px(U/ml)	315.353 [c]	350.001 [b]	390.35 [a]	383.333 [a]	348.684 [b]	345.175 [b]	4.882	0.097	<0.001
MDA (nmol/ml)	3.098	3.013	2.842	3.077	3.184	3.034	0.056	0.713	0.535

Note: [a.b.c] means within the same row with no common superscript differ significantly ($p < 0.05$). T-AOC: total antioxidant capacity; CAT: catalase; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; MDA: malondialdehyde; [1] standard error of the means; [2] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of BLE treatment; [3] CON: basal diet, BLE1, BLE2, BLE3, BLE4 and BLE 5 group, basal diet adding 1.0, 2.0, 3.0, 4.0 and 5.0g/kg BLE, respectively.

3.5. Antioxidant Index of Liver

The effect of dietary BLE on the liver antioxidant index is shown in Table 6. Except for SOD in BLE4 group, the T-AOC and SOD activities in BLE supplementation groups were significantly higher than in the CON group ($p < 0.05$). Compared with the CON group, the CAT activity in BLE4 and BLE5 groups, and GSH-Px in the BLE5 group significantly improved ($p < 0.05$). In addition, linear ($p = 0.028$, and $p < 0.001$) and quadratic ($p = 0.009$, and $p = 0.007$) increasing relationships between BLE level and T-AOC and CAT activities were observed, and there was a linear ($p = 0.010$, and $p = 0.011$) increase in SOD and GSH-Px activities as BLE supplementation increased, and SOD activity in BLE2 and BLE5 groups was significantly higher than in the BLE4 group ($p < 0.05$). Except for the BLE1 group, the MDA concentration was significantly decreased by BLE supplementation ($p < 0.05$); a linear ($p = 0.014$) and quadratic ($p = 0.018$) decrease effect was presented with increasing BLE. Moreover, a numerical minimum MDA concentration of liver was observed in the BLE2 group.

Table 6. Effect of dietary BLE on liver antioxidant index of broilers.

Item	Diet Treatment [3]						SEM [1]	*p* Value	
	CON	BLE1	BLE2	BLE3	BLE4	BLE5		Linear [2]	Quadratic [2]
T-AOC (U/mg prot)	2.099 [b]	2.479 [a]	2.753 [a]	2.527 [a]	2.565 [a]	2.545 [a]	0.053	0.028	0.009
CAT (U/mg prot)	11.209 [c d]	12.164 [b c]	11.129 [d]	12.089 [b c d]	12.473 [b]	13.976 [a]	0.171	<0.001	0.007
SOD (U/mg prot)	516.916 [c]	556.453 [a b]	577.177 [a]	555.953 [a b]	537.516 [b c]	583.752 [a]	5.293	0.010	0.250
GSH-Px (U/mg prot)	63.602 [b]	66.128 [a b]	66.280 [a b]	70.900 [a b]	70.038 [a b]	72.897 [a]	1.190	0.011	0.928
MDA (nmol/mg prot)	1.733 [a]	1.468 [a b]	1.082 [b]	1.214 [b]	1.210 [b]	1.266 [b]	0.061	0.014	0.018

Note: [a.b.c.d] means within the same row with no common superscript differ significantly ($p < 0.05$). T-AOC: total antioxidant capacity; CAT: catalase; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; MDA: malondialdehyde; [1] standard error of the means; [2] Orthogonal polynomials were used to evaluate linear and quadratic responses to the levels of BLE treatment; [3] CON: basal diet, BLE1, BLE2, BLE3, BLE4 and BLE 5 group, basal diet adding 1.0, 2.0, 3.0, 4.0 and 5.0g/kg BLE, respectively.

3.6. Antioxidant Enzyme Gene Expression in the Liver

As shown in Figure 1, the SOD, GSH-Px and CAT mRNA expressions were all up-regulated with BLE supplementation as compared to the CON group ($p < 0.05$). In addition, the GSH-Px mRNA expression in BLE5 was significantly higher than that in BLE1 and BLE2 groups ($p < 0.05$), and CAT mRNA expression in BLE3, BLE4 and BLE5 groups was significantly higher than that in BLE1 and BLE2 groups ($p < 0.05$).

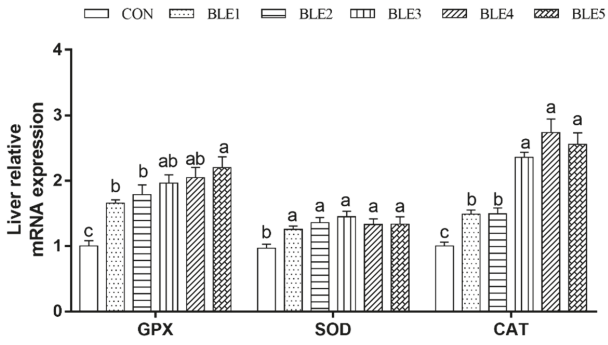

Figure 1. Effects of dietary BLE on antioxidant enzymes mRNA expression in liver of broilers. GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; CAT: Catalase. Note: $^{a.b.c}$ means within the same gene of the histogram with no common superscript differ significantly (<0.05); CON: basal diet; BLE1, BLE2, BLE3, BLE4 and BLE 5 group, basal diet adding 1.0, 2.0, 3.0, 4.0 and 5.0g/kg BLE, respectively.

3.7. Cholesterol Metabolism Related Gene Expression of Liver

Compared with the CON group, the cholesterol 7- alpha hydroxylase (CYP7A1) and low-density lipoprotein receptor (LDLR) mRNA expressions were significantly up-regulated by BLE supplementation ($p < 0.05$), and the CYP7A1 mRNA expression in BLE3 was significantly higher than in other BLE supplementation groups ($p < 0.05$). The highest LDLR mRNA expression was observed in the BLE4 group, and BLE2, BLE3 and BLE5 groups also showed favorable mRNA expression of LDLR compared with the BLE1 group ($p < 0.05$). In addition, the 3-hydroxy3-methyl glutamate coenzyme A reductase (HMGCR) mRNA expression was downregulated significantly except in the BLE1 group (Figure 2). Also, HMGCR mRNA expression in the BLE5 group was significantly lower than that in BLE2 and BLE3 groups ($p < 0.05$).

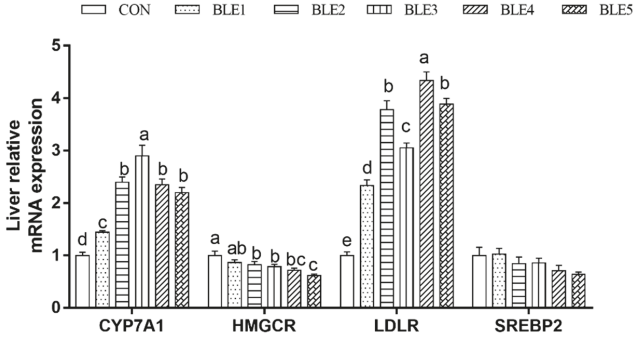

Figure 2. Effects of dietary BLE on cholesterol metabolism-related gene expression in liver of broilers. CYP7A1: cholesterol 7- alpha hydroxylase; HMGCR: 3-hydroxy3-methyl glutamates coenzyme A reductase; LDLR: low-density lipoprotein receptor; SREBP-2: sterol regulatory element binding transcription factor 2. Note: $^{a.b.c.d}$ means within the same gene of the histogram with no common superscript differ significantly ($p < 0.05$); CON: basal diet; BLE1, BLE2, BLE3, BLE4 and BLE 5 group, basal diet adding 1.0, 2.0, 3.0, 4.0 and 5.0g/kg BLE, respectively.

4. Discussion

Muscle and fat are the main traits of carcass yield, while excessive fat deposition is a problem in the current poultry industry. It will not only affect broiler processing and feed conversion but also decrease carcass quality and the acceptance of consumers [20]. The present study indicated that BLE

supplementation linearly improved eviscerated yield. Yang [21] reported that diet supplemented with 1.6 g bamboo leaf flavonoids per kg feed could improve carcass yield in broilers. As reported in the literature, flavonoids have a mild estrogen-like effect [22], which may contribute to muscle deposition of broilers. Bamboo leaf flavonoids are the major active components of BLE. It is reasonable to suggest that the linear improvement of eviscerated yield was attributed to increasing concentration of BLE. In addition, abdominal fat deposition was linearly decreased with BLE inclusion in the present study. It is well established that flavonoids regulate fat deposition and metabolism in animals. Li [23] reported that hawthorn leaf flavonoids reduced fat deposition in broilers in a dose-dependent manner. Most phytogenic flavonoids have a similar structure and functions [24]. Furthermore, Yang [25] demonstrated that BLE is confirmed to possess adipocyte differentiation properties. According to the present results, it is suggested that the flavonoids in BLE may contribute to reduce abdominal fat deposition. Although flavonoids in BLE play a major role in improving growth and fat metabolism, the lipid-lowering effect of polysaccharide in BLE cannot be ignored as it is reported that bamboo leaf polysaccharide could significantly decrease the liver fat content in mice [5], and the quadratic decline percentage of liver weight rate may result from the decrease of liver fat content in the present study.

Oxidative stress is a key factor leading to cardiovascular diseases, such as atherosclerosis, hyperlipidemia, inflammation and other chronic diseases [26]. When the redox state of the body is out of balance, the accumulation of reactive oxygen species will cause damage to vascular endothelial cells. On the other hand, the elevated TC and LDL-c in serum are the arch-criminal cause of cardiovascular diseases [27]. Thus, there are important links between antioxidant status, cholesterol metabolism and cardiovascular disease.

The antioxidant parameters in liver and serum indicate the antioxidant capacity of the organisms. Antioxidant enzymes like SOD, CAT and GSH-Px cooperate to eliminate excess free radicals and maintain homeostasis. The T-AOC refers to total antioxidant capacity, while MDA is one of the lipid peroxidation metabolites, reflecting the degree of oxidative stress. It is worth mentioning that CAT and GSH-Px activities in both serum and liver presented a dose-dependent enhancement as BLE increased. Zhang [28] reported that BLE could enhance GSH-Px enzyme activity in serum of aged rats. In addition, Zhang [29] demonstrated that liver-injured mice supplemented with bamboo leaf flavonoids showed improved GSH-Px enzyme activity and alleviated liver injury. As reported in the literature, the antioxidant effects of flavonoids in living systems are ascribed to their capacity to transfer electron free radicals, chelate metals catalysts, activate antioxidant enzymes, and inhibit oxidases [30]. It is reasonable to suspect that the flavonoids in BLE play a major part in improving antioxidant enzyme activities. However, the GSH-Px enzyme of serum in BLE2 and BLE3 groups was significantly higher than in other BLE groups. According to the research, BLE has peroxide scavenging capacity [31,32], so we speculated that with the increasing inclusion of BLE in the diet, the antioxidant capacity was mainly attributed to BLE itself. Our results showed that the MDA in liver tissue was linearly and quadratically decreased as BLE increased. Flavonoids and polyphenols play a role in inhibiting the formation of thiobarbituric acid-reactive substances [33]. In addition, studies showed that broilers supplemented with plant extracts rich in flavonoids, polyphenols and polysaccharide, such as *Ginkgo biloba* leave extract [34], and *Artemisia annua* extract [35], could enhance free radical scavenging capacity. In some animal models, like hyperlipemia [36], gastric mucosal damage [37] and myocardial ischemia reperfusion [38] rats, when supplemented with BLE remedy, the oxidative damage caused by these interventions was alleviated, accompanied by a low MDA concentration in serum or liver. According to our present results, it is suggested that BLE supplementation in broiler chickens improved antioxidant capacity and alleviate oxidative stress. Elisabeth [39] explained that a variety of electrophilic compounds including polyphenol and plant-derived constituents trigger the nuclear factor erythroid 2-related factor 2 pathway response. The SOD, CAT and GSH-Px are downstream genes of this pathway. Our results showed that mRNA expression in the liver was significantly up-regulated. It is reported that BLE can activate hepatic phase II enzymes [40] or the AKT pathway [41] to improve inflammation and oxidative stress, all related to antioxidant effects.

With the strictly demonstrated antioxidant effect of BLE in vitro studies [31,42], it is well-founded to speculate that BLE could both increase antioxidant enzymes and decrease chain breaking in broiler chickens, and these effects explain the linear improvement of T-AOC as BLE increased.

Cholesterol homeostasis is very important for broilers, as its metabolism dysfunction will lead to atherosclerosis, bile duct blockage or gallstones and other diseases [43,44]. The TC, TG, HDL-c, and LDL-c contents in serum are important indicators of cholesterol metabolism. In the present study, the TC and HDL-c concentrations in plasma were not affected by BLE supplementation. As reported in the literature, HDL-c is mainly secreted by the liver and small intestine, which plays a major role in transporting cholesterol and maintains a relative stable concentration itself [45]. Ding [12] and Liu [36] also found that BLE did not affect HDL-c concentration in hyperlipemia rats. These findings are similar to the present results. In addition, we speculated that the non-affected TC concentration with BLE supplementation in the present study may be attributed to the high content of TC in broilers [46]. The TG content in serum influences the accumulation of fat deposition. Our results present a quadratic decrease in TG and LDL-c contents in serum with BLE supplementation. According to the research, the TG, and LDL-c contents in serum were reduced significantly with BLE supplementation [13] in some hyperlipemia rat models. Furthermore, Yang [41] reported that BLE possesses an anti-inflammatory function in macrophages and inhibits adipogenic differentiation. A large number of studies have shown that flavonoids and polyphenols have regulatory effects on fat deposition and cholesterol metabolism in animals. Genistein [47], hawthorn leaf flavonoids [23], and soy isoflavone [48] reduce blood lipid and improve cholesterol metabolism in broilers. It is reasonable to speculate that bamboo leaf flavonoid possesses the same function, and supplementation of BLE could decrease the TG and LDL-c contents in serum of broilers and improve cholesterol metabolism. LDL-c is susceptible to free radicals, and high free radical scavenging capacity could reduce the oxidized LDL-c content [49]. It is speculated that the reduction of LDL-c concentration with dose may result from the antioxidant capacity improvement.

Although the lipid-lowering effects of BLE were strictly demonstrated, little research has been conducted on the effects of BLE on cholesterol metabolism and related mRNA expression in broiler chickens. The absorption, transformation, and synthesis of cholesterol metabolism are involved with some key regulators. For further investigating the effect of BLE on cholesterol metabolism, Quantitative Real-Time PCR was performed for these regulators. HMGCR is a rate-limiting enzyme in the whole process of cholesterol synthesis; increasing HMGCR activity will promote endogenous biosynthesis of cholesterol in the liver [50]. CYP7A1 catalyzes the conversion of cholesterol to bile acid; up-regulating CYP7A1 activity could decrease cholesterol levels [51]. LDLR mediates plasma LDL-c, which is ingested into cells for metabolism and degradation, and approximately 75% of plasma LDL-c was cleared in the liver [52]. Chen [47] reported that 50 mg/kg genistein significantly decreased HMGCR and CYP7A1 mRNA expression levels and increased LDLR mRNA expression in broiler liver. In addition, dietary of soy isoflavone showed the same outcomes in high-cholesterol diets rat [48]. Similarly, genistein exhibited the same effect of inhibiting HMGCR activity in cells [50]. Bamboo leaf flavonoid is the main compound of BLE, with a similar structure to genistein and soy isoflavone [30,53], and the present results are consistent with abovementioned studies in terms of HMGCR and LDLR mRNA expression. Furthermore, BLE showed an excellent inhibitory effect on HMGCR mRNA expression, indicating that BLE may reduce cholesterol synthesis and promote the clearance of LDL-c in serum. Studies on CYP7A1 activity regulation are not identical; both exogenous and endogenous cholesterol affect the expression of CYP7A1, and bile acids also have a negative feedback effect on CYP7A1. Our results showed significant up-regulated mRNA expression of CYP7A1, and higher CYP7A1 activity may contribute to the conversion of absorbed LDL-c to bile acid in the liver. By combining serum lipid parameters with the expression of liver cholesterol metabolism genes, it is deduced that BLE improved cholesterol metabolism by up-regulating LDLR and CYP7A1 mRNA expression to promote the conversion of LDL-c into bile acid, and down-regulated HMGCR expression to reduce cholesterol synthesis. However, due to the complicated mechanism of cholesterol metabolism, our study only

presented a basic result of the effect of BLE on cholesterol metabolism, and the mechanism still needs to be further studied.

5. Conclusions

In conclusion, broiler chickens supplemented with BLE presented a linear improvement of eviscerated yield and reduced abdominal fat deposition. BLE supplementation improved antioxidant capacity by enhancing SOD, GSH-Px and CAT mRNA expression and reducing lipid oxidation, and a dosage of 2.0 to 3.0 g/kg presented the best outcome. Supplemental BLE decreased LDL-c concentration in serum, up-regulated mRNA expression of CYP7A1 and LDLR, and down-regulated mRNA expression of HGGCR. Supplementation of BLE improved cholesterol metabolism of broilers to some extent, but the specific mechanism needs further investigation.

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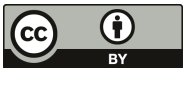

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Denatonium Benzoate-Induces Oxidative Stress in the Heart and Kidney of Chinese Fast Yellow Chickens by Regulating Apoptosis, Autophagy, Antioxidative Activities and Bitter Taste Receptor Gene Expressions

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Simple Summary: Denatonium benzoate is a strong bitter taste receptor agonist, extensively used for its activation of different cell pathways. Taste signals have been associated to food recognition and avoidance, and bitter taste provokes an aversive reaction and is assumed to protect chickens from consuming poisons and harmful toxic substances. The results of the study revealed that dietary supplementation with medium and high doses of denatonium benzoate damaged the epithelial cells of the heart and kidneys by inducing apoptosis and autophagy and reduced the growth of chickens, respectively. However, mRNA expressions of bitter taste receptors, downstream signaling effector genes, apoptosis-, autophagy- and antioxidant-related genes were higher on day 7, while these expressions were subsequently decreased on day-28 in the heart and kidney of Chinese Fast Yellow chickens in a dose-response manner.

Abstract: The sense of taste which tells us which prospective foods are nutritious, poisonous and harmful is essential for the life of the organisms. Denatonium benzoate (DB) is a bitter taste agonist known for its activation of bitter taste receptors in different cells. The aim of the current study was to investigate the mRNA expressions of bitter taste, downstream signaling effectors, apoptosis-, autophagy- and antioxidant-related genes and effector signaling pathways in the heart/kidney of chickens after DB dietary exposure. We randomly assigned 240, 1-day-old Chinese Fast Yellow chicks into four groups with five replicates of 12 chicks and studied them for 28 consecutive days. The dietary treatments consisted of basal diet and feed containing DB (5, 20 and 100 mg/kg). The results revealed that dietary DB impaired ($p < 0.05$) the growth performance of the chickens. Haemotoxylin and eosin staining and TUNEL assays confirmed that medium and high doses of DB damaged the epithelial cells of heart/kidney and induced apoptosis and autophagy. Remarkably, the results of RT-PCR and qRT-PCR indicated that different doses of DB gradually increased ($p < 0.05$) mRNA expressions of bitter taste, signaling effectors, apoptosis-, autophagy- and antioxidant- related genes on day 7 in a dose-response manner, while, these expressions were decreased ($p < 0.05$) subsequently by day-28 but exceptional higher ($P < 0.05$) expressions were observed in the high-dose DB groups of chickens. In conclusion, DB exerts adverse effects on the heart/kidney of chickens in a dose-response manner via damaging the epithelium of the heart/kidney by inducing apoptosis, autophagy associated with bitter taste and effector gene expressions. Correlation analyses for apoptosis/autophagy showed agonistic relationships. Our data provide a novel perspective for understanding the interaction of bitter taste, apoptosis, autophagy and antioxidative genes with bitter taste strong activators in the

heart/kidney of chicken. These insights might help the feed industries and pave the way toward innovative directions in chicken husbandry.

Keywords: denatonium benzoate; bitter taste receptors; apoptosis; autophagy; heart; kidney; chicken

1. Introduction

Taste is well-known biological descriptor for sweet, bitter, sour, salty receptors and ion channels, which plays a critical role in the life and nutritional status of chickens and other organisms. Bitter taste perception provides animals with critical protection against the ingestion of poisonous and harmful toxic compounds [1]. Taste signals have been associated to food avoidance and recognition, as well as feed or liquid intake in different species of animals [2–4]. With the simultaneous inflation in the cost of animal feed and higher standards of livestock products, people endeavor to discover novel feed additives and effective alternatives to traditional antibiotics [5,6]. Efforts have been made to extract incredible numbers of potential additives from natural plants, and they often display bitter taste. However, bitter taste receptors are part of a superfamily which includes more than twenty members. This makes very difficult to do functional studies for each bitter taste receptor. In addition, bitter taste receptors expressions are not limited to taste buds but also exist in extra-gustatory organs, so it is critical to determine their extra-gustatory functions. Several studies have already revealed that bitter taste receptors exert a variety of functions in different cells and tissues [7,8]. Chickens have only three bitter taste receptors: ggTas2r1, ggTas2r2 and ggTas2r7 [9]. Due to their low number of bitter taste receptors, chickens are a good minimalistic model for understanding the functions of bitter taste receptors in non-gustatory tissues [9]. Denatonium benzoate as a bitter taste receptor stimulus can bind to the bitter taste receptors to activate bitter taste signaling. Furthermore, it has been reported that this receptor family plays a critical role in the heart [10], thyroid [11], and gastrointestinal muscle [12]. However, their roles in the kidney has never been determined. Therefore, our present project aimed to examine the roles and underlying mechanisms of bitter taste transduction signaling associated with mRNA expression patterns of bitter taste receptors, apoptosis-, autophagy- and antioxidant-related genes in the hearts and kidneys of chickens.

Cysteine aspartate-specific proteases (CASPs or caspases) serve as intrinsic initiators of apoptosis by cleaving substrates at aspartate residues [13]. In mammals and humans the caspase protein family currently consists of 13 and 11 isoenzymes, respectively [14]. The function of caspases is closely associated to the initiation and execution of apoptosis, with caspases categorized as either initiator or effector caspases. CASP2, CASP8, CASP9 and CASP10 are initiator caspases, and CASP3, CASP6 and CASP7 are effector caspases [15]. Known as interleukin 1β-converting enzyme, CASP1 plays an important role in both apoptosis and inflammation [16]. Apoptosis is regulated by stepwise activation of caspases for the processing or cleaving of other caspases [17].

Programmed cell death or apoptosis has a vital role in various biological events. This is a process of single cell death controlled by the activation of specific genes, the elimination of unwanted or damaged cells by apoptosis is an indispensable action that occurs via several mechanisms which maintain cellular homeostasis, and normal regulation of the immune system [18]. An earlier study showed that denatonium benzoate enhanced intracellular Ca^{2+}, damaged mitochondria and induced apoptosis in airway epithelial cells, respectively [19]. Programmed cell death is activated by intracellular stresses and developmental cues. Well-known representative intrinsic regulators, the extended BCL2 family proteins, play crucial roles in cell death regulation and are able to regulate several cell death mechanisms, including apoptosis, necrosis and autophagy [20–22]. Caspase-independent mechanisms lead to the release of apoptosis-inducing factor (AIF) from the mitochondria, inducing large-scale DNA fragmentation in several cell types including heart and kidney cells to induce apoptosis caused by AIF [22]. BCL2 family proteins contain at least one of four BCL2 homology (BH) domains (i.e.,

BH1, BH2, BH3 or BH4), and the number of BH domains included in proteins is associated with their apoptotic functions [23].

Autophagy is the pathway involved in forming an organelle called an autophagosome. This pathway moves something from the cytoplasm of the cell into the lysosome for degradation. The term, which derives from the Greek words 'auto' meaning self and 'phagos' meaning to eat, is defined as a catabolic pathway involving the degradation of cellular components via the lysosomal machinery [24,25]. Autophagy is the natural process by which the cells can clear out damaged mitochondria, recycle proteins, and get rid of intracellular pathogens. However, all organisms need a balance of autophagy with anabolic processes. There are about 30+ different proteins involved in the formation of the autophagosome. Researchers are still actively figuring out how all of the bits and pieces of the process go together, but recent genetic studies have shed a lot of light on the pathway. A family of genes known as the autophagy-related genes, whose abbreviations start with A/TG, code for several of the proteins integral to autophagy. Several of these genes have variants that have been studied in reference to pathogen susceptibility, autoimmune diseases, cancer, and sepsis [26–28].

There is a complex system containing natural enzymatic and non-enzymatic antioxidants that protect the body from oxidative damage. Briefly, the antioxidant enzymes (SOD, MnSOD, CAT, GSH, and GSH-Px) appear to be the first line of defense during oxidative stress, and exert beneficial effects preventing oxidative damage in poultry raising [29–31]. Catalase (CAT) participates in defense mechanisms against oxidative stress by converting H_2O_2 into water and molecular oxygen [32]. The antioxidant glutathione peroxidase enzymes (GPX) are implicated in the protection of cells against oxidative damage by reducing H_2O_2 and other organic peroxidases to water with reduced glutathione [32].

Denatonium benzoate (DB), one of the most bitter-tasting substances known, is described as extremely unpleasant at a lower amount and can cause perceptible bitterness [33]. DB has been demonstrated extensively as a bitter taste agonist and used to activate bitter taste receptors in many cell types, including taste cells [34]. DB is intensely bitter and non-toxic, and can be detected by human taste receptors [35]. For these reasons DB has been broadly added to liquid detergents, cosmetics, plastic toys and personal care products to avoid the consumption of harmful substances [36]. Aside from its strong bitter taste, DB also exerts biologic effects on various physiological systems in different organisms. Exposure to DB quickly suppressed ongoing intake and delayed gastric emptying in rodents [37,38].

However, there is limited knowledge about the biologic effects of DB and its potential molecular mechanisms in chicken heart and kidneys and no studies have been conducted to date to investigate the relationship between bitter receptors, signaling effectors, and apoptosis-, autophagy- and antioxidant-related genes in the chicken heart and kidney. Therefore, the objective of this study was to investigate the mRNA expressions of bitter taste and its downstream signaling effectors, apoptosis-, autophagy-, and antioxidant-related genes and transduction signaling pathways in chicken heart and kidney to DB dietary exposure in a dose-response manner. The results were confirmed by RT-PCR, qRT-PCR, haemotoxylin and eosin, TUNEL assay, correlation of apoptosis, autophagy and bitter taste receptors and its associated downstream signaling pathway, complete amino acid sequence alignments of related genes, selected gene heat mapping and the potential molecular mechanisms of action of dietary exposure to denatonium benzoate on the heart and kidney cells of Chinese Fast Yellow chickens were determined.

2. Materials and Methods

2.1. Chemicals

Denatonium benzoate (98%) was purchased from Adamas Reagent Co. Ltd. (Nanjing, China) and then stored at room temperature until the end of the experiments. RNA0se (phenol 38%), isoprophyl alcohol, trichloromethane, 100% absolute alcohol, DNA/RNAase-free water all were purchased collectively from RNA detection from (Takara Bio Inc., Shiga, Japan), Cat. # RR047A v

201810Da PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) and Cat. # RR420A v201810Da, TB Green™ Premix Ex Taq ™ (Tli RNase H Plus), were both purchased from TaKaRa (Dalian, China).

2.2. Birds Management and Treatments

A total of 260 1-day-old local Chinese Fast Yellow chicks were housed individually in the Animal Room of Nanjing Agricultural University under standard conditions, and were randomly divided into four (4) treatment groups: Control group (Control), low-dose DB-treated group (5 mg/kg, Low-Den), Medium-dose DB-treated group (20 mg/kg, Medium-Den) and high-dose DB-treated group (100 mg/kg, High-Den), respectively. The control group was fed with basic corn-soybean diet according to NRC (1994) (Table 1). The Low-Den, Medium-Den and High-Den treatment groups were fed the same commercial diet with denatonium added at 5, 20 and 100 mg/kg, respectively. Each treatment includes five cages, and each cage consisted of 12 chicks. Chicks were reared in a ventilated chicken house in which the lighting regime was 16-h light:8-h dark, the relative humidity was approximately 50 ± 5% and chickens were offered formulated feed (Table 1) ad libitum with freely available tap water for 28 consecutive days. Average body weight was calculated on day 1, day 07, day 14 and day 28, respectively. The experimental protocol and procedures were designed and approved in accordance with the Guidelines for the Care and Use of Animals prepared by the Institutional Animal Care and Ethical Committee for Nanjing Agricultural University (Permit number: SYXK (Su) 2019-0047), Nanjing, China.

2.3. Feed Mixing and Formulation

A basic corn-soybean diet with the ingredients listed in Table 1 was purchased from ADM (Nanjing, China).

Table 1. Feed formulation for the entire period of experiment (d 1–d 28).

Item	Diet 1 to 07 d	07 to 28 d
Ingredient (%)		
Corn	61.52	74.00
Soybean meal	29.00	12.00
Soybean oil	2.44	2.60
Corn gluten meal	2.00	7.32
Dicalcium phosphate	1.68	1.02
Premix	1.50	1.00
Limestone	1.15	1.05
Lysine sulfate	0.51	0.80
Methionine	0.20	0.21
Total	**100**	**100**
Calculation of nutrients		
Metabolizable energy, MJ/kg	11.92	12.13
Crude protein, %	21.00	19.00
Lysine, %	1.10	0.97
Methionine, %	0.46	0.40
Methionine + Cystine, %	0.80	0.72
Calcium, %	1.00	0.90
Available phosphorus, %	0.70	0.65
Denatonium benzoate mg/kg	5, 20, 100	5, 20, 100

The complete diet provided the following contents (% per kilogram): vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin E, 30 IU; vitamin K3, 2.65 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; nicotinic acid, 50 mg; pantothenic acid, 12 mg; vitamin B_6, 4 mg; folic acid, 1.25 mg; vitamin B_{12}, 0.025 mg; biotin, 0.25 mg; Fe, 50 mg; Zn, 75 mg; Mn, 100 mg; Cu, 8 mg; I, 0.35 mg; Co, 0.2 mg; and Se, 0.15 mg.

The feed was mixed with a manual electric mixing machine available in the Nanjing Agricultural University animal house, according the experimental design.

2.4. Sample Collection

On days 7 and 28 of age (starter and grower stages), 10 chickens with body weights near the average of their group were slaughtered via exsanguination. The heart and kidneys were gingerly separated, and immediately cut up into two sections. One section was promptly fixed in 4% paraformaldehyde for histological analyses, whereas the second section was stored at −80 °C for the analysis of gene expression.

2.5. TUNEL Assay

The terminal deoxynucleotidyl transferase-mediated deoxy uridine triphosphate nick-end labeling (TUNEL) assay was carried out referring to the kit manufacturer's instructions. Heart and kidney apoptosis were determined using a TUNEL Bright Green Apoptosis Detection Kit (A112, Vazyme Biotech, Nanjing, China). According to the protocol, the paraffin sections of heart and kidney were deparaffinized, rehydrated and then incubated with Proteinase K (20) at room temperature for 20 min. Later, the sections were incubated with TdT enzyme buffer containing double distilled H_2O, eecombinant TdT enzyme, equilibration buffer and Bright Green Labeling Mix at 37 °C for 60 min under dark conditions. Finally, after washing three times in PBS, the sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) staining solution (C1005, Beyotime Biotechnology, Shanghai, China) for 5 min under dark conditions. The negative control was prepared as above without incubation of the TdT enzyme buffer to ensure no non-specific reaction occurred during the experiments. Images were taken through a LSM 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). The total numbers of apoptotic cells (green color) and total cells (blue color) were counted using the Image-Pro Plus software 6.0. The apoptotic index was defined as the ratio of apoptotic cells to total cells.

2.6. Body and Organ Weight Measurements

All chicken live body weights were measured at four different stages on days, 1, 7, 14 and 28 of the experiment and a total 10 chicken in each group (two chicken/replicate) were used in each stage of killing to collect and measure heart and kidney weights at day 07 (starter stage) and at day 28 (grower stage), respectively (Figure 1).

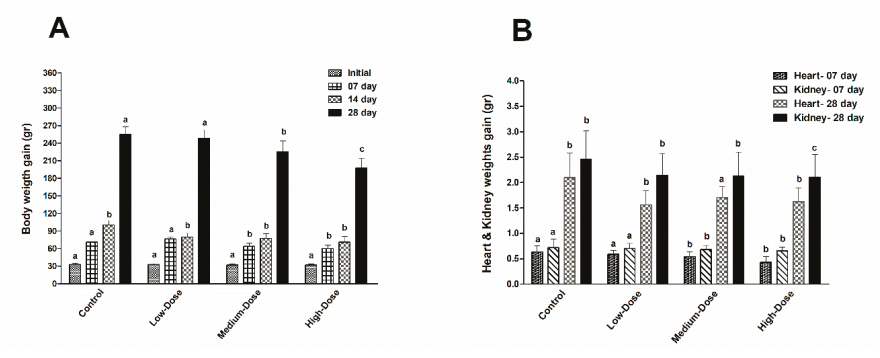

Figure 1. Effects of DB supplementation on the body and organ weight gain (gr) of chickens. (**A**) Body weight of chickens at 0, 07, 14, 28 days of age (n = 10). (**B**) Organ weights of chickens at day 7 and day 28 of age (n = 10). Data are presented as mean value ± SEM. Values without the same mark (a, b, c) represent statistically significant differences ($p < 0.05$). Subfigure A uses line graphs with experimental days on the *X*-axis for body weights, and subfigure B separates the data into two bar graphs for heart and kidney, respectively.

2.7. Haemotoxylin and in Eosin Staining (Histological Observations)

To observe histological changes, heart and kidney samples were fixed in 4% paraformaldehyde for 12 h, then dehydrated through a graded ethanol series, cleared with xylene, embedded in paraffin wax, and serially sectioned at 4-μm thickness. The sections were stained with H&E, and histopathological changes were then viewed under a YS100 microscope (Nikon, Tokyo, Japan). Four (4) sections from each stage were observed for determination of apoptotic and autophagic cells in the tissues as reported previously [39].

2.8. Total RNA Extraction and mRNA Quantification

The total RNA from heart and kidney for RT-PCR and real-time PCR was extracted and purified from frozen collected tissues using RNAose (Takara Bio Inc,), which includes gDNA Eraser (Perfect Real Time) for elimination of genomic (g) DNA according to the manufacturer's protocols. The concentration and quality of total RNA was identified by a micro-spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The samples with the 260/280 ratios of 1.8–2.0 and 260/230 ratios of 2.0–2.2 were chosen for further RT and qRT-PCR reactions. Afterward, mRNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, Cat. # RR047A v201810Da) in accordance to the manufacturer's instructions. Gene-specific primers for bitter taste receptors (ggTas2Rs), apoptosis related genes, autophagy genes and antioxidant genes were generated with aid of the nucleotide database of The National Center for Biotechnology Information (NCBI) [40]. according to their published cDNA sequences (Table 2). The target genes and the housekeeping gene were synthesized by Sangon (city, country) and applied for real-time PCR and their primer sequence is shown in Table 2. Amplicon lengths for real-time PCR were between 92 and 200 bp. The PCR final reaction mixture of 20 μL included 10 μL of TB Green Premix Ex Taq (Tli RNase H Plus) (2×), 0.4 μL of ROX Reference Dye 1 or Dye 2 (50×), 2 μL of cDNA template diluted in ratio of 1:3, 0.4 μL of each primer (10 μM) and 6.8 μL of double distilled H2O (TaKaRa). PCR reactions were performed in 96 well reaction plates on a 7500 Real-time PCR instrument (Applied Biosystems, ABI, Beverly, MA, USA), all genes were assayed three times. under the following conditions: hold stage (95 °C for 30 s), PCR stage (40 cycles of 95 °C/2 min, 60 °C/34 s) apparently, to verify the amplification of a single product, a stage with a temperature increment (Melt Curve Stage) was conducted to generate a melting curve under the following conditions: (95 °C/15 s, 60 °C/1 min), followed by a temperature increment of 95 °C/15 s. Relative gene expression levels were analyzed by the 2***** method after normalization against β-actin.

2.9. Statistical Analysis

Body and Organs Weights Measurements

Body weight and two (2) selected organs weight measurements were described previously [41]. Significant differences between treatment groups and control group were analyzed by one-way analysis of variance (ANOVA) followed by a Duncan post hoc test. A $p < 0.05$ value was considered as statistically significant, marked with (a–d). Value =MEAN ± SEM, weight unit (g).

2.10. Gene Expression Profile

For relative gene expression analysis apoptosis, autophagy, antioxidant, downstream signaling effectors and bitter taste receptor genes for each organ at different growing stages were compared to he chosen control gene (β-actin) in two tissues (heart and kidney) using one-way ANOVA. In addition, multiple comparison among means of mentioned target genes and β-actin gene levels in each group were calculated using Dunnett's test (marked with a–e) as shown in the respective figures. An alpha level of 0.05 was set for all tests. Results were described as the mean ± standard error of the mean (SEM). $p < 0.05$ was considered to be statistically significant. These statistical analyses were conducted

with GraphPad Prism 6 (IBMP Crop, Unites States), and IBM SPSS Statistics, version 20 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Body and Organ Weights Measurements

After continuous feeding for 28 consecutive days with DB-containing diets or control, the body weights of Chinese Fast Yellow chickens were significantly decreased in the medium-dose (20 mg/kg) and high-dose (100 mg/kg) groups ($p < 0.05$), however, no differences were observed between control and the low-dose (5 mg/kg) groups (Figure 1). After 28 days of dietary exposure, all DB-treated groups showed significantly reduced body weights ($p < 0.05$; Figure 1A,B). However, the average body weight of control and low-dose treated groups were almost similar and no major changes were observed during the experiment. The heart and kidney weight gains were recorded twice during the time of experiment, the heart weight gains on day 07 in the control, low-dose and medium-dose of DB-treated groups were significantly ($p < 0.05$) similar, but reduced in the high-dose DB group, respectively, whereas, the heart weight gain on day-28 in all DB-treated groups were significantly ($P < 0.05$) reduced compared to the control group. In addition, the kidney weight gain on day-07 was similar in all DB-treated groups, interestingly the kidney weight gaining in comparison to heart weight gaining was higher in all groups. Moreover, the kidney weight gain on day-28 was equally reduced in all three DB-treated groups (low-dose, medium-dose and high-dose) but significantly ($p < 0.05$) increased in the control group, respectively (Figure 1A,B).

3.2. Histological Observations

3.2.1. Histological Changes in the Heart of Chicken

To evaluate whether dietary DB exposure caused pathological changes in the hearts of chickens, we examined the heart tissue cellular morphology on days 07 and 28 via H&E staining and observed apoptotic changes. Compared to the control group, there was moderate necrosis, myocardial inflammatory infiltration and pyknotic cells, but no morphological distortion of cardiac cells in the low-dose of DB group (Figure 2A,B). In the medium-dose DB groups, there were greater changes compared to the low-dose ones, while severe changes were observed in the high DB dose groups. We also observed cell necrosis, apoptotic cells (cell damage/death), pyknotic cells, and distortion of the normal morphological characteristics of the cells due to the toxic effects of DB for all respective doses. When the DB dietary treatment was continued for 28 consecutive days these effects were increased, with karyorrhexis and karyolysis due to severe necrosis which caused autophagy, shrinkage of fibroblasts leading to condensation, and the number of apoptotic cells in the hearts of chickens was increased (Figure 2A,B).

The results indicate that a high dose of DB can cause severe necrosis of the cells, shrinkage, distortion and changes in the characteristics of the cells which finally lead to apoptosis and autophagy, respectively.

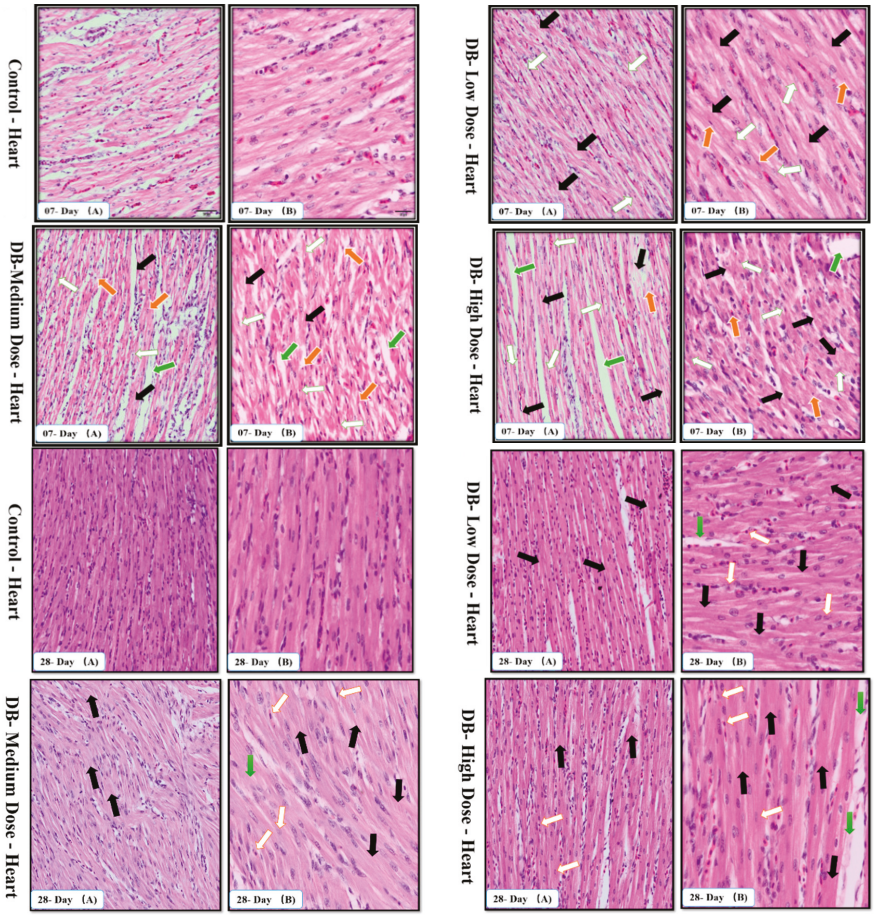

Figure 2. Effects of Denatonium benzoate supplementation on chicken heart histomorphology on days 07 and 28 (**A**,**B**). On day-07 necrosis (red arrow), apoptotic cells (white arrow), pyknotic cells (yellow arrow) and distortion of the morphological characteristics of the cells (green arrow) due to the toxic effect of DB were seen. On day-28, karyolysis due to severe necrosis which caused autophagy (black arrow), apoptotic cells (white arrow) shrinkage of fibroblasts which led to condensation (green arrow) indicate the effect of DB.

3.2.2. Histological Changes in the Kidney of Chicken

Histological changes are a direct indication of kidney damage. In our study, microstructure studies were performed on chicken kidneys. The chicken kidney microstructure is shown in Figure 3A,B.

By comparing the control group results with the groups treated with three different doses of DB, we found several histological changes in the kidney of chickens on days 07 and 28 (Figure 3A,B). Major changes were observed in the high-dose DB group on day-07 of dietary exposure to DB. These changes included Proximal Convoluted Tubules (PCTs) with swelling due to hydrophiid degeneration and influencing the smaller in size collecting tubes. Inflammed infiltrated cells were also observed and separation of basement membrane of Distal Convoluted Tubules (DCTs) which led to karyolysis, apoptosis and autophagy. No live cells were found in the damamged areas of kidneys due to the toxic effects of medium and high doses of DB (Figure 3A,B). In addition, the chickens were more sensitive

during the initial days of DB dietary exposure, therefore the major histological effects were visible on day-07 of exposure rather than day-28 and there were no major differences in terms of histopathological observations on day-07 and day-28 (Figure 3A,B). The results indicated that the chicken kidney is a vital organ for the filtration and excretion of hydrophilic substances inside the body. Therefore, swelling of nephrotic cells were observed which can lead to nephrosis and dysfunction of nephrons, the glomerulus is sensitive to toxic compounds and this can easily lead to inflammation and damage of nephrotic cells.

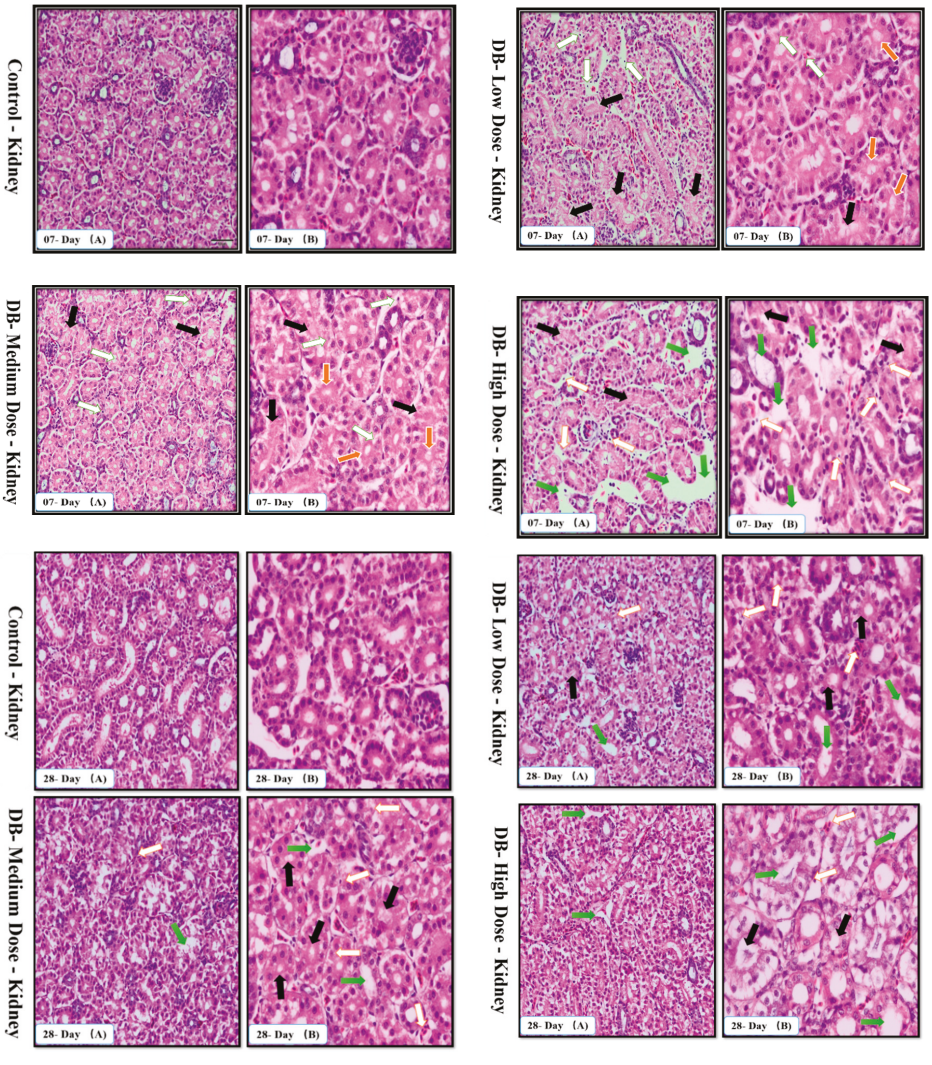

Figure 3. Effects of DB supplementation on the histomorphology of chicken kidney on day-07 and day-28 (**A**,**B**). On day-07, the black arrow shows that PCTs became swollen (hydrophiid degeneration). The white arrow indicates inflamed infiltrated cells and the green arrow indicates separation of the basement membrane of DCTs; Karyolysis occurred where no cells were found due to the toxic effects of DB. The effects on day 28 showed no major differences.

3.3. Confirmation of Apoptosis in the Heart and Kidney of Chicken by TUNEL Assay

The results after staining samples with a TUNEL assay kit are shown in Figure 4A, BTUNEL-positive cells with green colored nuclei represent apoptotic cells.

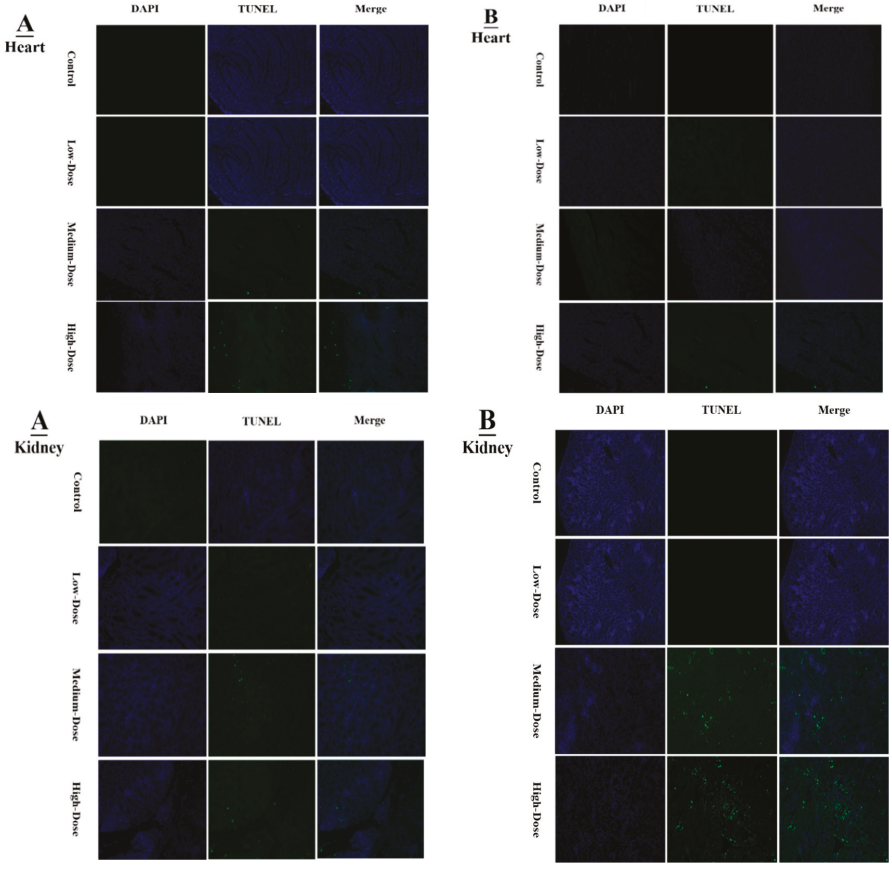

Figure 4. Tunnel assay of heart and kidneys at 07 days (**A**) and at 28 days (**B**) of age by immunofluorescence. The blue color represents the total cells in the heart and kidneys, and the green color represents the apoptosis cells in the heart and kidneys with three different doses of DB (low-dose, medium-dose and high-dose).

These apoptotic cells were observed on day-07 and day-28 in the hearts and kidneys of chicken. However, the numbers of apoptotic cells in both hearts and kidneys were higher on both day-07 and day-28 in the medium- and high-dose DB-treatment groups compared to control group ($p < 0.05$; Figure 4A,B). In comparison with the medium-dose group, the high-dose group exhibited a greater percentage of apoptotic cells on day-28 in both hearts and kidneys of the chickens ($p < 0.05$). No significant pathological differences were observed in apoptotic cells in the low-dose group in contrast with the control group on days 07 and 28, respectively.

3.4. Determination of Real Time (RT) and Quantitative Real-Time (qRT- PCR) Expressions

3.4.1. The mRNA Expressions of Bitter Taste and Downstream Signaling Effectors in the Heart

RT and qRT-PCRs results showed that after dietary exposure to DB the expressions of ggTas2R1, ggTas2R2 and ggTas2R7 in the hearts of chickens were significantly ($p < 0.05$) close on days 07 and 28 for all three different DB doses, with some slight subsequently higher expressions in the BD high and medium dose groups, respectively (Figure 5A,B). We observed however correlated expressions of bitter taste receptor genes, which were gradually up-regulated in a dose-dependent manner, with exception in ggTas2R7 on day 28, while the rest of the genes were similarly up-regulated (Figure 5A,B).

The expressions of downstream signaling effectors (α-gustducin, PLCβ2, IP3R3, TRPM5) in the heart of chickens on day 07 were significantly ($p < 0.05$) higher in most of the DB-treated groups than control (Figure 5A,B). whereas, the low- and high-dose DB groups on day-07 displayed significantly ($p < 0.05$) enhanced expressions of α-gustducin, PLCβ2 and IP3R3, while only the low-dose group displayed higher expressions of TRPM5 (Figure 5A,B), respectively.

The expressions of downstream signaling effectors on day-28 were only significantly ($p < 0.05$) up-regulated for α-gustducin, in the medium and high dose DB groups, while PLCβ2, IP3R3 and TRPM5 were significantly ($p < 0.05$) down-regulated in all DB-treated groups compared to control (Figure 5A,B). Nevertheless, the high DB (100 mg/kg) groups still exhibited significantly ($p < 0.05$) increased mRNA expressions of ggTas2R1, ggTas2R2, ggTas2R7, α-gustducin, PLCβ2, IP3R3, TRPM5 in contrast with the control group ($p < 0.05$; Figure 5A,B).

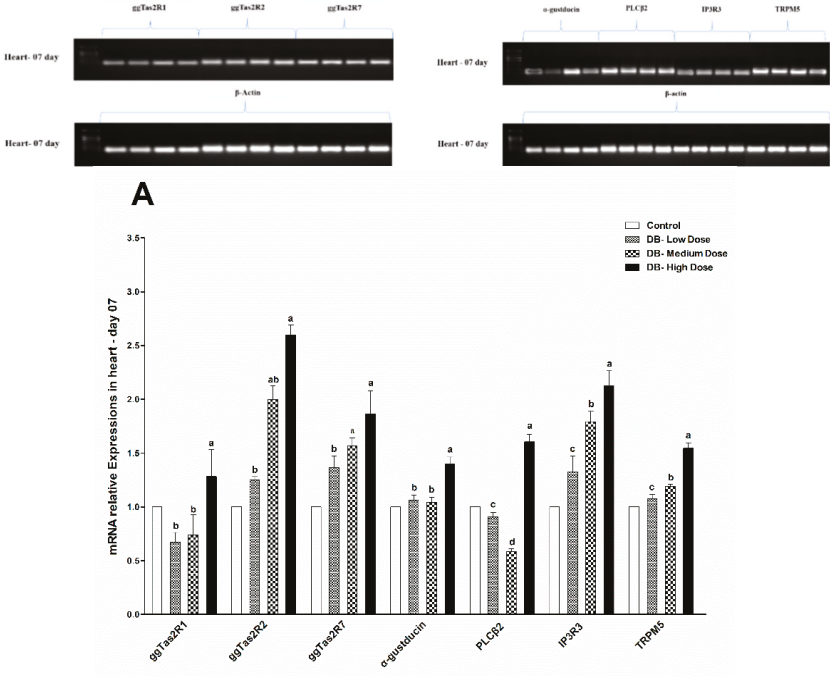

Figure 5. *Cont.*

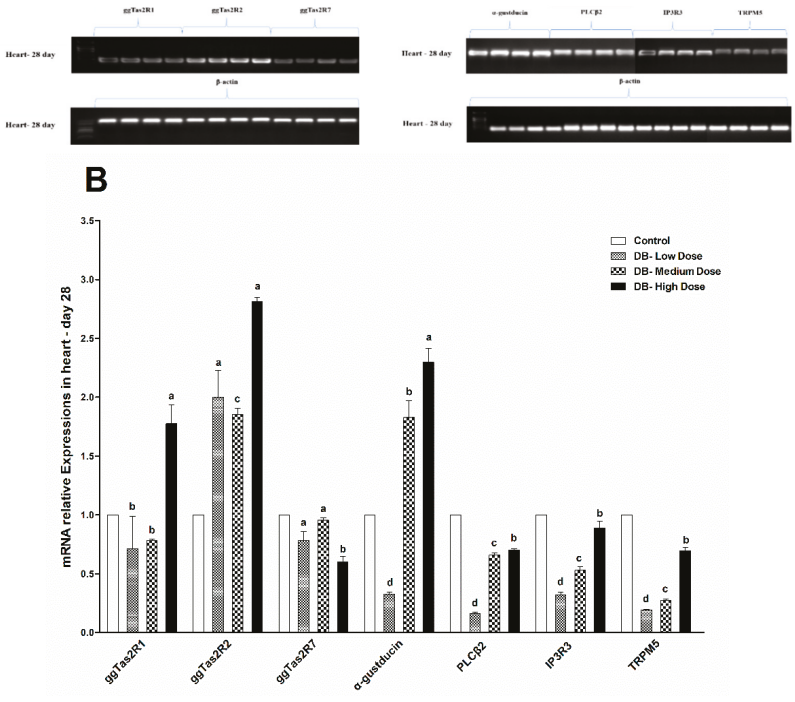

Figure 5. RT and qRT-PCR showed effects of DB supplementation on heart mRNA expressions of bitter taste receptors and downstream effectors at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). gg, Gallus gallus; PLCβ2, phospholipase Cβ2; IP3R3, type 3 inositol-1,4,5-trisphosphate receptor; Denatonium benzoate- Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate- High dose treated group.

3.4.2. Correlation Analysis

Correlation analyses (Tables 2 and 3), exhibited a highly positive correlation among bitter taste receptors and downstream signaling gene sets separately, which may be due to their similar biological function. Moreover, a weak negative correlation among bitter taste genes-and signaling effectors-related genes was observed, which suggests that bitter receptors and signaling effectors may function frequently antagonistically.

Table 2. Correlation analysis of bitter taste and downstream signaling effectors related genes in the heart of chicken.

Gene	ggTas2R1	ggTas2R2	ggTas2R7	α-Gustducin	PLCβ2	IP3R3	TRPM5
ggTas2R1		0.857 *	0.717 *	0.263 *	0.169 *	−0.117 *	0.857 *
ggTas2R2	0.857 *		0.877 **	0.611 *	0.294 *	0.230 *	0.717 **
ggTas2R7	0.717 *	0.877 **		0.877 **	0.720 **	0.322 *	0.272 *
α-gustducin	0.263 *	0.611 *	0.877 **		0.263 *	0.611 *	0.720 **
PLCβ2	0.169 *	0.294 *	0.720 *	0.263 *		0.629 *	0.681 *
IP3R3	−0.117 *	0.230 *	0.322 *	0.611 *	0.629 *		0.169 *
TRPM5	0.857 *	0.717 **	0.272 *	0.720 **	0.681 *	0.169 *	

** Correlation is highly significant at the 0.01 level (2-tailed), while * correlation is less significant.

Table 3. Correlation analysis of bitter taste and downstream signaling effectors related-genes in the kidney of chicken.

Gene	ggTas2R1	ggTas2R2	ggTas2R7	α-Gustducin	PLCβ2	IP3R3	TRPM5
ggTas2R1		0.857 *	0.717 **	0.263 *	0.169 *	−0.117 *	0.857 *
ggTas2R2	0.857 *		0.877 **	0.611 *	0.294 *	0.230 *	0.717 *
ggTas2R7	0.717 *	0.877 **		0.720 *	0.322 *	0.272 *	0.263 *
α-gustducin	0.263 *	0.611 *	0.720 *		0.629 *	0.681 *	0.169 *
PLCβ2	0.169 *	0.294 *	0.322 *	0.629 *		0.863 *	−0.117 *
IP3R3	−0.117 *	0.230 *	0.272 *	0.681 *	0.863 *		0.230 *
TRPM5	0.857 *	0.717 **	0.263 *	0.169 *	−0.117 *	0.230 *	

** Correlation is highly significant at the 0.01 level (2-tailed), while * correlation is less significant

3.4.3. The mRNA Expressions of Bitter Taste and Downstream Signaling Effectors in the Kidney

ggTas2Rs showed significantly ($p < 0.05$) higher expressions in the kidney of chickens compared to the medium dose chickens, contrary to ggTas2R7, which was expressed higher in the DB high dose group on day-07 and then gradually lower expressed in day-28 with slight differences in ggTas2R7 (Figure 6A,B), respectively.

The results indicate that ggTas2R1, ggTas2R2, ggTas2R7 have contrary expressions in kidney on day-07 and day-28 (Figure 6A,B). It was found that the expressions of ggTas2Rs were dose-dependent on DB different doses on day-07, while ggTas2R1 expressions were highly dose-dependent in the DB medium dose group among all treated groups in day-07 (Figure 6A,B). However, on day-28 the expressions of bitter taste-related genes (ggTas2Rs) were significantly ($p < 0.05$) down-regulated and there were dose-independent correlations with expressions (Figure 6B).

On day-07 the expressions of two downstream signaling effectors genes (IP3R3, TRPM5) were significantly higher in the low, medium and high DB dose groups compared to α-gustducin and PLCβ2 (Figure 7A). However, on day-07 the high dose (100 mg/kg) DB group showed significantly higher expressions among all treated groups, exhibiting a high dose-dependent correlation (Figure 6A). In addition, on day-28 the downstream signaling effectors individually up-regulated the expression of α-gustducin in the medium and high dose DB groups, while PLCβ2, IP3R3 and TRPM5 were significantly ($p < 0.05$) down-regulated in all DB- treated groups compared to control (Figure 7B). Therefore, we concluded that the downstream signaling effector gene expressions were quite similar on day-07 and day-28 and no major differences were observed between them (Figure 6A,B)

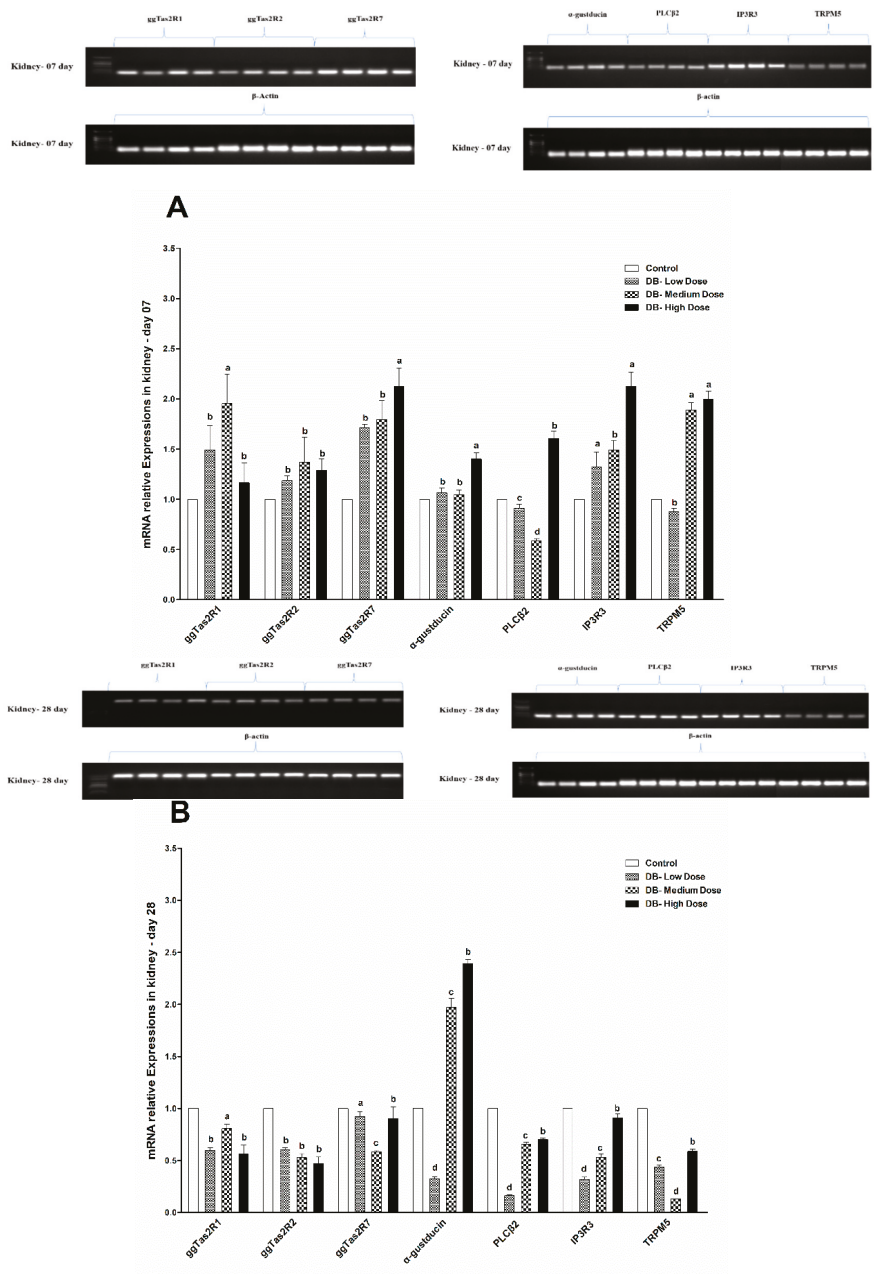

Figure 6. Effects of DB supplementation on kidney mRNA expressions of bitter taste receptors and downstream effectors at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). gg, Gallus gallus; PLCβ2, phospholipase Cβ2; IP3R3, type 3 inositol-1,4,5-trisphosphate receptor; Denatonium benzoate- Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate- High dose treated group.

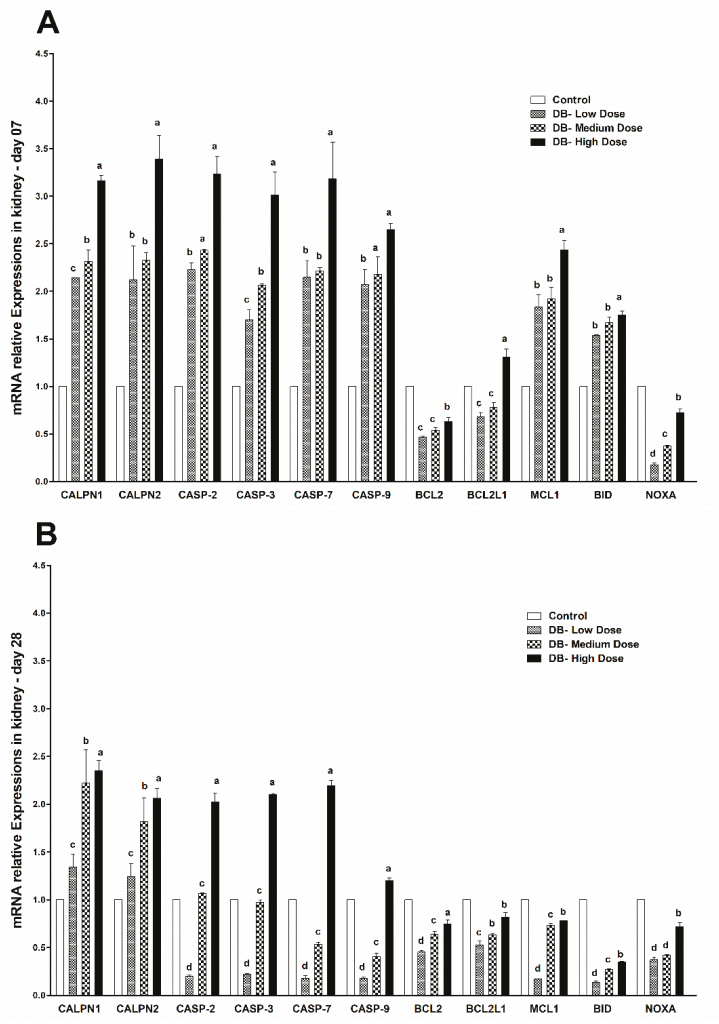

Figure 7. Effects of DB supplementation on kidney mRNA expressions of apoptosis-related genes at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). CALPN1, Calpain 1; CALPN2, Calpain 2; CASP-2, Caspase 2; CASP-3, Caspase 3; CASP-7, Caspase 7; CASP9, Caspase 9; BCL2, B-cell CLL/lymphoma 2; BCL2L1, BCL2 like 1; MCL1, myeloid cell leukemia sequence 1; BID, BH3 interacting domain death agonist; NOXA, similar to ATL-derived PMA-responsive peptide; Denatonium benzoate-Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate-High dose treated group.

3.5. Determination of Real Time (RT) and qRT-PCR Expressions of Apoptosis Related Genes

3.5.1. The mRNA Expressions of Apoptosis Related Genes in the Heart of Chicken

The qRT-PCR results showed significant ($p < 0.05$) changes in the expressions of apoptosis-related genes in both day-07 and day-28, whereas lower expressions were observed in the heart of chicken on day-28, respectively (Figure 8A,B). Comparing the CALPN1 and CALPN2 caspase family genes (CASP2, CASP3, CASP7 and CASP9) and BCL2, BCL2L1, MCL, BID and NOXA on day-07,

a significantly ($p < 0.05$) higher expression pattern was observed in CALPN1 among the selected genes, that gradually reduced the CASP family-related genes and consequently the BCL2 family (Figure 8A). Lower expressions were observed in BCL2 and NOXA genes among others (Figure 8A). Therefore, the expression levels of apoptosis-related genes in day-07 were dose-dependent in the heart of chicken (Figure 8A).

On day-28 of the experiments, the apoptosis-related gene expression results revealed higher expressions of both CALPN1 and CASP9 among the genes (Figure 8B), while other genes were also significantly ($p < 0.05$) expressed, but the expressions were still lower in contrast with day-07 (Figure 8A,B). Remarkably, lower expressions on day-28 in the heart of chicken were observed in NOXA among apoptosis-related genes in the low-dose DB group (Figure 8B). To conclude, the expressions in both stages in the heart of chicken were dose- and time-dependent, hence, we found a dose-dependent relation but a time-independent relation in expressions was exhibited. Therefore, higher expressions were observed in CALPN and CASP family genes against a high dose (100 mg/kg) of DB but lower in the low-dose chickens. Thus, these genes might have similar sensitivity against DB in the heart of chicken.

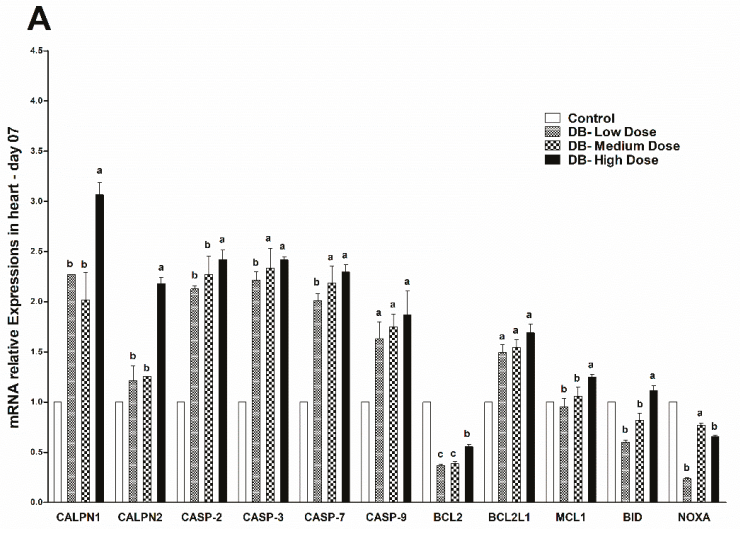

Figure 8. *Cont.*

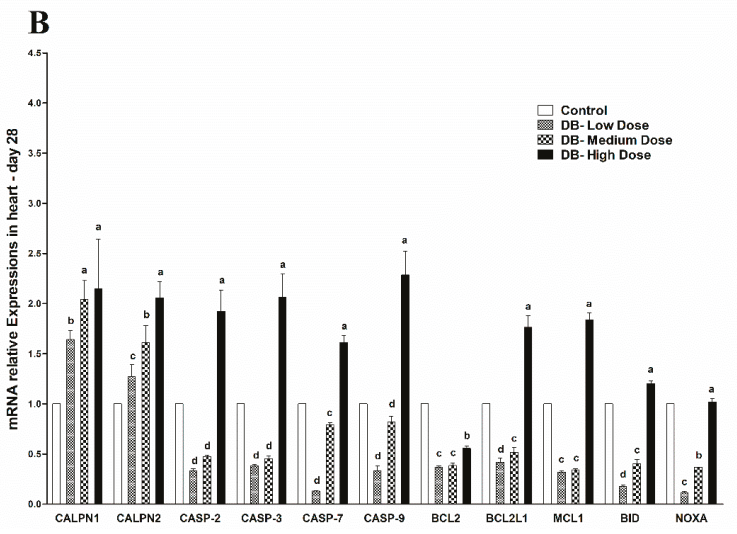

Figure 8. Effects of BD supplementation on heart mRNA expressions of apoptosis-related genes at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). CALPN1, Calpain 1; CALPN2, Calpain 2; CASP-2, Caspase 2; CASP-3, Caspase 3; CASP-7, Caspase 7; CASP9, Caspase 9; BCL2, B-cell CLL/lymphoma 2; BCL2L1, BCL2 like 1; MCL1, myeloid cell leukemia sequence 1; BID, BH3 interacting domain death agonist; NOXA, similar to ATL-derived PMA-responsive peptide; Denatonium benzoate-Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate-High dose treated group.

3.5.2. Correlation Analysis

Correlation analyses (Tables 4–7), exhibited a highly positive correlation among apoptosis and autophagy-related gene sets in the heart and kidney of chicken, separately, which may be due to their similar biological function in selected organs. Moreover, a strong positive correlation among different apoptosis and autophagy-related genes was observed, which suggests that different apoptosis genes (CASP and BCL2 families) may function agonistically, while a very weak correlation was also observed among some genes, which suggests that they may function anti-agonistically, as shown in Tables 4–7, respectively.

Table 4. Correlation analysis of apoptosis related-genes in the heart of chicken.

Gene	CALPN1	CALPN2	CASP2	CASP3	CASP7	CASP9	BCL2	BCL2L1	MCL1	BID	NOXA
CALPN1		0.972 **	0.946 **	0.350 *	0.493 *	0.783 **	0.972 **	0.893 **	0.184 *	0.360 *	0.756 **
CALPN2	0.972 **		0.946 **	0.893 **	0.587 *	0.693 *	0.817 **	0.184 *	0.587 *	0.895 **	0.448 *
CASP2	946 **	0.946 **		0.493 *	0.360 *	0.693 *	0.895 **	0.494 *	0.783 **	0.756 **	0.817 **
CASP3	0.350 *	0.893 **	0.493 *		0.972 **	0.946 **	0.350 *	0.493 *	0.783 **	0.972 **	0.893 **
CASP7	0.493 *	0.587 *	0.360 *	0.972 **		0.184 *	0.360 *	0.756 **	0.946 **	0.893 **	0.587 *
CASP9	0.783 **	0.693 *	0.693 *	0.946 **	0.184 *		0.693 *	0.817 **	0.350 *	0.184 *	0.587 *
BCL2	0.972 **	0.817 **	0.895 **	0.350 *	0.360 *	0.693 *		0.895 **	0.448 *	0.493 *	0.360 *
BCL2L1	0.893 **	0.184 *	0.494 *	0.493 *	0.756 **	0.817 **	0.895 **		0.693 *	0.895 **	0.494 *
MCL1	0.184 *	0.587 *	0.783 **	0.783 **	0.946 **	0.350 *	0.448 *	0.693 *		0.783 **	0.756 **
BID	0.360 *	0.895 **	0.756 **	0.972 **	0.893 **	0.184 *	0.493 *	0.895 **	0.783 **		0.817 **
NOXA	0.756 **	0.448 *	0.817 **	0.893 **	0.587 *	0.587 *	0.360 *	0.494 *	0.756 **	0.817 **	

** Correlation is significant at the 0.01 level (2-tailed), while * correlation is less significant.

Table 5. Correlation analysis of apoptosis related-genes in the kidney of chicken.

Gene	CALPN1	CALPN2	CASP2	CASP3	CASP7	CASP9	BCL2	BCL2L1	MCL1	BID	NOXA
CALPN1		0.972 **	0.946 **	0.350	0.493	0.783 **	0.972 **	0.893 **	0.184	0.360	0.756 **
CALPN2	0.972 **		0.946 **	0.893 **	0.587	0.693 *	0.817 **	0.350	0.184	0.587	0.895 **
CASP2	0.946 **	0.946 **		0.448	0.493	0.360	0.693 *	0.895 **	0.494	0.783 **	0.756 **
CASP3	0.350	0.893 **	0.448		0.817 **	0.448	0.494	0.972 **	0.946 **	0.350	0.493
CASP7	0.493	0.587	0.493	0.817 **		0.783 **	0.972 **	0.893 **	0.184	0.360	0.756 **
CASP9	0.783 **	0.693 *	0.360	0.448	0.783 **		0.946 **	0.893 **	0.587	0.693 *	0.817 **
BCL2	0.972 **	0.817 **	0.693 *	0.494	0.972 **	0.946 **		0.350	0.184	0.587	0.895 **
BCL2L1	0.893 **	0.350	0.895 **	0.972 **	0.893 **	0.893 **	0.350		0.448	0.493	0.360
MCL1	0.184	0.184	0.494	0.946 **	0.184	0.587	0.184	0.448		0.693 *	0.895 **
BID	0.360	0.587	0.783 **	0.350	0.360	0.693 *	0.587	0.493	0.693 *		0.494
NOXA	0.756 **	0.895 **	0.756 **	0.493	0.756 **	0.817 **	0.895 **	0.360	0.895 **	0.494	

** Correlation is highly significant at the 0.01 level (2-tailed), while * correlation is less significant

Table 6. Correlation analysis of autophagy-related-genes in the heart of chicken.

Gene	ATG5	Beclin-1	Dyanin	LC3-I	LC3-II	mTOR	ATG5
ATG5		0.972 **	0.946 **	0.350 *	0.493 *	0.783 **	0.972 **
Beclin-1	0.972 **		0.893 **	0.184 *	0.360 *	0.756 **	0.946 **
Dyanin	0.946 **	0.893 **		0.587 *	0.693 *	0.817 **	0.350 *
LC3-I	0.350 *	0.184 *	0.587 *		0.292 *	0.589	0.057 *
LC3-II	0.493 *	0.360 *	0.693 *	0.292 *		0.693 *	0.895 **
mTOR	0.783 **	0.756 **	0.817 **	0.589 *	0.693 *		0.494 *
ATG5	0.972 **	0.946 **	0.350 *	0.057 *	0.895 **	0.494 *	

** Correlation is highly significant at the 0.01 level (2-tailed), while * correlation is less significant

Table 7. Correlation analysis of autophagy-related-genes in the kidney of chicken.

Gene	ATG5	Beclin-1	Dyanin	LC3-I	LC3-II	mTOR	ATG5
ATG5		0.972 **	0.946 **	0.350 *	0.493 *	0.783 **	0.972 **
Beclin-1	0.972 **		0.893 **	0.184 *	0.360 *	0.756 **	0.946 **
Dyanin	0.946 **	0.893 **		0.587 *	0.693 *	0.817 **	0.350 *
LC3-I	0.350 *	0.184 *	0.587		0.895 **	0.448 *	0.493 *
LC3-II	0.493 *	0.360 *	0.693 *	0.895 **		0.494 *	0.783 **
mTOR	0.783 **	0.756 **	0.817 **	0.448 *	0.494 *		0.494 *
ATG5	0.972 **	0.946 **	0.350 *	0.493 *	0.783 **	0.494 *	

** Correlation is highly significant at the 0.01 level (2-tailed), while * correlation is less significant

3.5.3. The mRNA Expressions of Apoptosis-Related Genes in the Kidney of Chicken

After 28-days of continuous DB dietary exposure of the chickens, the results of both PCRs confirmed the expressions of apoptosis related-genes in the kidney of chickens (Figure 7A,B). On day-07, the expressions of CALPN2, CALPN1, CASP2, CASP3, CASP6, CAPS9 were significantly much higher than other apoptosis genes, respectively (Figure 8A). However, on day-07, the lowest expressions were found in NOXA among the genes in the kidney of chicken (Figure 7A). On day-28 of exposure, the highest significant expressions of apoptosis related-genes in the kidney of chicken were observed in CALPN1 and CAPS7 among the selected genes, but lower expressions were confirmed for BID, respectively (Figure 7B). The results indicate that the apoptosis-related gene expressions in the heart and kidney of chicken at the two stages of exposure were dose-dependent while we found an independent correlation with time (Figures 7A,B and 8A,B).

3.6. Determination of Real Time (RT) and qRT-PCR Expressions of Autophagy-Related Genes

3.6.1. The mRNA Expressions of Autophagy-Related Genes in the Heart of Chicken

To determine autophagy-related genes expression in the heart of chicken, we performed RT and qRT-PCR to examine their expressions. On day-07, we observed that the lowest expressions for all DB-treated groups were for ATG5 and Beclin-1 among six selected autophagy-related genes (ATG5, Beclin-1, Dyanin, LC3-I, LC3-II, mTOR) respectively (Figure 9A,B). Meanwhile, the highest expressions of autophagy-related genes were found in LC3-II, Dyanin and LC3-I among the genes gradually (Figure 9A). Furthermore, significantly higher expressions for autophagy-related genes on day-07 was observed in the high dose (100 mg/kg) DB groups as shown in Figure 9A, whereas, the lowest expressions were exhibited in the Low-dose (5 mg/kg) DB-treated group and then in the medium-dose (20 mg/kg) DB groups (Figure 9A). We concluded from the above results that the expression levels of autophagy-related genes are directly correlated with the DB dose, therefore the expressions were dose-dependent, which lead to their higher and lower expressions in a dose-dependent manner. To evaluate the effects of DB on the autophagy-related gene expression profile in the heart of chicken we detected the expressions of six autophagy-related genes (ATG5, Beclin-1, Dyanin, LC3-I, LC3-II, mTOR) using RT and qRT-PCR techniques to confirm the expression levels. The results of both PCRs on day-28 showed that higher expressions were found in Dyanin and the lowest expression was found for Beclin-1 among all selected genes, respectively (Figure 9B). however, the expressions in ATG5, LC3-I, LC3-II and mTOR were almost similar and no major differences were observed among them (Figure 9 A,B). Notably, there was no significant differences in the expressions of ATG5, Beclin-1, LC3-I, LC3-II and mTOR and the expressions level was dose-dependent for those genes, with the only exception of mTOR, where we found a lower expression in the high dose DB group (Figure 9B). Remarkably, the expression of Dyanin was significantly higher at both stages (day-07 and day-28) in the heart of chicken among the six autophagy-related genes (Figure 9A,B). These results indicated that DB benzoate exposure aggravated ER stress and increased autophagy and the autophagic effects are dose dependent.

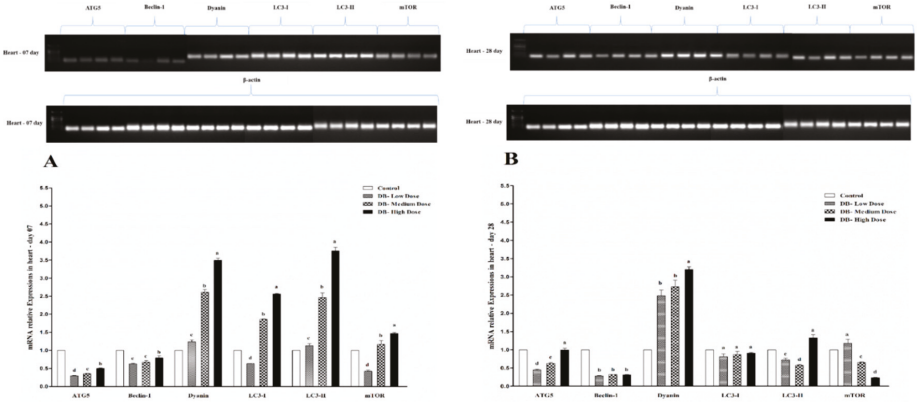

Figure 9. Effects of DB supplementation on heart mRNA expressions of autophagy- related genes at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). ATG5; Beclin 1; Dynein; LC3-I; LC3-II, mTOR; Denatonium benzoate- Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate- High dose treated group.

3.6.2. The mRNA Expressions of Autophagy-Related Genes in the Kidney of Chicken

The autophagy-related gene expression figures indicated that among six examined autophagy-related genes in the kidney of chicken, the expression level of mTOR gene on day-07 is dramatically increased (Figure 10A), while the rest of the genes were significantly almost equally expressed in the kidney of Chinese Fast Yellow chickens, but the higher expressions were observed in the DB high dose group for all six autophagy-related genes of the experiment with the exception of ATG5 (Figure 10A). These findings indicate that, there is direct relationship between dose and autophagy-related gene expressions in the kidney of chickens.

On day-28, higher expressions were found in Dyanin like on day-07, but lower expressions were displayed for Beclin-1 among autophagy-related genes, respectively (Figure 10B). However, the expression profile of autophagy-related genes in the kidney of chicken was dose-dependent and showed significantly gradually changing expressions (Figure 10B). Therefore, the results suggest that, autophagy-related gene expressions are dose-dependent on both day-07 and day-28 in the kidney of chicken and high dose (100 mg/kg) of DB was found to be the dose with the highest effects among the treatments.

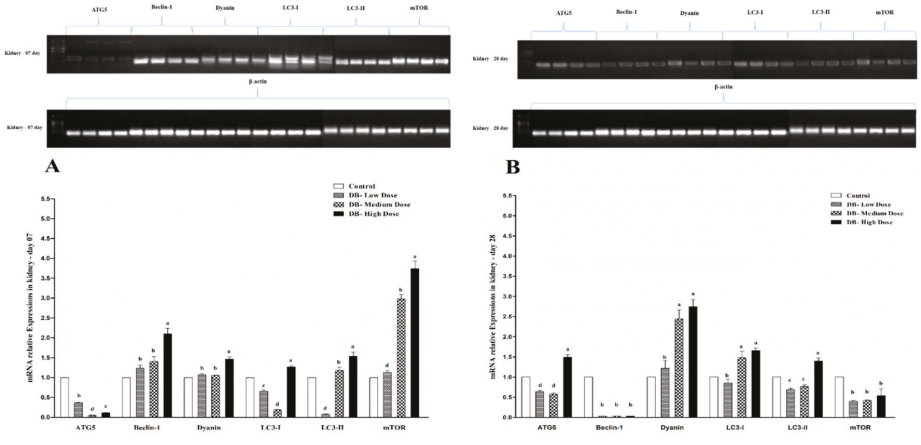

Figure 10. Effects of DB supplementation on kidney mRNA expressions of autophagy- related genes at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). ATG5; Beclin 1; Dynein; LC3-I; LC3-II, mTOR; Denatonium benzoate- Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate- High dose treated group.

3.7. Determination of Real Time (RT) and qRT-PCR Expressions of Antioxidant Genes

3.7.1. The mRNA Expressions of Antioxidant-Related Genes in the Heart of Chicken

Chicken heart is one of the organs susceptible to oxidative processes, and its oxidation state can be reflected by the levels of antioxidant gene expression, for example, glutathione peroxidase (GPx1) and catalase (CAT), which are responsible for the clearance of hydroxyl radicals [42]. Our results showed that the expression profile of oxidative stress-related genes in the heart of chicken on day-07 was significantly ($p > 0.05$) increased in a dose-dependent manner and we found high level expressions in the DB high dose (100 mg/kg) group among the three selected doses (low-dose, medium-dose and high-dose), however the expression pattern of GPX1 gene was significantly higher compared to other antioxidant genes (CAT, SOD1) (Figure 11A). On day-28, the expression level of three antioxidant-related genes were significantly ($p > 0.05$) increased consequently and higher-level expressions were detected in the DB high dose chickens, the gene expressions for GPX1, SOD1 and CAT were dose-dependent

and CAT showed higher expression among them (Figure 11B). The results suggest that the heart is sensitive to oxidative stress and shows significant ($p > 0.05$) elevated expressions of antioxidative stress genes in a dose-dependent manner on both day 07 and day 28, while low-dose chickens displayed inconspicuous decreased expressions, respectively (Figure 11A,B).

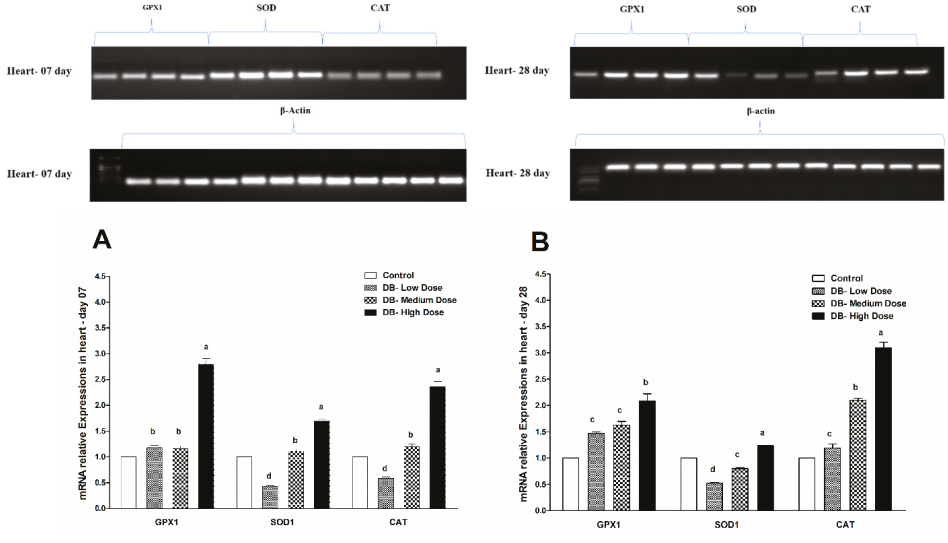

Figure 11. Effects of DB supplementation on heart mRNA expressions of antioxidant-related genes at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). GPX1, glutathione peroxidase 1; SOD, superoxide dismutase 1; CAT, catalase; Denatonium benzoate- Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate- High dose treated group.

3.7.2. The mRNA Expressions of Antioxidant-Related Genes in the Kidney of Chicken

Because of the high sensitivity of chicken kidney to oxidative stress, the oxidation state could be reflected by the level of antioxidant genes expression profile. Most enzymatic components of this antioxidant defense system are commonly known as "antioxidant enzymes" (e.g., catalase, superoxide dismutase, glutathione peroxidase). We evaluated the expressions of such antioxidant enzymes at two different ages of chicken as a parameter to assess oxidative stress in selected organs (heart and kidney). The experimental data revealed that the expressions of three antioxidant genes (GPX1, SOD1, CAT) were expressed with approximately equally significance ($p > 0.05$) and there were slight differences in the expression level among the genes, which indicates that the expressions and sensitivity of genes related to antioxidative activity in the kidney of chicken are correlated with the dose of DB, the same as was shown in the heart of chicken, respectively (Figures 11 and 12A,B).

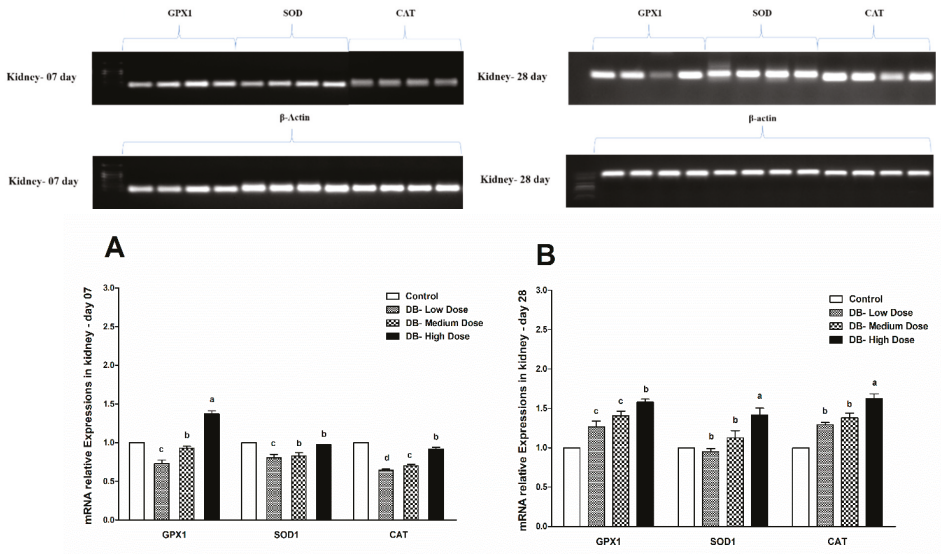

Figure 12. Effects of DB supplementation on kidney mRNA expressions of antioxidant- related genes at 07 days (**A**) and 28 days (**B**) of age. Data are presented as mean value ± SEM (n = 6). Values without the same mark (a–d) represent statistically significant differences ($p < 0.05$). GPX1, glutathione peroxidase 1; SOD, superoxide dismutase 1; CAT, catalase; Denatonium benzoate- Low Dose treated group, Denatonium benzoate- Medium Dose treated group, Denatonium benzoate- High dose treated group.

3.8. Amino Acid Sequences Complete Alignment

We performed complete amino acid sequence alignment for the experiment selected genes. First, we searched for the amino acid complete sequence through exploring the NCBI database, then we did a complete alignment using two bioinformatic tools (ClustalX and Gene-Doc). The amino acid sequence alignment showed us that those genes which are identical have black color while those which are similar with each other have gray color for the alignment indications. The empty area means there is no similarity and identity among the genes, respectively. Interestingly, we found more identical and similar genes in bitter taste receptors family (ggTas2Rs), downstream signaling effectors genes and antioxidant genes, while there was little similarity among apoptosis- and autophagy- related genes. The illustrated alignment figures for bitter taste receptors genes, downstream signaling effectors genes, apoptosis related genes, autophagy related genes and antioxidant genes are shown in the Supplementary Materials.

3.9. Heat Map of Selected Genes

We performed a heat map analysis for all selected genes of the experiment (in Figure 13). In this heat map analysis, we precisely showed all gene expressions levels and confirmed the same results as described earlier in the Results section.

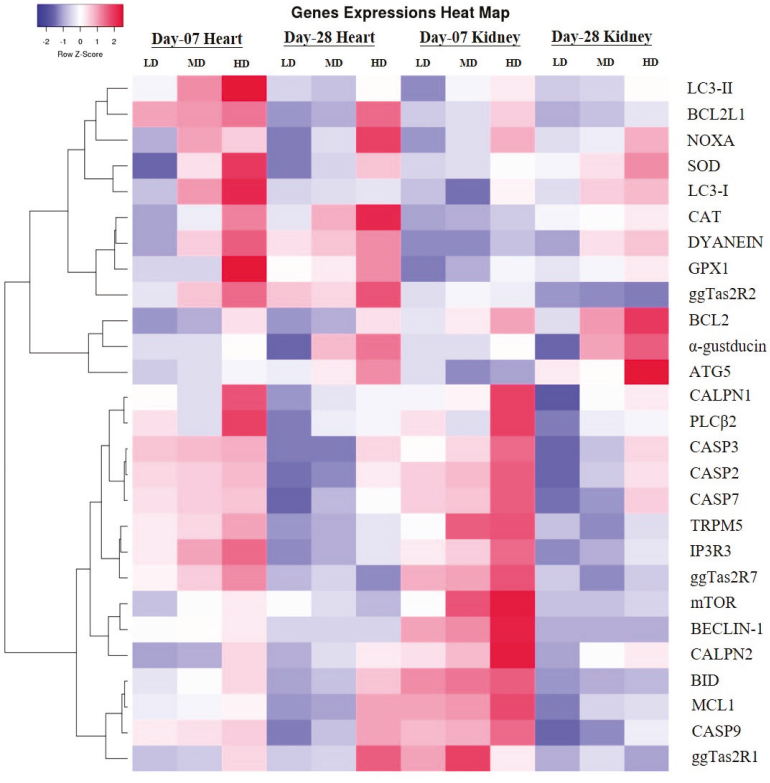

Figure 13. Heat map of expression profiles (bitter taste, downstream signaling effectors, apoptosis, autophagy and antioxidant genes) in supplementation with three different doses of DB in two stages (day-07 and day-28) in the heart and kidney of chicken.

4. Discussion

In the present study, we investigated the biological effects of denatonium benzoate (DB) on growth performance, mRNA expressions of bitter taste receptors, its downstream signaling effectors genes and related pathway, apoptosis, autophagy, antioxidant related genes, histological changes and correlations among genes expressions in the heart and kidney of Chinese Fast Yellow chickens on both day-07 and day-28 of the experiments using RT, qRT-PCR, Hematoxylin and Eosin and TUNNEL assays. We found that DB induced apoptosis, autophagy and increased the expressions of antioxidant-related genes in the heart and kidney of the chickens. However, the expressions of bitter taste receptors genes and its downstream signaling effectors were significantly higher on day-07 compared to day-28 for different DB doses, but the High-dose DB had more potential effects on apoptosis, autophagy, antioxidant, bitter taste receptors and its downstream signaling effectors gene expression than other doses, which significantly induced apoptosis and autophagy in the heart and kidney of chicken on both day-07 and day-28. Remarkably, we also found that bitter taste receptors and the associated signaling effectors, apoptosis, autophagy and antioxidant gene expressions were dependent in a dose-response manner. These findings suggest that the bitter taste receptors have a potential role among the extra-gustatory organs of the chicken, and high-dose DB causes severe necrosis via apoptosis and may result in autophagy in chicken heart and kidneys, while these symptoms were obviously observed on day-07, which proves that the chicken were more sensitive to DB exposure at the beginning of the experiments and later they adapted accordingly.

DB (485–740 mg/kg) exhibited a low toxicity rate in acute oral LD_{50} tests in rats and rabbits, while chronic toxicity studies have indicated that gavage of 16 mg/kg/day resulted in no compound-related toxicity in monkeys and rats [43,44]. In our current study, the average exposure amount of DB in the Low-dose (5 mg/kg), Medium-dose (20 mg/kg) and High-dose (100 mg/kg) groups was calculated on a daily basis for the feed and was less than the doses above. Therefore, DB dietary exposure for 28 days significantly reduced the growth performance and organ (heart and kidney) weights of the chickens. In agreement with our results, four weeks of treatment with bitter agonists like DB or quinine resulted in decreases in body weight gain associated with decreased feed intake [45]. Moreover, DB has been shown to influence ongoing interdigestive behavior, food intake and gut peptide secretion in healthy volunteers and DB may be able to suppress the contraction of smooth muscles, which inevitably affects the nutrient integration, palatability, digestibility and impairs body weight gain [33,46]. Interestingly, our results indicated that at day-07 and day-28 the ggTas2r2 expressions were higher among three tested bitter taste receptors, and separately, the higher expressions were found in the DB High-dose (100 mg/kg) group, which indicate that the expressions were dose-dependent. Overall, the expressions were significantly decreased on day-28 in contrast do day-07. This finding indicates that chicken sensitivity to DB decreased consequently.

The avian circulatory system is the main transport system of the body. It is the means by which nutrients, enzymes and other important needs for the proper functioning of body systems, organs, tissues and cells as well as body defense components are transported to where they are required. The heart is the most significant and vital organ of the avian circulatory system and its main function is blood supply/pumping of the blood [47]. To our knowledge this is the first time the potential mechanism underlying the heart pathological changes caused by different doses of DB has been determined. We performed haemotoxylin and eosin staining as well as TUNNEL assay examinations on day-07. In the haemotoxylin and eosin staining expreiments, we found particular pathological changes which alter cell necrosis, apoptosis, pyknotic cells, and distortion of morphological characteristics of the cell due to toxic effect of denatonium on the heart of chicken. Interestingly, these changes were more due to medium and high dose DB exposure. However, on day-28, we observed severe necrosis which caused apoptosis, autophagy and some shrinkage of the fibroblasts which can lead to condensation due to the effect of a high dose of BD (Figure 2A,B).

The urinary system is very complex because of its function. The kidneys maintain the water balance by removing excess water from the blood stream. Additionally, the kidneys maintain the electrolyte balance, and eliminate metabolic wastes, particularly nitrogen products. In addition, they need their own supply of nutrients for the maintenance of their own tissues and cells. When the kidneys are diseased or damaged and unable to carry out their functions efficiently, the animal becomes debilitated and death often occurs quickly [48]. The present study showed that, on day-07 and day-28, after exposure to different doses to DB the PCTs swell due to hydrophiid degeneration, the basement membrane of DCTs becomes separated and karyolysis occurred in the kidney of chickens due to the toxic effects of medium and high dose DB, respectively (Figure 3A,B). These findings were obviously more severe on day-28 compared to day-07 and suggest that after long term treatment with DB, the chicken kidney may suffer dysfunction.

Apoptosis is involved in cellular growth and development, and is important for the turnover of heart and kidney epithelial cells and tissue homeostasis [49]. Severe apoptosis is harmful for the heart and kidney, and can lead to cellular dysfunction [49,50]. It is reported that BD inhibits airway epithelial cell proliferation, decreases the number of cells and promotes cell apoptosis in a dose-dependent manner via a mitochondrial signaling pathway [19]. We performed TUNNEL assays to confirm pathological changes caused by denatonium benzoate in the heart and kidneys of chicken. As described previously, the apoptosis-related genes showed higher expressions in the heart and kidney of chicken, pathological changes that were also confirmed by haemotoxylin and eosin staining. In the present study, we detected more serious apoptosis in the heart and kidney epithelial cells of the medium and high dose DB groups. In addition, we found greater number of apoptotic cells in the high dose DB

group than in the medium-dose group at 28 days, these findings revealed that denatonium benzoate amplified apoptosis in a dose-effect manner. Consistent with our results, a previous study indicated that DB inhibited airway epithelial cell proliferation, and increased cell apoptosis in a dose-effect manner [19]. Other studies also demonstrated that bitter-tasting compounds induced apoptosis in cancer cells [51]. In our study, high-dose DB exerted seriously negative effects on the heart and kidney of Chinese Fast Yellow chickens. Interestingly, low-dose DB reduce the body weight without affecting the heart and kidney epithelium after long-term adaptation. We hypothesize that low-dose DB could be added into the feed for obese layers to control the body weight due to obesity-induced dysfunctions in layers [52]. This hypothesis requires further investigations to evaluate.

The downstream signaling effectors genes (α-gustducin, PLCβ2, IP3R3 and TRPM5) of bitter taste receptors displayed similar expression patterns as the bitter taste receptors. However, these expressions were higher in day-07, while the age of chicken increased the amplified genes' expressions (bitter and downstream signaling) in the heart and kidney of chicken were attenuated in both low-dose and medium-dose groups apparently. The results indicate that heart and kidney have a better tolerance to bitter stimuli after long-term of exposure to low and medium doses of DB. Taste transduction gene mRNA expression showed variations in the heart and kidney, through administration of DB, which suggests possible extra-gustatory effects for these genes on heart and kidney cell function of the chicken which require further investigations.

The transduction of taste is a fundamental process that allows animals to discriminate nutritious from noxious substances. Three taste modalities, bitter, sweet, and amino acid, are mediated by G protein-coupled receptors that signal through a common transduction cascade: activation of phospholipase Cβ2, leading to a breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol and inositol 1,4,5-trisphosphate, which causes release of Ca2+ from intracellular stores. The ion channel, TRPM5, is an essential component of this cascade; however, the mechanism by which it is activated is unknown. The bitter taste signaling transduction requires the involvement of Ca^{2+} influx [53,54]. It is clarified that increased cytosolic [Ca^{2+}] is reversed by Ca**-ATPase [55,56]. Ca^{2+}-ATPase is responsible for actively maintaining the balance of Ca^{2+} concentration within the cytoplasm and cellular organelles [57]. In the present study, the reduced activity of Ca^{2+}-ATPase revealed that the function of the Ca^{2+} pump was affected in the heart and kidney. In addition, in humans and rodents, mitochondrial dysfunction and oxidative damage could cause Ca^{2+}-ATPase damage [58,59]. Hence, in order to further understand the exact mechanism of Ca^{2+}-ATPase damage in chicken further studies are required. Likewise, in agreement with our results in this experiment, the Ca^{2+}-ATPase activity in low-dose DB and medium-dose DB groups were recovered with an adaptation to denatonium after 28 consecutive days of exposure. Moreover, excessive Ca^{2+} concentration is able to activate the Ca^{2+}-dependent cysteine proteases (calpain family) [60]. The major calpain isoforms are calpain 1 and calpain 2, which are expressed in different tissues including heart and kidney of the chicken [61]. Calpain is demonstrated to be capable of inducing the activation of caspase family, which results in apoptosis [56,62]. The activation of calpain could cause tissue damage, apoptosis and autophagy [62,63]. Calpain 1 (u-calpain) and calpain 2 (m-calpain) require micromolar [Ca^{2+}] and millimolar [Ca^{2+}] to activate, respectively [60]. We speculate that long-term of bitter taste receptor agonist caused [Ca^{**}]$_c$ to increase in heart and kidney epithelial cells in micromolar degree according to the result of enhanced CAPN1 expression and invariant expression of CAPN2. Elevated gene expressions of CAPN1 and apoptosis executioners (BCL2, BCL2L1, Caspase 2, Caspase 3, Caspase 7, Caspase 9, MCL1, BID, NOXA) in high-dose group indicated that high-dose DB induced more apoptosis in the heart and kidney of chicken. The apoptosis result was validated by a TUNEL assays. These data increase the possibility that after administration of DB, bitter taste receptors expressed in the heart and kidney of chicken are involved in the process of apoptosis via a calpain/caspase-dependent mechanism.

Autophagy is an evolutionary conserved catabolic process that includes different forms of digestive pathways, namely macro-autophagy, micro-autophagy, chaperone-mediated autophagy and non-canonical autophagy, regulating the degradation of a cell's own components through the lysosomal

machinery [64]. Dramatically it plays a key homeostatic role in every cell type to maintain the balance between the synthesis, degradation, and consequent recycling of cellular components [65]. Currently, more than thirty different autophagy-related genes have been identified by genetic screening in yeast, and many of these genes are conserved in plants, flies and mammals, respectively [21,66]. Particularly, Bcl-2, a major apoptosis inhibitor, binds Beclin-1 to prevent its interaction with AGT5, thus resulting in the inhibition of autophagic initiation [67]. Conversely, when cleaved by caspase-3, Beclin-1 loses its ability to promote autophagy but renders cells sensitive to apoptosis [68]. Some reports indicate that autophagic degradation prevents apoptosis by eliminating harmful cellular wreckages [69,70], whereas others suggest that boosted autophagy results in increased apoptotic vulnerability [68,71]. However, data in birds are rare. Here, we report our results for the first time to indicate the autophagy-related genes expressions in the heart and kidney of chicken exposed to dietary DB treatment for 28 consecutive days. All selected autophagy-related genes in this experiment (ATG5, Beclin-1, Dyanin, LC3-I, LC3-II and mTOR) had high basal expression levels in the two examined tissues from chicken both at day-07 and day-28, respectively. However, the expressions of autophagy related genes were confirmed by RT and qRT-PCR analysis. Moreover, the expressions level of ATG5, Beclin-1 and mTOR were significantly lower in day-07 and day-28, while, we visualized higher significant expressions for Dyanin, LC3-I and LC3-II in both experimental stages in the heart of chicken. Interestingly, these expressions were in contradiction with the kidney of chicken data, where we observed higher significant expressions of Beclin-1, Dyanin and mTOR at both day-07 and day-28 among other selected genes, respectively. These results suggest that autophagy may play a crucial role in regulating many toxicity- and apoptotic-related complications which may be due to exposute to BD. On the other hand, limited knowledge is available on the role of the effect of the modulation of the autophagy process in the DB exposure context in chickens.

The endogenous cellular defense system consists of a number of antioxidant enzymes and proteins that maintain the cellular redox status, which is critical for various biological processes and functions. Most enzymatic components of this antioxidant defense system are commonly known as "antioxidant enzymes" (e.g., catalase, superoxide dismutase, glutathione peroxidase). Additionally, several experimental works evaluate the activity and expression of such antioxidant enzymes in different physiological conditions as a parameter to assess oxidative stress in a given system. As reported by Yuzhalin and Kutikhin [72]. Long-term accumulation of ROS and high levels of reactive oxygen species (ROS) may enhance oxidative damage at the DNA level. This process may affect several genes responsible for the regulation of proliferation, growth, survival, apoptosis, autophagy, invasion, leading to genomic instability and deregulation of several pathways [72]. Several enzymes, such as super oxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), nitric oxide synthase (NOS), and paraoxon's (PON), function to prevent damage caused by ROS [73].

Therefore, in the present study, our results indicated different expressions level in RT and qRT-PCR analysis for the confirmation of oxidative stress in the heart and kidney of chicken due to exposure to different doses of denatonium benzoate for 28 consecutive days. However, the expressions of GPX1, SOD and CAT were almost similar on both day-07 and day-28 in the heart of chicken but we observed higher significant ($p < 0.005$) expressions among them in the BD high-dose treatment groups. This indicates that the oxidative genes expressions are dose-responsive, and it confirms our previous apoptosis and autophagy results, while similar expression patterns were observed in the kidney of chicken in day-07 and day-28 of the experiment. Remarkably, GPX1, SOD and CAT expressions were significantly ($p < 0.005$) similar, while there were slightly higher expressions in the DB high-dose treatment groups, respectively. These results suggest that oxidative stress damage is correlated with apoptotic and autophagic changes in the heart and kidney of chicken in a dose-responsive manner.

5. Conclusions

In summary, exposure to DB for 28 consecutive days impaired the growth performance of chickens. The present study demonstrates that dietary DB has adverse effects on the heart and kidney

epithelial cells of chickens in a dose-response manner via apoptosis, autophagy and antioxidative status involving bitter taste transduction. DB can increase the oxidative stress and promote the mitochondrial apoptotic pathway via regulating ATP synthesis and mitochondrial apoptosis. Our data provide a novel perspective for understanding the interaction of heart and kidney with strong bitter taste receptor agonist. This might open a new window and maybe helpful for deeper studies of the roles and underlying mechanisms of bitter taste receptors in chickens and could have a great contribution for the improvement of chicken feedstuffs.

Supplementary Materials: The following are available at http://www.mdpi.com/2076-2615/9/9/701/s1.

Author Contributions: Conceived and design the experiments: the work was conceived by E.H. Performed the experiment: E.H., Z.S. & Z.L. Analyzed the data: Data was analyzed by E.H., A.Z. and M.M.R. Contributed reagents/materials/analysis tools: Z.S., Z.L., Q.W. Manuscript writing: Manuscript writing was performed by E.H. All authors reviewed and approved the manuscript.

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Conflicts of Interest: The author declares no conflict of interest is present in this manuscript

Abbreviations

DB	Denatonium Benzoate
ggTas2R1	Gallus Gallus Taste 2 Receptor 1
ggTas2R2	Gallus Gallus Taste 2 Receptor 2
ggTas2R7	Gallus Gallus Taste 2 Receptor 7
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time; Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid

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The Effect of Dietary *Camelina sativa* Oil or Cake in the Diets of Broiler Chickens on Growth Performance, Fatty Acid Profile, and Sensory Quality of Meat

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Simple Summary: Feeding broiler chickens components rich in polyunsaturated fatty acid (PUFA), especially n-3 family fatty acid (*Camelina* oil or expeller) can be an effective way to improve both animal health and meat quality. The rate of mortality was the lowest in the group fed *Camelina* oil or expeller. Broiler chicken meat enriched with bioactive PUFA n-3 can be an alternative source of these fatty acids in the human diet. Introduction to the broiler diet of 40 g/kg *Camelina* oil, as well as 100 g/kg *camelina* expeller cake, significantly increased PUFA n-3 fatty acid and lowered PUFA n-6/PUFA n-3 fatty acid ratio. Furthermore, meat of chickens fed with *Camelina* oil was characterized by better juiciness.

Abstract: The aim of the present study was to determine the effect of supplementing the diets of broiler chickens with *Camelina sativa* oil or cake as a source of polyunsaturated fatty acids (PUFAs) on their growth performance, fatty acid profile, and sensory quality of meat. The 456 Ross 308 broilers aged 21–42 days were divided into 3 groups with 4 replicates of 38 birds in each. Chickens in the control group I (CTR) were fed a standard grower–finisher feed mixture containing 60 g/kg rapeseed oil. The experimental components, *C. sativa* oil—CSO (group II) or cake—CSC (group III), were included in a diet based on wheat and soybean at 40 and 100 g/kg, respectively. The use of *Camelina* oil and cake as feed components did not have a significant effect on the growth performance of the chickens. Analysis of the fatty acid profile in the lipids of the breast muscles showed that *Camelina* oil and cake reduced the content of monounsaturated fatty acids ($p < 0.05$) but increased the content of n-3 polyunsaturated fatty acids, especially α-linolenic acid (C18:3) ($p < 0.01$). Furthermore, both components reduced the ratio of n-6/n-3 PUFAs in the breast muscles ($p < 0.01$). Sensory analysis revealed that *Camelina* oil had a beneficial effect on meat juiciness, whereas *Camelina* cake slightly worsened the flavor and tastiness of the meat. In conclusion, supplementing the diet of broiler chickens with *Camelina* oil or cake can be an efficient method for modifying the fatty acid profile of the meat lipids in a beneficial way, without any negative impact on the growth performance of the chickens. According to the dietetic recommendations for humans, broiler chicken meat with a higher level of PUFA n-3 can be a good alternative source of these fatty acids in the human diet. Furthermore, *Camelina* oil improved the juiciness of breast meat.

Keywords: *Camelina* oil; *Camelina* cake; polyunsaturated fatty acids; growth performance; broiler chicken

1. Introduction

The ratio of n-6/n-3 polyunsaturated fatty acids (PUFAs) in the feed mixtures used for fast-growing broiler chickens plays a significant role in the prevention of metabolic disturbances [1] and heart

failure, which is a cause of sudden cardiac death [2–4]. Numerous studies have indicated that the main cause of the sudden death of birds is the high content of n-6 PUFA in the feed mixtures. The results showed that the serum and heart muscle of these birds contained increased amounts of arachidonic acid (AA; C20:4) and a reduced total level of n-3 PUFA, especially eicosapentaenoic acid (EPA; C20:5n-3) [3,5,6]. Furthermore, the fatty acid profile of meat lipids is a significant factor determining meat quality. The ratio of n-6/n-3 PUFAs in chicken meat ranges from 7:1 to 15:1, and the breast muscles (*Pectoralis major*) are characterized by the most beneficial proportion [7,8]. The wide variation of this ratio is the result of using feed mixtures based on cereal seeds (corn, barley, wheat, and triticale) and plant oils (sunflower, corn, and soybean), as well as oilseeds characterized by a high content of n-6 linolenic acid [9].

Studies have revealed that the meat of chickens, similar to other monogastric organisms, can be efficiently enriched with n-3 PUFA by using an appropriate diet [10,11]. It was found that the use of feed mixtures supplemented with oils as a source of n-3 PUFA during the second growth phase of broiler chickens modified the fatty acid profile of meat lipids in a beneficial way [12,13]. The introduction of oil rich in α-linolenic acid (ALA) in the feed mixture used for broiler chickens increased the concentration of this acid and its long-chain derivatives, including EPA, DPA, and docosahexaenoic acid (DHA) in the meat lipids, which resulted in a decreased proportion of n-6/n-3 PUFAs [8,12]. Compared to mammals, broiler chickens have a greater ability to convert ALA to long-chain derivatives due to higher activity and wider substrate specificity of elongases responsible for the conversion of DPA to C24:5n-3 and then to DHA [14,15].

According to Haug et al. [8], the inclusion of poultry meat as a potential source of n-3 PUFA in the diet of contemporary populations can contribute to reducing the risk of cardiovascular diseases. In their experiment, the authors modified the fatty acid profile of the leg and breast muscles in broiler chickens by using a feed supplemented with a mixture of rapeseed and flax oil as a source of ALA. People consuming such enriched meat for 4 weeks showed an increased concentration of EPA in the serum. The results of other authors have also indicated that the meat of broiler chickens can be included as a potential source of n-3 PUFA in the human diet [11,16,17].

The oil of *Camelina sativa* is one of the richest known plant sources of ALA of the n-3 group [18,19]. *Camelina sativa* is an oil plant that attracts renewed interest of industry and agriculture after it was replaced in the post-war period by higher yielding rapeseed. The renewed interest in *Camelina sativa* results from a higher demand for fat raw materials necessary for production of biofuels. *Camelina* belongs to the oldest crop plants of the *Brassicacea* family. Soil requirements of this plant are modest; it can grow on poor soils and is resistant to drought and frost [20]. Moreover, it requires a lower fertilization rate than rapeseed and is resistant to insect pests [19,20]. *Camelina* oil is considered valuable mostly due to its nutritional values and chemical composition [19]. In particular, the cold-pressed oil is characterized by a high content of PUFAs and natural antioxidants such as tocopherols (791 mg/kg) [20] that make it exceptionally durable and fit for human consumption for 6 months [18]. It is also distinguished from other oils by a special taste and pleasant clear flavor of medium intensity. *Camelina* oil of a domestic variety has been shown to have high efficacy in modifying the fatty acid profile of meat lipids in broiler chickens [21–23]. In addition, it was observed to have a beneficial effect in reducing the ratio of n-6/n-3 PUFAs [22,23]. On the other hand, *Camelina* cake is characterized by high protein content (up to 45%) with a beneficial composition of amino acids [24] and fat content with a high proportion of ALA [19,25]. The energy value of *Camelina* cake in poultry, pig, and cattle was estimated at 8.0, 14.0, and 15.0 MJ ME/kg DW, respectively [24]. However, due to the presence of non-starch polysaccharides and glucosinolates, adding a high percentage of *Camelina* cake in the feed mixture can adversely affect the growth performance of broiler chickens [26,27].

Therefore, the aim of the present study was to determine the effect of *C. sativa* oil or expeller cake included as components in the diets of broiler chickens on the growth performance, fatty acid profile of lipids in the breast muscle (*Pectoralis major*), and sensory quality of meat.

2. Materials and Methods

2.1. Birds, Housing, and Feeding

The experiment was carried out according to the guidelines of the Ethics Committee for the Use of Animals in Research. No explicit approval of the committee was needed because the birds were only fed different diets (none of them were toxic—the regulation 68/2013 (16 January 2013) of the European Union Commission allowed the use of *C. sativa* seeds and products obtained by their processing, including oil and *Camelina* meal, as a feed component in animal diets) and no invasive procedures were performed on them. A total of 456 Ross 308 broiler chickens (hens and cockerels) were raised in group pens on litter from 1 to 42 days of age under standard housing conditions with free access to feed and water. During the first growth phase (1–21 days of age), the chickens were fed a starter feed mixture which did not contain the tested additives. In the second period of rearing (22–42 days of age), chickens were randomly divided into 3 groups with 4 replicates of 38 birds each. The chickens in the control group CTR (group I) were fed a standard grower diet containing 60 g/kg of rapeseed oil, whereas birds in the experimental groups were fed a feed mixture containing 40 g/kg of cold-pressed oil obtained from the seeds of spring *C. sativa* var. Borowska—CSO (group II) and 20 g/kg of rapeseed oil, or 100 g/kg of expeller cake—CSC obtained from *Camelina* of the same variety and 50 g/kg of rapeseed oil (group III). The composition and nutritional value of the grower feed mixtures used for the broiler chickens are presented in Table 1. Diets were formulated to provide nutrients according to the Polish recommendations for broilers [28] and to contain the same amount of metabolizable energy and crude protein within each set.

Table 1. Composition and the calculated nutrient contents of grower–finisher feed mixtures (g/kg).

Components (g/kg)	Group		
	CTR	**CSO**	**CSC**
Maize, ground	480.0	480.0	500.0
Wheat, ground	104.6	104.6	49.5
Soybean meal (46% protein)	315.0	315.0	260.0
Rapeseed oil	60.0	20.0	50.0
Camelina oil	–	40.0	–
Camelina cake	–	–	100.0
Ground limestone	11.5	11.5	12.5
Phosphate 2-Ca	17.0	17.0	16.0
NaCl	3.5	3.5	3.5
DL-Methionine	2.1	2.1	1.8
L-Lysine HCl	1.3	1.3	1.7
Vitamin–mineral premix (0.5%) *	5.0	5.0	5.0
Metabolic energy (MJ)	13	13	13
Total protein (g)	200.0	200.0	200.0
Lysine (g)	11.5	11.5	11.5
Methionine (g)	5.2	5.2	5.2
Ca (g)	9.2	9.2	9.2
P available (g)	4.0	4.0	4.0

* Vitamin–mineral premix provided the following (per kg of feed mixture): Retinyl acetate—10,000 IU; cholecalciferol—2000 IU; tocopherol—20 mg; menadione sodium bisulfite—2.0 mg; thiamine—1.5 mg; riboflavin—5 mg; pyridoxine—3 mg; cyanocobalamin—0.02 mg; Ca-pantothenate—12 mg; folic acid—1 mg; biotin—1 mg; niacin—25 mg; choline chloride—400 mg; manganese—100 mg; iodine—0.8 mg; zinc—65 mg; selenium—0.2 mg; and copper—8 mg. Abbreviations: CTR, control group; CSO, *C. sativa* oil group; CSC, *C. sativa* cake group.

2.2. Data Collection and Chemical Analyses

The individual body weight of the chickens was determined at 1, 21, and 42 days of age, and the number of dead birds was noted throughout the experiment. Feed intake by the groups was determined for each pen. On the basis of the experimental data collected, the following basic parameters of

production were calculated: Body weight gain (BWG), feed conversion ratio (FCR) per kilogram BWG, and mortality of birds. At the end of the experiment, at 42 days of age, 8 birds from each group (4 cocks and 4 cockerels) were slaughtered using a method adapted to their age, species, and body weight. The procedure was carried out in accordance with the Annex IV of European Parliament and Council Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes. If no pain or suffering was inflicted during the trial, the regulations allowed sacrificing the experimental birds before sampling. Accordingly, the birds were electrically stunned and then decapitated. The mass of fresh carcasses (after slaughter), as well as mass of cold carcasses (after cooling it for 24 h in temperature of +4 °C for 24 h), were determined. The simplified slaughter analysis of the carcasses was performed after cooling them at +4 °C for 24 h [29]. Samples of the breast muscles were collected for further analysis.

The diets were analyzed for the profile of higher fatty acids (HFA) and the content of tocopherols and tocotrienols. The content of HFA was determined using the modified method by Loor and Herebain [30] based on ISO 12966–2:2011. The fatty acids were separated in the form of methyl esters and determined using a VARIAN 3400 gas chromatograph with a flame ionization detector (250 °C, range = 11; carrier gas: helium, 3 mL/min; gas injection: 0.7 µL) and an RTX™-2330 capillary column (105 m × 0.32 mm, 0.2 µm). Tocopherols and tocotrienols were determined using liquid chromatography, according to the method described by Manz and Philip [31], with a Merck-Hitachi HPLC system equipped with a LiChroCART® 250–4 Superspher® 100 RP-18 cartridge on a 4-µm column and an FL detector (Ex. 295 nm and Em. 350 nm).

The collected samples of the breast muscles (*musculus Pectoralis major*) were analyzed for basic chemical composition, fatty acid profile, sensory parameters, and malonaldehyde content after 90 days of frozen storage (−20 °C). The basic chemical composition of the breast meat was determined according to the AOAC method [32]. The content of fatty acids in meat lipids was determined in the form of methyl esters using gas chromatography according to the procedures validated by the Central Laboratory of the National Research Institute of Animal Production in Aleksandrowice, Poland. Fat was extracted from the samples with a mixture of chloroform and methanol (2:1) according to the modified method of Floch et al. [33], and the extract was evaporated at 65 °C under nitrogen. The residue was saponified with 0.5 NaOH in methanol (80 °C, 20 min), and then esterified with BF in methanol [34] at 80 °C for 10 min, followed by the addition of hexane. After salting out with a saturated NaCl solution, the hexane layer was collected into a chromatographic vial and subjected to gas chromatography using a VARIAN 3400 system with an RTX-2330 capillary column (105 m × 0.32 mm, 0.2 µm; detector range = 11, 250 °C; carrier gas: helium, 3 mL/min), a Varian 8200 CX Autosampler, and Varian Star 4.5 software package for data analysis.

The TBA values in the breast meat were expressed as milligrams of malonaldehyde (MDA) per kilogram of meat. To determine TBA, meat samples were prepared according to a modified version of the method of Salih [35], as modified and described by Pikul [36]. TBA values were measured by the colometric method at the presence of 2-thiobarbituric acid.

Breast meat samples held for sensory analysis were frozen at –20 °C until evaluation. Breast meat was analyzed after cooking to determine the sensory impact of the tested components used on flavor and tastiness quality. The sensory evaluation of meat samples was conducted by eight internal panelists. The day before the analysis, 200 g of each sample was thawed at 4 °C, and cooked individually in a covered container in 400 mL of 0.6% saline, until the temperature inside the meat reached 70 °C. The temperature was measured using a special thermometer. After cooling, the meat samples were evaluated within 10 min. The flavor, juiciness, tenderness, and tastiness of the meat were evaluated based on a 5-point scale, where 5 meant strong appreciation and 1 an extreme dislike, on the basis of the method described earlier by Matuszewska and Baryłko-Pikielna [37].

2.3. Statistical Analysis

For evaluating the growth performance during the experimental period of 21–42 days, a total of 162 birds per treatment with 4 replications each were considered. As the other results were analyzed by including 8 replications per treatment, statistical analysis of the obtained indices was performed using a one-way analysis of variance. The significance of differences between the experimental groups was evaluated using the multiple-range Duncan test. The differences were deemed statistically significant at a confidence level of $p < 0.05$. The procedures were carried out using the SAS statistical package (version 9.2), procedure GLM.

3. Results

The addition of *Camelina* seed oil or expeller to the grower feed mixture used for broiler chickens influenced its PUFA profile (Table 2).

Table 2. Fatty acid profile of the feed mixtures used for broiler chickens (% of the sum of fatty acids).

Item	Group		
	CTR	CSO	CSC
C16:0	13.764	12.270	9.062
C16:1	0.265	0.199	0.176
C18:0	2.990	2.832	2.095
C18:1	66.191	45.169	42.387
C18:2	9.074	23.655	31.440
γC18:3	0.113	0.247	0.171
C20:0	0.567	0.595	0.445
C18:3	1.353	11.593	12.648
C20:4	0.043	0.494	0.379
C22:1	0.444	1.368	0.788
SFA	19.208	16.141	11.781
MUFA	66.900	46.736	43.351
PUFA	33.891	37.122	44.868
PUFA n-6	9.230	24.397	31.989
PUFA n-3	1.353	11.593	12.648
MUFA/SFA	3.483	2.895	3.680
PUFA/SFA	0.723	2.300	3.808
PUFA n-6/PUFA n-3	6.824	2.104	2.529

The use of *C. sativa* oil or expeller cake reduced the content of saturated fatty acids (SFAs) from 19% (found in the feed mixture used for control group) to 16.1% and 11.7%, respectively, in the feed mixture used for experimental groups, and increased the content of n-3 PUFA (from 1.5% to approximately 12%), especially ALA. The supplemented feed mixtures were characterized by a narrow ratio of n-6/n-3 PUFAs amounting from 2.1 to 2.5 compared with the control feed mixture (6.8). The contents of α-tocopherol in the feed were similar in all the groups. Supplementing the diet with *Camelina* oil, and especially cake, increased the content of γ-tocopherol by 20.4% and 110%, respectively, and slightly decreased the level of β-tocopherol (Table 3).

Table 3. Contents of natural antioxidants in the feed mixtures used for broiler chickens (mg/kg of feed).

Parameter	Group		
	CTR	CSO	CSC
α-Tocopherol	40.01	39.42	39.48
β-Tocopherol	2.67	3.03	2.31
γ-Tocopherol	30.4	36.6	63.9
δ-Tocopherol	3.26	4.01	5.7
α-Tocotrienol	2.44	3.03	3.08
β-Tocotrienol	7.5	8.18	4.82
γ-Tocotrienol	1.79	1.86	3.72
δ-Tocotrienol	0.36	0.35	0.46

The level of gamma-tocopherol in the feed mixture used for experimental groups was 36.6 (CSO group) and 63.9 mg/kg (CSC group), while that in the feed used for the control group was 30.4 mg/kg. The content of delta-tocopherol in the feed containing *Camelina* oil (4.01 mg/kg) or cake (5.7 mg/kg) was higher compared with the feed used for the control group (3.26 mg/kg) by approximately 23% and 75%, respectively.

Rearing results of broiler chickens are shown in Table 4. At the first period of rearing, there were no significant differences in the body weight of chickens and feed consumption, as well as in feed consumption ratio between the groups. Mortality rate in each group was similar and ranged from 0.91 to 1.29%. The addition of *Camelina* oil or cake to feed mixtures during the second growth phase did not significantly affect the BWG of the experimental groups compared with the control group. Moreover, the feed intake and conversion per kilogram of BWG remained at a similar level in all of the groups. Most birds that died during the second growth phase and the whole experimental period belonged to the CTR group (Table 4).

Table 4. Production parameters of broiler chickens. Abbreviations: BWG, body weight gain.

Variable	Age (d)	Groups			SEM	*p*-Value
		CTR	CSO	CSC		
BWG (g/bird)	1–22	666	671	679	5.17	0.210
	22–42	1856	1834	1734	26.35	0.117
	1–42	2522	2505	2413	24.35	0.509
Feed intake (g/bird)	1–22	934	957	955	7.95	0.288
	22–42	3075	3025	2959	27.41	0.654
	1–42	4019	3937	3977	23.44	0.684
Feed conversion ratio (kg/kg BWG)	1–21	1.40	1.43	1.34	0.02	0.208
	22–42	1.66	1.62	1.75	0.03	0.393
	1–42	1.59	1.57	1.65	0.02	0.259
Percentage of dead and culled birds (%)	1–22	1.28	1.29	0.91	-	-
	22–42	1.31	0.65	0.56	-	-
	1–42	2.60	1.31	1.74	-	-

The addition of *Camelina* oil or cake to the diet did not affect the carcass weight and slaughter yield (Table 5). However, a reduction in the percentage of abdominal fat was noted in the carcasses in the CSO and CSC groups. The carcasses of chickens fed *Camelina* oil (CSO) were characterized by the largest weight and the greatest percentage of breast muscles ($p < 0.05$) and the lowest fat content. The carcasses of chickens in the CSO and CSC groups also showed the lowest percentage of the liver compared with the CTR group. A significant ($p < 0.05$) reduction in proportion of skin with subcutaneous fat was observed in the carcasses of chickens from the CSO group.

Table 5. Slaughter analysis of the carcasses of broiler chickens.

Item	Group			SEM	*p*-Value
	CTR	CSO	CSC		
Fresh carcass weight (g)	1865.00	1887.50	1798.75	44.06	0.615
Cold carcass weight (g)	1793.75	1816.25	1725.00	38.05	0.574
Carcass yield (%)	71.79	72.53	72.74	1.85	0.481
Percentage of muscles (%)					
-Breast	28.25 [a]	28.69 [a]	26.13 [b]	2.03	0.020
-Leg	21.29	21.71	21.52	1.73	0.259
Percentage of the liver (%)	2.57	2.48	2.48	0.22	0.888
Percentage of abdominal fat (%)	1.67	1.58	1.52	0.03	0.777
Weight of the skin with subcutaneous fat (%)	6.10 [a]	5.88 [b]	5.91 [a]	0.38	0.759

a, b—the mean values in a row marked with different letters differ statistically significantly at $p < 0.05$.

The use of *Camelina* oil and cake as grower diet components for broiler chickens did not significantly affect the dry mass and the content of total protein and crude fat in the breast muscles (*Pectoralis major*) (Table 6).

Table 6. Results of the chemical analysis (%) of the breast muscles (*Pectoralis major*).

Item	Group			SEM	*p*-Value
	CTR	CSO	CSC		
Dry mass	25.72	25.25	25.90	0.10	0.449
Total protein	23.89	23.47	23.81	0.13	0.521
Crude fat	1.06	1.10	1.15	0.08	0.183

The results of the analysis of PUFAs in the lipids of the breast muscles (*Pectoralis major*) demonstrated that *Camelina* oil and cake caused a highly statistically significant ($p < 0.01$) increase in the content of n-3 PUFA, especially ALA (Table 7).

In addition, a significant increase ($p < 0.01$) in EPA was noted in the CSO group, which was fed with the diet supplemented with 4% *Camelina* oil. In the CSO group, as well as in the CSC group, the content of AA (C20:4) belonging to n-6 PUFA was significantly reduced ($p < 0.05$). The ratio of PUFA/SFA was significantly higher and the ratio of n-6/n-3 PUFAs was significantly reduced ($p < 0.01$) in groups II and III, compared with the control group. In the CSO and CSC groups, the content of monounsaturated fatty acids (MUFAs) was statistically significantly reduced ($p < 0.01$) compared with the CTR group, while the level of erucic acid (C22:1) was significantly increased ($p < 0.01$). In the CSC group receiving the *Camelina* oil-supplemented diet, the SFA content was significantly increased compared to the CTR and CSC groups ($p < 0.05$), mostly due to the increased levels of palmitic acid (C16:0) ($p < 0.01$) and stearic acid (C18:0) ($p < 0.05$). Among the acids of this group, significant increases were observed in AA (C20:0) ($p < 0.01$) and behenic acid (C22:0) ($p < 0.05$). The addition of 4% *Camelina* oil to the diet also significantly increased the content of conjugated linoleic acid (CLA) in the CSO group ($p < 0.01$).

Table 7. Fatty acid profile of the lipids of the breast muscles (*Pectoralis major*) (% of the sum of acids).

Item	Group			SEM	*p*-Value
	CTR	CSO	CSC		
C16:0	19.97 ^{A,B}	21.42 ^{A}	19.59 ^{B}	1.282	0.0001
C18:0	8.24 ^{a,b}	8.63 ^{a}	7.56 ^{b}	0.885	0.045
C16:1	1.57	1.81	1.57	0.140	0.367
C18:1	41.24 ^{A}	34.58 ^{C}	37.95 ^{B}	3.210	0.0001
C18:2	17.45 ^{B}	17.61 ^{A,B}	18.94 ^{A}	0.928	0.009
γC18:3	0.88	0.94	0.94	0.004	0.793
C20:0	0.15 ^{B}	0.21 ^{A}	0.21 ^{A}	0.021	0.006
C18:3	3.57 ^{B}	8.07 ^{A}	7.26 ^{A}	0.954	0.0001
C22:0	0.13 ^{b}	0.29 ^{a}	0.24 ^{a,b}	0.017	0.029
C20:4	3.88 ^{a}	3.04 ^{b}	2.91 ^{b}	0.793	0.035
C22:1	0.08 ^{B}	0.14 ^{A}	0.14 ^{A}	0.002	0.007
EPA	0.62 ^{B}	1.00 ^{A}	0.71 ^{B}	0.031	0.0001
DHA	1.03	1.02	0.98	0.139	0.952
SFA	29.84 ^{a,b}	31.79 ^{a}	28.68 ^{b}	2.163	0.029
MUFA	42.89 ^{A}	36.53 ^{C}	39.66 ^{B}	2.031	0.0001
PUFA	27.27 ^{B}	31.68 ^{A}	31.65 ^{A}	1.643	0.0001
PUFA n-6	21.43	20.75	21.94	1.416	0.155
PUFA n-3	5.21 ^{C}	10.09 ^{A}	8.94 ^{B}	0.577	0.0001
PUFA/SFA	0.92 ^{B}	1.00 ^{A,B}	1.11 ^{A}	1.448	0.006
PUFAs n-6/n-3	4.12 ^{A}	2.08 ^{C}	2.46 ^{B}	0.369	0.0001
CLA	0.63 ^{B}	0.86 ^{A}	0.77 ^{A,B}	0.010	0.0009
C18:1/C18:0	5.00 ^{a}	4.00 ^{b}	5.02 ^{a}	0.098	0.028

a, b—the mean values in a row marked with different letters differ statistically significantly at $p < 0.05$; A, B, C—the mean values in a row marked with different letters differ statistically significantly at $p < 0.01$.

After 3 months of frozen storage (−20 °C), the content of malonaldehyde was found to be reduced (Figure 1) by 19% and 20%, respectively, in the meat of the broiler chickens in the CSO and CSC groups compared with the CTR control group. However, statistical analysis did not confirm the significance of these differences.

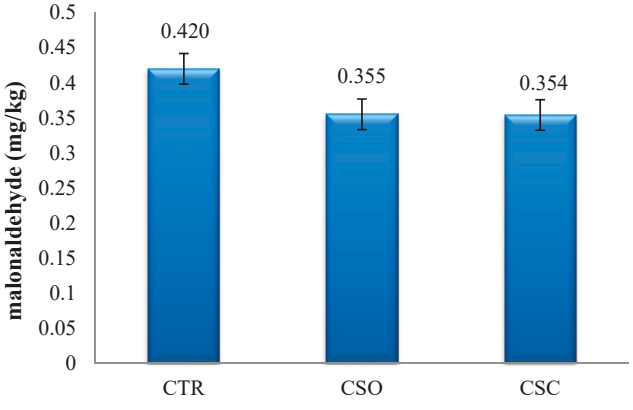

Figure 1. Malonaldehyde content (mg/kg of sample) in the breast muscles (*Pectoralis major*) of broiler chickens fed with rapeseed oil—CTR group, *Camelina* oil—CSO group, and *Camelina* expeller cake—CSC group.

Supplementation of the diet of broiler chicken with *Camelina* oil (CSO group) significantly ($p < 0.05$) influenced the juiciness of the cooked meat compared with the control group (Table 8). On the other hand, the meat of the CSC group chickens fed *Camelina* cake-supplemented diet was characterized by an inferior tastiness and flavor.

Table 8. Results of the sensory analysis of the breast muscles (*Pectoralis major*) of broiler chickens (according to a 5-point scale: 5—the highest score; 1—the lowest score).

Parameter	Group			SEM	*p*-Value
	CTR	CSO	CSC		
Flavor	4.40	4.35	4.05	1.850	0.679
Juiciness	4.05 ^{b}	4.62 ^{a}	4.25 ^{a,b}	1.550	0.029
Tenderness	4.20	4.35	4.26	0.126	0.735
Tastiness	4.15 ^{a,b}	4.20 ^{a}	3.88 ^{b}	0.948	0.043

a, b—the mean values in a row marked with different letters differ statistically significantly at $p < 0.05$.

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

4. Discussion

The quality and nutritional value of oilseeds, oil pressed from them, and the by-products of oil production depend on their chemical compositions, fatty acid profile, and especially, the contents of antinutrients [26,27,38]. Recent studies have indicated that the oil and cake of *C. sativa* can be used as a source of PUFAs, mostly of the n-3 group, and natural antioxidants such as tocopherols without any impairment of the sensory quality of the poultry products [39–42]. Antinutrients contained in *Camelina* seeds include mostly glucosinolates, the content of which mainly depends on the variety and environmental conditions that prevail during the plant vegetation [38]. Moreover, the seeds contain crude fiber which can also have a negative impact on the production performance of chickens. Thus, the efficiency of production depends on the choice of *Camelina* variety and the percentage of seeds or by-products of oil pressing in the feed mixtures.

In the present study, the addition of 4% *Camelina* oil to the diet of broiler chickens did not significantly affect their growth performance. Moreover, *Camelina* oil had no effect on the final body weight and FCR. Similar results were shown by Pietras and Orczewska-Dudek [23], who investigated the effect of the addition of 3% and 6% dietary *Camelina* oil to broiler diet. In addition, Jaśkiewicz et al. [21] demonstrated that the addition of *Camelina* oil to broiler diet both during the first (1.43%) and second phase of growth (2.16%) did not adversely affect the production parameters. Analogous results were reported by Jaśkiewicz et al. [22] who used 6.91% *Camelina* oil in the starter diet and 4.07% *Camelina* oil in the grower diet.

The addition of *Camelina* cake to feed mixtures caused a slight reduction in growth rate, and thus led to a lower BWG in chickens. Feed conversion per kilogram of BWG was also found to be slightly increased in this group; however, the differences were not confirmed as significant by statistical analysis. Similar results were obtained by Aziza et al. [42,43] who supplemented the feed with 2.5, 5, and 10% *Camelina* cake. These authors indicated that the addition of *Camelina* cake to the diet of broiler chicken at 10% did not impair the production performance. On the other hand, Pekel et al. [44] demonstrated a negative effect of *Camelina* cake supplement on the production performance of chickens. They found that the addition of 10% *Camelina* cake in the feed mixture used for broiler chickens diminished their growth between 15 and 37 days of age, reducing feed intake, which resulted in a significant reduction of the final body weight. This could probably have resulted from a higher content of glucosinolates in *Camelina* cake used in their experiment, a higher level of fiber, and a limitation of nutrient availability [38].

Valkonen et al. [45] showed that increasing the content of *Camelina* cake (from 0 to 25%) in the feed mixtures used for broiler chickens produced a linear negative effect on the feed consumption, body weight, and feed conversion, but a beneficial effect in lowering the mortality. The authors indicated that the best growth performance was obtained using 5% and 10% *Camelina* cake in the feed. Moreover, the study by Widyaratne [46] demonstrated that when the percentage of *Camelina* cake in the feed mixtures used for broiler chickens was increased from 3 to 15%, the BWG of chickens and feed intake decreased in direct proportion to the increase. *Camelina* cake also had a negative effect on feed conversion (kg/kg BWG). Furthermore, when *Camelina* seeds were added to feed mixture at 30%, a slower growth rate and a low final body weight were observed. It was observed [41] that the addition of 5% and 10% *Camelina* cake to the diets of turkeys led to both growth depression and reduction of feed intake in the birds. These discrepancies between the present study and the above-mentioned studies could probably be due to the use of different varieties of *Camelina* grown under different climatic conditions. This was confirmed by the study of Waraich et al. [20], who indicated that the *Camelina* variety, climatic conditions during vegetation, and fertilization program influenced the contents of fatty acids, vitamins, and glucosinolates in seeds.

The lower percentage of dead birds noted during the second growth phase in groups fed with the diet containing *Camelina* oil or cake was in agreement with the results obtained by Aronen et al. [47]. It can be expected that the beneficial effect of *Camelina* oil is associated with its high content of ALA, which contributes to a reduction in the formation of pro-inflammatory eicosanoids when chickens are fed a diet deficient in ALA but containing high levels of phytosterols. Moreover, it was found that both *Camelina* oil and cake are a rich source of ALA of n-3 group and caused a reduction in the ratio of n-6/n-3 PUFAs in the feed mixtures used for broiler chickens which, according to Chen et al. [48], propitiously increased the level of antioxidant enzymes in the heart muscle and improved the immunological function of the thymus. In addition, Świątkiewicz et al. [49] suggested that n-3 PUFA improved the immunological functions in animals; in particular, they reduced the prevalence of acute and chronic inflammatory response generated towards harmful factors.

The present study demonstrated that the addition of 10% *Camelina* cake significantly reduced the proportion of the breast muscle in the carcasses by 7.5% compared with the control group. However, Aziza et al. [42,43] did not notice such a relationship. These authors concluded that irrespective of the percentage, *Camelina* cake did not have any negative effect on the quality and tissue composition of the chicken carcasses. In our study, it was observed a reduction in the content of abdominal fat in the carcasses of chickens that received the feed mixture supplemented with 4% *Camelina* oil or 10% cake. These results are in accordance with the data obtained earlier in the study by Pietras and Orczewska-Dudek [23], who also observed a reduction in the percentage of abdominal fat and weight of skin with subcutaneous fat in the carcasses of chickens fed with diet containing 6% *Camelina* oil.

On the other hand, Jaśkiewicz et al. [22] did not notice any significant effect of 2.04% *Camelina* oil added in the feed mixture used for chickens on the content of abdominal fat in the carcasses. According to Valkonen et al. [45], the amount of abdominal fat in the carcasses of chickens that were fed the diets supplemented with *Camelina* cake linearly decreased with an increase in the content of *Camelina* cake in the feed (from 5 to 10%). Similar results were reported by Crespo and Esteve-Gracia [13] and Ferrini et al. [50], who revealed that PUFAs reduced the accumulation of abdominal fat in contrast to SFAs and MUFAs. According to Takeuchi et al. [51] and Sanza et al. [52], PUFAs inhibited the synthesis of lipids in the liver and enhanced the processes of thermogenesis. This mechanism explains why PUFAs reduce fat in the abdomen and other parts, and as a consequence, decrease the total content of fat in a carcass [52,53]. In our study, both *Camelina* oil and cake did not affect the content of crude fat in the breast muscles of the broiler chickens. This is in line with the reports of other authors [13,54], who concluded that the source of fat in feed mixtures and their fatty acid profile did not influence the content of crude fat in the meat samples of broiler chickens.

The results of the analysis of HFA in the lipids of the breast muscles (*Pectoralis major*) indicate that both *Camelina* oil and cake significantly reduced the percentage of MUFAs, especially oleic acid, and

increased the percentage of PUFAs, mostly of the n-3 group. According to the dietetic recommendations for humans, reducing the ratio of n-6/n-3 PUFAs is desirable because a narrow ratio of n-6/n-3 PUFAs is beneficial for maintaining a proper balance between eicosanoids formed from both groups of fatty acids [55,56]. The obtained results confirmed the data reported by other authors [22,23,42,43]. According to Thacker and Widyaratne [46], the addition of 15% *Camelina* cake induced a statistically significant increase in the content of n-3 PUFA and beneficially narrowed the ratio of n-6/n-3 PUFAs. In addition, Nain et al. [57] indicated that feeding broiler chickens with both a mixture enriched with 24% *Camelina* cake for 28 days and 16% *Camelina* cake for 42 days significantly increased the content of n-3 PUFA in the lipids of the breast and leg muscles, exceeding the content of 300 mg/100 g of meat.

In the present investigation, the lowest level of MUFAs was observed in the breast muscles from chickens that were fed with the mixture supplemented with 4% *Camelina* oil. In addition, the desaturation index SCD-1 (C18:1/C18:0) was significantly lower in this group, which can indicate a reduction in the activity of stearoyl-CoA desaturase which catalyzes the synthesis of MUFAs in the liver [58]. Moreover, Paton and Natambi [59] and Green et al. [60] confirmed the role of this enzyme as an inhibitor of the synthesis of MUFAs from SFAs, especially of the transformation of stearic acid into oleic acid and palmitic acid into palmitoleic acid. In the present study, the content of EPA was found to be statistically significantly increased in the experimental groups. The increase in EPA resulted from the elevated content of ALA, a precursor of long-chain fatty acids, which was confirmed by the studies Azcon et al. [61] and Jiang et al. [62]. It was found from the experiment that the addition of *Camelina* oil or cake to broiler diet significantly modified the fatty acid profile of the lipids of the breast muscle (*Pectoralis major*), leading to a significant increase in ALA (C18:3n-3).

Other authors [11] also observed that an oil rich in PUFAs influenced the lipid metabolism, leading to a greater accumulation of ALA in tissue lipids and reduction of SFA. As explained earlier, the increase of SFAs in the lipids of breast muscles (*Pectoralis major*) of broiler chickens fed with a mixture enriched with 4% *Camelina* oil could be caused by the suppression of the transformation of SFAs to MUFAs due to the diminished activity of an enzyme participating in these reactions. It is supposed that the highest increase in CLA also observed in this group additionally contributed to the reduction in the activity of SCD-1, which significantly decreased the content of SFAs in the lipids of the breast muscle. The group that received the feed mixture supplemented with *Camelina* cake showed a significantly increased content of linoleic acid (C18:2 n-6). LA is biologically converted into AA, the level of which was found declined in the lipids of the breast muscle (*Pectoralis major*) of chickens fed with *Camelina* oil or cake. Betti et al. [63] also noted in their study that the increase in n-3 PUFA resulted in a reduction of AA in the phospholipids of the breast muscle.

The results of several studies have demonstrated that the meat of animals containing more PUFAs is more susceptible to oxidative processes [63], which has a negative impact on its organoleptic characteristics and shelf life [11]. Supplementation of α-tocopherol to the chicken diet increases its content in body tissues and limits the oxidation of fat in the breast muscles (*Pectoralis major*) [42,43,64]. It was also confirmed that malondialdehyde (MDA) content in the breast muscles (*Pectoralis major*) of broiler chickens in the experimental groups, measured after 3-month frozen storage, was lower by 6% compared with the control group. The high content of natural antioxidants such as tocopherols and tocotrienols in *Camelina* oil and cake increased tocopherol content in the feed, which resulted in its increase in cell membranes, thus slowing down the oxidation of the lipids in the breast muscle (*Pectoralis major*). Moreover, *Camelina* oil was found to contain high levels of phytosterols and phenolic compounds that also contribute to the limitation of PUFA oxidation.

The obtained results agree with those reported by Aziza et al. [42] who discovered that *Camelina* cake added to feed mixture at 10% efficiently restricted the oxidation of fatty acids and improved the oxidative stability of meat lipids. In the present study, supplementation of the feed mixture with 4% *Camelina* oil did not impair the organoleptic quality of the cooked meat, and even significantly improved its juiciness. The obtained results were consistent with the earlier observations of Pietrsa and Orczewska-Dudek [23]. However, these authors did not note the effect of 3% and 6% *Camelina*

oil on the juiciness of breast muscle (*Pectoralis major*) in chicken. The current study showed that the addition of 10% *Camelina* cake to the feed mixture used for broiler chickens had a less favorable effect on the sensory quality by worsening the flavor and tastiness of the meat. In contrast, a beneficial effect of 5% *Camelina* cake added as a supplement to the feed mixture used for broiler chickens on the tenderness and juiciness of the meat was documented [41]. Such an effect was not observed when the content of *Camelina* cake was increased to 10%, which was also noticed in presented study. In addition, Valkonen et al. [45] indicated that *Camelina* cake had a favorable effect on the sensory properties of the breast muscles.

5. Conclusions

On the basis of the obtained results, it can be concluded that the addition of *Camelina* oil or expeller to the diet of broiler chickens can be an efficient method for modifying the fatty acid profile of the meat lipids in a way that is beneficial according to the dietetic recommendations for humans, without compromising the growth performance of the birds.

A high content of tocopherols in *Camelina* oil and *Camelina* meal slows down the oxidative processes of breast meat lipids, which is reflected in a lower content of malondialdehyde.

Additionally, *Camelina* oil had a beneficial effect on meat juiciness, whereas *Camelina* cake slightly worsened the flavor and tastiness of the meat.

Camelina expeller cake can be a cheaper alternative source of polyunsaturated fatty acids, as well as natural antioxidants, but the level of *Camelina* expeller used in broiler chicken diet should be more thoroughly investigated in future.

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Effects of Dietary Threonine Levels on Intestinal Immunity and Antioxidant Capacity Based on Cecal Metabolites and Transcription Sequencing of Broiler

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Simple Summary: Threonine (Thr), an indispensable amino acid for animals and the third limiting amino acid of broilers, plays a vital role in the synthesis of gut mucosal proteins, which also has better effects on growth performance, biochemical indexes, antioxidant function, and gut morphology, as well as acting as a nutrient immunomodulator that affects the intestinal barrier function of broilers. However, it is not clear how it works in depth. The objective of the current study was to investigate the mechanism of effects of different dietary threonine levels on the antioxidant and immune capacity of broilers. Our findings suggest that a Thr level of 125% NRC (*Nutrient Requirements of Poultry*, 1994) recommendations had better effects on antioxidant and immune capacity, including resisting viruses and decreasing the abnormal proliferation of cells. As well as this, it also had better effects on maintaining the homeostasis of the body.

Abstract: This study aimed to determine the effects of different dietary threonine levels on the antioxidant and immune capacity and the immunity of broilers. A total of 432 one-day-old Arbor Acres (AA) broilers were randomly assigned to 4 groups, each with 6 replicates of 18 broilers. The amount of dietary threonine in the four treatments reached 85%, 100%, 125%, and 150% of the NRC (*Nutrient Requirements of Poultry*, 1994) recommendation for broilers (marked as THR85, THR100, THR125, and THR150). After 42 days of feeding, the cecum contents and jejunum mucosa were collected for metabolic analysis and transcriptional sequencing. The results indicated that under the condition of regular and non-disease growth of broilers, compared with that of the THR85 and THR150 groups, the metabolic profile of the THR125 group was significantly higher than that of the standard requirement group. Compared with the THR100 group, the THR125 group improved antioxidant ability and immunity of broilers and enhanced the ability of resisting viruses. The antioxidant gene CAT was upregulated. PLCD1, which is involved in immune signal transduction and plays a role in cancer suppression, was also upregulated. Carcinogenic or indirect genes PKM2, ACY1, HK2, and TBXA2 were down-regulated. The genes GPT2, glude2, and G6PC, which played an important role in maintaining homeostasis, were up-regulated. Therefore, the present study suggests that 125% of the NRC recommendations for Thr level had better effects on antioxidant and immune capacity, as well as maintaining the homeostasis of the body.

Keywords: threonine; broiler; metabolism; antioxidant capacity; immunity

1. Introduction

Threonine (Thr)—the third limiting amino acid in broilers after lysine and some sulfur-containing amino acids in which chickens themselves cannot synthesize [1]—is involved in many metabolic

processes, such as protein synthesis and degradation, the conversion of ammonia nitrogen, and the conversion of the carbon skeleton to glucose and energy, among other processes. [2]. However, for current poultry breeds, some publications have challenged the NRC (*Nutrient Requirements of Poultry,* 1994) recommendations for amino acids as being inadequate [3,4]. Part of the reason for needing to correct the NRC in time is the adaptive change of chickens in unstable environments, the change of nutrient transport and composition, and scientific feed management. Among the four primary livestock species, the poultry publication is the oldest revised edition of the NRC [3]. A number of studies have shown that adding a higher level of Thr in broiler diet than is recommended by the NRC improved antioxidant ability and immune function [5–9]. Debnath et al. [10] found serum glutathione peroxidase (GSH-PX) and catalase concentration and superoxide dismutase (SOD) level increased linearly with the increasing of Thr level, which was similar to Azzam et al. [9] and Liu et al. [7]. Thr may be a nutritional immunomodulator affecting intestinal barrier function, which can improve immunity and intestinal health of broilers, especially 125% NRC Thr level [11]. The imbalance of Thr has a significant effect on the activities of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in poultry serum [12]. Moreira et al. [13] suggested that Thr promoted intestinal health in broilers infected with *Salmonella enteritidis*. Moderately excessive levels of Thr could reduce the incidence of *Eimeria* infestation in broilers and improve the resistance in vivo [14]. Thr could reduce the number of colonies of *Escherichia coli* and *Salmonella* in the cecum, which might be related to the increase in intestinal mucosal protein synthesis and immunoglobulin secretion caused by Thr [15]. An adequate dietary level of Thr has been shown to enhance intestinal integrity in poultry [16,17].

Our team's previous study has shown that 125% of the NRC recommended [18] Thr level had better effects on growth performance, biochemical indexes, antioxidant function, and gut morphology of broilers [11]. However, little data focus on the further mechanism of Thr on broilers. Therefore, the present study in which metabonomics were used to identify group differential metabolites (the differential metabolites are closely related to amino acid metabolism, but few studies exist on the effect of excessive Thr on metabolites) and to associate transcriptional histology data, was undertaken to explain the in-depth mechanism of dietary Thr level on the immune function and oxidation resistance of poultry.

2. Materials and Methods

2.1. Experimental Design and Diets

The experiment was carried out under normal conditions in a layered cage with a concentration gradient in the amount of Thr, avoiding stress. The single factor complete random design was used in the experiment. Four levels of Thr were added to the basal diet. The levels of Thr in the diet were 85%, 100%, 125%, and 150% of the total Thr requirement of broilers, respectively. They were recorded in turn as THR85, THR100, THR125, and THR150. Four-hundred and thirty-two healthy Arbor Acres (AA) broilers of similar body weight were randomly divided into 4 groups with 6 replicates in each group and 18 chickens in each replicate. The experimental period lasted 42 days. The research techniques used on the living animals met the guidelines approved by the Institutional Animal Care and Use Committee (IACUC). The experimental diet was prepared according to AA broiler feeding requirements and the NRC standard. The basal diet was designed to provide all the nutrients except Thr, and its dietary composition and nutrient levels are listed in Table 1. The mass fraction of Thr in the basal diet was 0.69% and 0.62% in the early and late stages, respectively. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Northwest A&F University and were approved by the Animal Ethics Committee of Northwest A&F University (NWAFU-314020038).

Table 1. Ingredients and nutrient composition of the basal diet (as-fed basis).

Items (%)	Starter (0 to 21 d)	Finisher (22 to 42 d)
Ingredients		
Corn	31.92	32.54
Soybean meal	26.25	18.40
Wheat	25.00	30.00
Peanut meal [1]	5.00	5.00
Soy bean oil	4.20	4.80
Distillers dried grains with solubles	3.00	5.00
Dicalcium phosphate	1.65	1.35
Limestone	1.10	1.30
Premix [2]	1.00	1.00
L-lysine	0.32	0.28
Salt	0.25	0.25
DL-methionine	0.18	0.13
Choline chloride	0.10	0.10
Antioxidant	0.03	0.03
L-threonine	0.00	0.00
Total	100.00	100.00
Calculated composition		
Metabolizable energy (kcal/kg)	3000.00	3100.00
Total phosphorus	0.68	0.62
Available phosphorous	0.45	0.40
Calcium	0.90	0.90
Sodium chloride	0.30	0.30
Analyzed composition		
Crude protein	20.87	19.10
Lysine	1.24	1.11
Methionine	0.50	0.42
Isoleucine	0.67	0.58
Threonine [3]	0.69	0.62
Tryptophan	0.23	0.20
Valine	0.85	0.74
Arginine	1.32	1.14

[1] Per kilogram of peanut meal (g/kg): crude protein, 523 g; ash, 62.4 g; water, 54.5 g; aspartic acid, 65.2 g; threonine, 14.7 g; serine, 24.7 g; glutamic acid, 100.4 g; glycine, 29.2 g; alanine, 20.9 g; valine, 20.0 g; methionine, 5.6 g; isoleucine, 16.8 g; leucine, 33.6 g; tyrosine, 21.4 g; phenylalanine, 26.4 g; lysine, 16.3 g; histidine, 12.1 g; arginine, 64.3 g; and proline, 25.8 g. [2] Provided per kilogram of diet: vitamin A, 45000 IU; cholecalciferol, 14000 IU; vitamin E, 90 IU; vitamin K3, 10 mg; thiamin, 7.36 mg; riboflavin, 25.6 mg; pyridoxine, 19.68 mg; vitamin B12, 0.1 mg; nicotinamide, 158.4 mg; calcium pantothenate, 46 mg; folic acid, 3.325 mg; biotin, 0.7 mg; Cu, 7.25 mg; Fe, 72 mg; Zn, 74.52 mg; Mn, 71.232 mg; Se, 0.3 mg; I, 0.5 mg; Co, 0.2 mg. [3] The starter (0 to 21 d) poultry requirements for Thr (%) in the four groups (THR85, THE100, THR125, and THR150 in turn) are listed: 0.59%, 0.69%, 0.86%, and 1.04%; the finisher (22 to 42 d) poultry requirements for Thr (%) in the four groups (THR85, THR100, THR125, and THR150 in turn) are listed: 0.53%, 0.62%, 0.78%, and 0.9%.

2.2. Sample Collection and Preparations

After reaching 6 weeks of age, and after fasting for 12 h, one bird from each replicate was chosen according to average body weight and slaughtered. The cecum contents were divided into four tubes and cryopreserved at −20 °C, and 24 cecum content samples were collected and measured (Wuhan Anlong Kexun Technology Co., Ltd., Wuhan, China). A comprehensive report of the metabolites in the cecum samples was obtained.

The jejunum mucosa was washed with normal saline, divided into four tubes, and cryopreserved at −20 °C. A total of 24 jejunal mucosa samples were obtained. The total RNA of the jejunum tissue was extracted, and three of the several samples from THR85-THR150 were randomly selected and assessed in terms of the quality of total RNA, which was extracted with a QIAGEN kit(Beijing

Novosource Science and Technology Co., Ltd., Beijing, China) (all samples OD 260/280 should be between 1.8–2.2; the amount of all samples should be greater than 3 μg), and the degree of degradation and contamination of the RNA was measured by 1% agarose gel electrophoresis for detection (agarose imaging should be satisfied in no less than three bands: 28 S, 18 S, and 5 S; the brightness of 28 S is more than twice that of 18 S). The sequence quantity and quality of the constructed library were evaluated, then the effects of Thr on antioxidant and immune activity were studied.

2.3. Sample Analysis

The analysis of the cecum contents is herein briefly explained. Ultrasonic extraction and centrifugation were undertaken after the sample was dissolved in pure water and then filtered. The filtrate was then extracted. Finally, 50 μL 3-(Trimethylsilyl) propanesulfonic acid (DSS)standard solution (Anachro, Toronto, Canada) was added. The upper layer of the liquid was extracted, and the spectra were collected.

The analysis of the jejunum mucosa is briefly explained here. RNA was extracted according to the specific process in the Total RNA Kit I (Takara Bio Inc., Tokyo, Japan). First, RNA quantification and qualification were evaluated. The RNA degradation and contamination were monitored on 1% agarose gels. The RNA purity was checked using a NanoPhotometer spectrophotometer (IMPLEN, Westlake Village, CA, USA). The RNA concentration was measured using a Qubit RNA assay kit in a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Second, the library was prepared for transcriptome sequencing. Finally, clustering and sequencing were determined.

2.4. Data Analysis

First, we discuss the process of data analysis for the cecum content. The free induction decay (FID) signal was imported into Chenomx nuclear magnetic resonance (NMR) suite software to achieve 1H NMR analysis, and the Fourier transformation, phase correction, and baseline realignment were conducted automatically. The concentration and spectrum area of the peak at 0.0 ppm were defined as the standard, and the names and corresponding concentrations of 45 metabolites were obtained. The information on the intestinal content sample was included in the variable matrix as the source data for partial least squares discrimination analysis (PLS-DA), and the Ggplot 2 package was used to visualize the data analysis.

Next, we introduced the process of data analysis for the jejunum mucosa. Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA, USA) was used to sequence the two ends of the sample, and the base was connected to read the sequence. The original sequence was filtered, and a high-quality sequence was obtained. The genomic localization results of all reads were combined, and the reads were assembled with the help of Cufflinks. Twelve intestinal mucosal tissues were compared and annotated using Tophat (v2.0.13) (UC, California, USA) On the basis of the results of the Tophat reference genome alignment, Cufflinks (v2.2.0) (UC, California, USA) was used to obtain the spliced transcripts for each biological replicate. Cuffmerge was used to calculate the expression of the transcripts and analyze the differential expression. The alignment sequence (mapped reads) and the spliced transcripts were submitted to Cuffdiff to search for the differentially expressed genes to complete the analysis on biological function. Finally, sequencing results were verified by qPCR.

The mRNA was extracted before cryopreservation, and the concentration of total RNA was determined by Nano-200 using 1 μg of RNA as the template. The first cDNA chain was synthesized by reverse transcription with a Super RT cDNA synthesis kit (CWBIO, Jiangsu, China). Using reverse transcriptase as a template, a matching fluorescent quantitative reagent was used to configure the sample system for analysis. Fluorescence quantitative PCR was performed using a kit. By observing the data and the final result of the real-time display on the instrument, we were able to analyze and compare the differences in gene expression. The reverse transcription reaction (20 μL) was conducted in a mixture containing 10 μL of 2 × UltraSYBR mixture (High ROX), 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 1 μL of cDNA, and 8.2 μL of ddH_2O. The primer sequences used

for PCR and their gene bank accession numbers are listed in Table 2. The fluorescence quantitative PCR reaction was carried out under the conditions in a Bio-Rad iQTM5 Thermal Cycler (Bio-Rad Lab., Calif., Philadelphia, PA, USA).

Table 2. Nucleotide primer sequences of the PCR primers.

Gene Symbol	Ensemble Accession No.	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product Size/bp
ABCC2	ENSGALG00000007395	TGTCCTTACCATTGCCCACC	CCACTTACATCCGCTCCACC	197
CAT	ENSGALG00000030187	GAACGCCGCATAGTAAGA	GAGGGTCACGAACAGTAT	414
PKM2	ENSGALG00000001992	ATGTCGAAGCCCCATAGTGAA	TGGGTGGTGAATCAATGTCCA	118
G6PC	ENSGALG00000030034	GCGTCTGGTATGTAATGG	AGAATAACTTGATGAGGGA	181
PLCD1	ENSGALG00000005805	CCAGTGAACGAGCCAAGAAG	TTCCACACAGCGGCAACCTT	245
GAPDH	ENSAPLG00000013511	TCTTCACCACCATGGAGAAG	CAGGACGCATTGCTGACAAT	154

3. Results

3.1. Searching for Differential Metabolites

Based on 1H NMR analysis, the metabolites were identified. Through a PLS-DA analysis to find the differential metabolites, we obtained the corresponding score plots and loading plots, as shown in Figures 1–3. In the score plots, the degree of dispersion can reflect the reproducibility of the samples and the similarity of the metabolic profiles among the sample groups. The results showed that the compared groups tended to be independently separated from each other. However, the group THR100 and THR125 were more distinct than the others. In the VIP diagram, differential metabolites with VIP values greater than 1 were selected (significant difference among the variables with VIP values greater than 1): glutamate, acetate, proplonate, ethanol, butyrate, and valeate (Table 3).

Table 3. The VIP scores of THR100 and THR125 for partial least squares discrimination analysis (PLS-DA). [1]

Metabolite	VIP Scores
Propionate	3.82660
Butyrate	3.58300
Glutamate	2.89960
Acetate	1.40370
Ethanol	1.28460
Valerate	1.05500
Aspartate	0.78458
Glucose	0.77806
Lysine	0.69175
2-Oxoglutarate	0.62168
Hypoxanthine	0.55743
3-Phenylpropionate	0.52454
Glycine	0.49846
Uridine	0.47948
Methionine	0.45836

[1] There were significant differences among the metabolites whose VIP value is greater than 1. There were six metabolites with a VIP score greater than 1: glutamate, acetate, proplonate, ethanol, butyrate, and valeate.

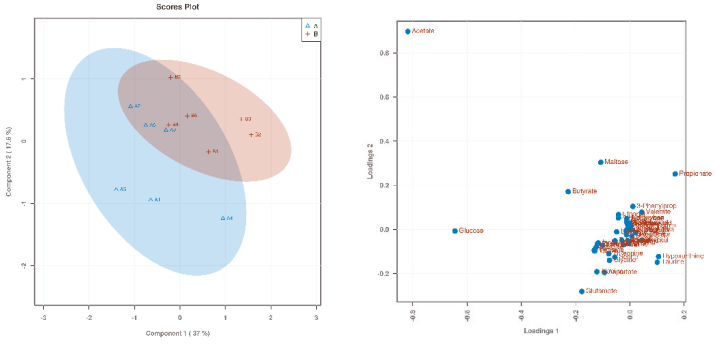

Figure 1. The score plot and loading plot of THR85 and THR100 from the partial least squares discrimination analysis (PLS-DA) analysis. In the above PLS-DA score chart, different groups are represented by different colors. The degree of dispersion can reflect the reproducibility of the samples and the similarity of the metabolic profiles among the sample groups. The comparison group tended to separate from each other, but the trend was not significant.

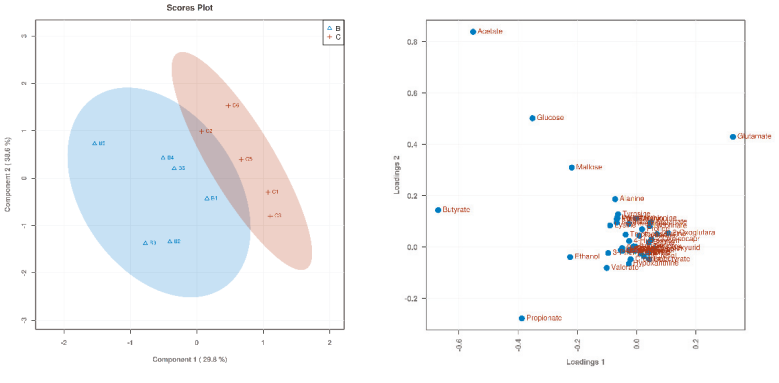

Figure 2. The score plot and loading plot of THR100 and THR125 from the PLS-DA analysis. The result is the picture below. The degree of separation in the score plot for this group was the most significant when compared with other groups.

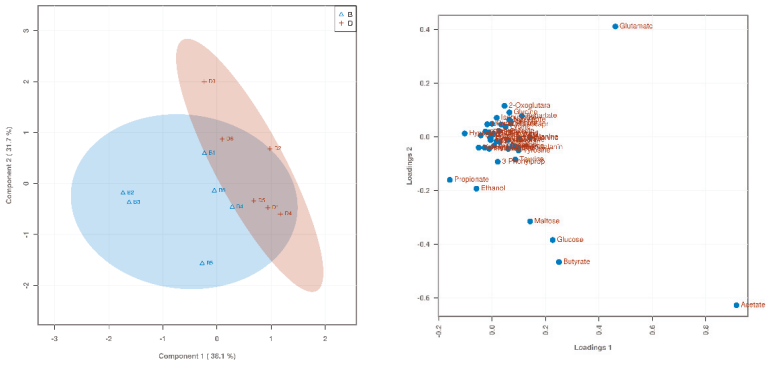

Figure 3. The score plot and loading plot of THR100 and THR150 from the PLS-DA analysis. In the above PLS-DA score chart, the comparison group tended to separate from each other, but the trend was not significant.

3.2. Sequencing of the Jejunal Mucosal Transcriptome

As shown in Figure 4, the total RNA extracted from the samples had good integrity, and the total RNA purity was high.

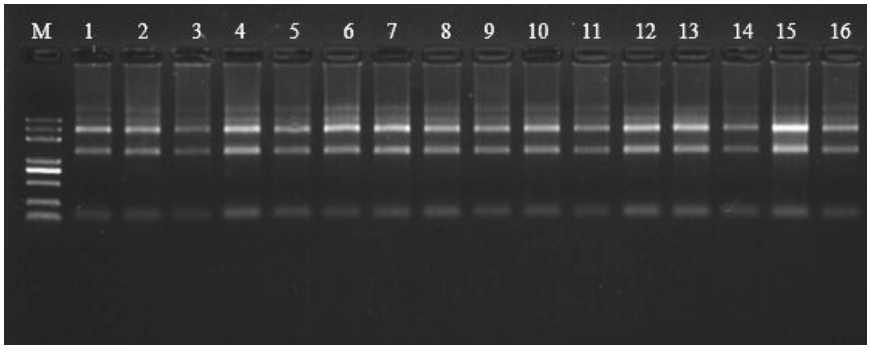

Figure 4. Quality report by 1% agarose gel electrophoresis. The marker is Trans 2K Plus. The figure corresponds to the following sequences: sequences 1, 2, 7, and 11 were diluted 20-fold; sequences 4, 8–10, 12–14, and 16 were diluted 10-fold; and the samples were 1 μL. Sequence 5 was diluted sevenfold, and the sample was 1 μL. Test parameters: gel concentration, 1%; voltage, 180 v; and electrophoretic time, 16 min. Agarose imaging was no less than three bands: 28 S, 18 S, and 5 S; the 28 S and 18 S bands were clear, and the brightness of 28 S was more than twice that of 18 S. The integrity of total RNA was better.

3.3. An Analysis of the Cross-Section of the Study Groups

On the basis of cross-group analysis, we combined the results of the metabolite data and transcriptome analysis of the mucosal samples. In the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of all differentially expressed genes (upregulated genes and downregulated genes) between the THR100 and THR125 groups (a total of 135 pathways involving all the differentially expressed genes), all the metabolic pathways that can produce the previously mentioned six metabolites were screened, resulting in a total of 12 pathways. There were 36 differentially expressed genes, 24 were upregulated genes, and 12 downregulated genes, as follows: ABC transporters (enriched 10 differentially expressed genes), alanine, aspartate and glutamate metabolism (enriched 6 differentially expressed genes), glycolysis/gluconeogenesis (enriched 7 differentially expressed genes), nicotinate and nicotinamide metabolism (enriched 4 differentially expressed genes), 2-oxocarboxylic acid metabolism (enriched 2 differentially expressed genes), sulfur metabolism (enriched 1 differentially expressed gene), glycosaminoglycan biosynthesis–heparan sulfate/heparin (enriched 2 differentially expressed genes), biosynthesis of amino acids (enriched 4 differentially expressed genes), terpenoid backbone biosynthesis (enriched 1 differentially expressed gene), glyoxylate and dicarboxylate metabolism (enriched 1 differentially expressed gene), arginine and proline metabolism (enriched 2 differentially expressed genes), and the calcium signaling pathway (enriched 9 differentially expressed genes).

The pathways in which glutamate was involved were ABC transporters, alanine, aspartate and glutamate metabolism, nicotinamide and nicotinamide metabolism, 2-oxocarboxylic acid metabolism, biosynthesis of amino acids, glyoxylate and dicarboxylate metabolism, arginine and proline metabolism, and the calcium-signaling pathway. The codes of the pathways in the KEGG database are gga02010, gga00250, gga00760, gga01210, gga01230, gga00630, gga00330, and gga04020. The pathway in which ethanol was involved was glycolysis/gluconeogenesis. The number of the pathway in the KEGG database is gga00010. The pathways in which acetic acid was involved included sulfur metabolism, glycosaminoglycan biosynthesis–heparan sulfate/heparin, terpenoid backbone biosynthesis and

glycolysis/gluconeogenesis. The corresponding codes of the pathways in the KEGG database are gga00920, gga00534, gga00900, and gga0010, respectively.

Expression profiles of differential genes between THR100 and THR125 are presented in Figure 5. There were 24 up-regulated genes and 12 down-regulated genes. Of the 36 genes, eight were screened for biological significance related to immune and antioxidant regulation, among which the upregulated genes were CAT, GPT2, PLCD1, GLUD2, and G6PC, and the down-regulated genes were PKM2, HK2, and TBXA2R.

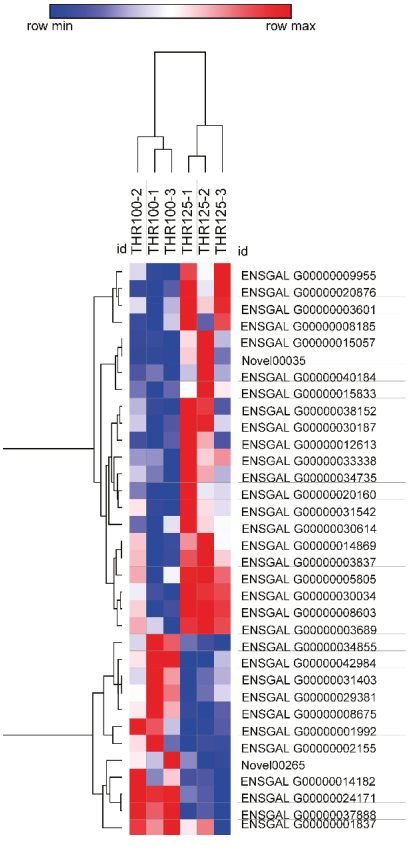

Figure 5. Expression profiles of differential genes between THR100 and THR125. The heat map showed the differential gene expression profiles in THR100 and THR125 groups. Each row represents the level of expression of each differentially expressed gene; each column represents a biological repetition. The expression level of each gene is expressed by fragments per kilobase of exon per million reads mapped (FPKM) value. The FPKM value for each differential gene was used for plotting. Red indicates that the gene is highly expressed, and blue indicates a low expression level.

3.4. Identification of the Differentially Expressed Genes by qPCR

To further verify the results of transcriptome sequencing, fluorescence quantitative polymerase chain reaction (real-time PCR) was performed. We selected five important genes (ABCC1, CAT, G6PC, PLCD1, and PKM2) for verification. The results showed that the gene expression pattern of these genes was consistent with the results of transcriptome sequencing and that the common phase relationship was 0.901 ($p < 0.01$). Therefore, the results of transcriptome sequencing were effective.

4. Discussion

Thr is the third limiting amino acid of broilers, which plays a key role in antioxidant and immune capacity. On the basis of group cross, we discovered the reason why the dietary Thr levels at 125% and 100% of the recommended NRC had different effects. Eight of the 36 genes in 12 pathways were screened for biological significance related to immune and antioxidant regulation. The upregulated genes were CAT, GPT2, GLUD2, G6PC, and PLCD1; the downregulated genes were PKM2, HK2, and TBXA2R.

In our study, the CAT expression was upregulated; Debnath et al. [10] came to the same conclusion—of great significance to the antioxidant ability of broilers—finding it to be higher in the THR125 group when compared with THR100. In broilers, Thr plays a vital role in the synergistic effects of the scavenging of reactive oxygen free radicals and the regulation of peroxidase on membrane lipid peroxidation. Liu et al. [7] and Debnath et al. [10] found that when the addition of THR was higher than the recommended amount, the activity of serum GSH-PX and total superoxide dismutasewas higher. Our results indicated that excessive Thr could significantly increase the antioxidant enzyme activity of broilers, which is consistent with Habte et al. [12] and Azzam et al. [9]. The improved oxidation state may be due to the simultaneously enhanced immune function, reduced colonization of pathogenic bacteria, and improved intestinal health.

The expression of GPT2 was upregulated, which is involved in the metabolism of proteins and amino acids. GPT2 is involved in gluconeogenesis, which converts a variety of non-sugar substances into glucose or glycogen and assists in amino acid metabolism [19]. GPT2 is one of the two major aminotransferases (other than glutamic oxaloacetic transaminase) in poultry, and plays an important role in the maintenance of homeostasis [20]. A high activity of aminotransferase is beneficial to amino acid metabolism in vivo. Liu et al. [7] used an automatic biochemical analyzer to measure the activity of GOT and GPT, and found that dietary levels of Thr above the recommendation by the NRC could significantly increase the activities of these two enzymes, and the same conclusion was reached by Gao et al. [21] and Habte et al. [12].

PLCD1 participates in the transduction of multiple immune signals. It can promote apoptosis and inhibit the invasion of tumor cells [22]. PLCD1 encodes enzymes that regulate energy metabolism, calcium homeostasis, and intracellular signaling pathways in animals. Many scientific findings indicate that it has been identified as a tumor suppressor gene for many types of cancer, including pancreatic cancer, esophageal squamous cell carcinoma, gastric cancer, chronic myeloid leukemia, and breast cancer [23–27].

G6PC deficiency can cause metabolic disorders such as glycogen storage disease Ia (GSD Ia) [28], which is a key rate-limiting glycosylation gene [29]. G6PC plays an important role in maintaining blood glucose stability, which is a key enzyme for the final reaction between hepatic glycosylation and glycogen degradation in broilers [30].

In our study, the results showed that 125% Thr supplementation, some oncogenes were downregulated, tumor proliferation was inhibited, immunity and the antioxidation capacity were enhanced, and the antitumor mechanism was induced. Satomura et al. [31] and Tomas et al. [32] reached similar conclusion, respectively. The four oncogenes that were downregulated have long been proven to have carcinogenic effects in humans, and further tests are needed to confirm on the similar effects on broilers.

PKM2, which can regulate apoptosis and proliferation of cancer cells and meet their high energetic and biosynthetic demands of rapid growth and proliferation is a marker of the metabolic transition of cancer cells [33–37], and also regulated by many oncogenes, with its upregulation promoting tumor formation [38,39]. Yang et al. [40] reported that nuclear translocation of the glycolic key enzyme, PKM2, helps cancer cells survive under metabolic stress. PKM2 is the link between oncogenes and metabolism, which plays a central role in the metabolic recombination of tumor cells, the activation of cancer metabolism, and proliferation of cancer cells [41].

HK2 downregulation inhibits the regulatory factors of tumor metabolism. Palmieri et al. [42] pointed out that tumor metastasis was associated with high expression of HK2. Tao et al. [43] proposed that HK2 could promote tumor proliferation and survival. HK2 is highly expressed in tumor cells and is the first to use of mitochondria-synthesized HK2 catalyzes the rate-limiting and first step of glucose metabolism. HK2 is highly expressed in many cancers, including breast cancer, ovarian cancer, and colon cancer [44].

TBXA2R is usually expressed in tumors, especially in invasive tumors, which has a potential effect on the metastasis of cancer cells through its metabolite TAX2, which has previously been reported to be found in various tumors, including prostate, glioma, and melanoma [45].

In our study, one of the differential pathways was the ATP-binding cassette transporter (ABC transport). There were 10 different genes, which included 9 with an upregulated expression: ABCA1, ABCA2, ABCB1, ABCB4, ABCC2, ABCC6, ABCD2, ABCG5, ABCG8, and with a downregulated expression, ABCB3 [46]. The results of this change demonstrate the correctness of our previous experiment—compared with the 100% NRC group, the concentration of serum total protein and globulin in the 125% NRC group was significantly higher than that of Debnath et al. [10], indicating that the total protein concentration and serum globulin level increased linearly. The conversion efficiency of some proteins affects the metabolic process. Sufficient levels of Thr promote the trans-membrane transport of antibodies and biological molecules, and the synthesis of proteins. Antibodies and immunoglobulins are all proteins, and Thr is an important limiting amino acid for poultry immunoglobulin molecules. The sufficient supplying of Thr contributes to the production of immunoglobulins and lymphocytes.

The imbalance of Thr leads to deamination, and, in addition, it interferes with the absorption and utilization of other amino acids. THR85 did not result in significant differences in metabolites compared with THR100, which may be because the Thr supply can meet the basic maintenance needs of the broilers and other amino acids act as Thr by binding to certain substances. The effect of THR150 was not satisfactory. Baker et al. [47] noted that excess Thr could be converted to glycine in vivo. At the same time, an excess of Thr could also result in a relative deficiency and low utilization of other amino acids.

In different growth and developmental stages or under different conditions, the expression of genes in the same cell or tissue is different. Gene expression has temporal and spatial specificity [48]. Therefore, the evaluation of the immunity and antioxidant capacity of broilers via gene expression in intestinal tissue has some limitations, but it is of great importance to studies on the optimal content of Thr in broiler feed. In this experiment, the results on the regulation of differential genes was combined with those from previous studies, fully indicating that the level of Thr at 125% of the standard recommended amount of the NRC could significantly improve the immunity and antioxidant capacity of broilers. This concentration of Thr can decrease the abnormal proliferation of resistant cells in broilers. The antioxidant gene CAT was upregulated, along with PLCD1, which is involved in immune signal transduction and plays a role in cancer suppression. Carcinogenic or indirect genes PKM2, ACY1, HK2, TBXA2, on the other hand, were down-regulated. The genes GPT2, glude 2, and G6PC, which played an important role in maintaining homeostasis, were up-regulated.

5. Conclusions

Thr level of 125% NRC recommendations had better effects on antioxidant and immune capacity, including resisting viruses and decreasing the abnormal proliferation of cells. Alongside this, it also had better effects on maintaining the homeostasis of the body.

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Valorization of Red Grape Pomace Waste Using Polyethylene Glycol and Fibrolytic Enzymes: Physiological and Meat Quality Responses in Broilers

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Simple Summary: Red grape pomace (GP) waste, although rich in beneficial phenolic compounds, is traditionally disposed in landfills and through incineration, resulting in environmental pollution. The revalorization of GP as a source of nutrients and bioactive compounds in chicken diets is an environmentally sustainable and lower-cost alternative to current disposal methods. This approach has the potential to improve food and nutrition security while providing health benefits to consumers of poultry products. Unfortunately, the amount of GP that can be included in broiler diets is limited by fiber and condensed tannins found in this agro-waste. These compounds reduce the digestibility of GP in chickens, resulting in poor bioavailability of the beneficial bioactive compounds. Strategies are, therefore, required to ameliorate the effects of fiber and condensed tannins. This study investigated whether pre-treating GP with polyethylene glycol (PEG) and a cellulolytic enzyme mixture (Viscozyme®) would improve feed intake, physiological parameters, carcass characteristics and meat quality parameters of broilers. It was concluded that PEG treatment successfully ameliorated the anti-nutritional effects of condensed tannins. However, the cellulolytic enzyme treatment was ineffective against GP fiber.

Abstract: The amount of grape pomace (GP) waste that can be included as a functional feed in broiler diets is limited by anti-nutritional compounds such as fiber and condensed tannins. This study evaluated the effect of pre-treating GP with polyethylene glycol (PEG) and a cellulolytic enzyme mixture on physiological and meat quality parameters of broilers. Cobb 500 broilers (249.2 ± 20.31 g live-weight) were reared on five isoenergetic and isonitrogenous diets: 1. Commercial chicken diet (CON); 2. CON containing untreated GP at 100 g/kg (dGP); 3. CON containing 100 g/kg GP pre-treated with PEG (50 g/kg) (dPEG); 4. CON containing 100 g/kg GP pre-treated with enzyme (1 g/kg) (ENZ); and 5. CON containing 100 g/kg GP pre-treated with PEG (50 g/kg) and enzyme (1 g/kg) (PENZ). Overall body weight gains were similar in broilers reared on the CON, dPEG, ENZ and PENZ diets but lower in dGP chickens. The meat of birds reared on dPEG, ENZ, dGP and CON had a similar water-holding capacity, which was lower than in PENZ chickens. Diets influenced the size of duodenum, ileum, jejunum and caeca. Polyethylene glycol treatment promoted similar body weight gains and hot carcass weights as the commercial control diet, suggesting that the anti-nutritional effects of condensed tannins were successfully ameliorated.

Keywords: blood parameter; broiler; growth performance; meat quality; polyethylene glycol; red grape pomace waste

1. Introduction

The traditional approach of disposing large volumes of red grape pomace waste (GP) in landfills and through incineration poses a major environmental challenge [1]. The valorization of GP could help maintain environmental equilibrium through waste reduction while providing additional economic benefits. Using this agro-waste as a functional feedstuff in broiler diets is a re-valorization and waste reduction strategy with the potential to improve food and nutrition security and environmental stewardship. Indeed, the alternative use of GP as a source of potentially beneficial phytochemical compounds in broiler diets has been evaluated with promising results [2,3]. These phytochemical compounds are reported to have antioxidative, antimicrobial and health-promoting effects [4,5]. Consequently, GP is a potential functional feedstuff for broilers with polyphenols that has been demonstrated to enhance the oxidative stability of meat and to promote the proliferation of beneficial intestinal bacteria [2,6]. However, the bioavailability of beneficial bioactive compounds in this by-product is rather low due to high fiber (43–75%) [7] and condensed tannin (20–30%) [8] content. Both condensed tannins and fiber are known to negatively affect the digestibility of diets [9], especially in simple non-ruminants such as chickens [10]. Azizi et al. [6] reported that high amounts of tannins in GP reduce feed intake and negatively affect feed efficiency in chickens. This, in turn, limits the amount of GP that could be included in broiler diets to the detriment of red grape pomace re-valorization and waste reduction efforts.

Strategies to counter the anti-nutritional effects of condensed tannins and fiber need to be identified and evaluated in order to improve the bioavailability of bioactive compounds from GP. Pre-treatment of GP with exogenous fibrolytic enzymes and/or polyethylene glycol (PEG) may allow for higher dietary inclusion levels of GP in chicken diets. While the utility of cell wall-degrading feed enzymes in improving the feed value of feed ingredients is generally well documented, their use in enhancing the utilization of GP in birds is limited to pectinases and cellulases [11]. On the other hand, PEG is known for its ability to bind tannins, thus freeing proteins and other dietary components for digestion and absorption [12]. Indeed, Besharati and Taghizadeh [13] reported that PEG breaks already formed tannin–protein complexes, due to its high tannin affinity. This was also confirmed by Hlatini et al. [14] when PEG was included in *Acacia tortilis* leaf meal diets for pigs. While the use of PEG to inactivate condensed tannins in ruminant diets has been widely investigated, there are no documented studies on its application to improve GP utilization in broiler chickens. The possible effects of inactivating condensed tannins using PEG on physiological and meat quality parameters of broilers are also unknown. In addition, this study is the first to explore possible additive effects of PEG and cell wall-degrading enzymes as a strategy to valorize red grape pomace waste for broilers. Therefore, this study was designed to determine the effect of PEG and Viscozyme® treatment of dietary GP on growth, blood parameters, and carcass and meat quality traits of broilers. The research hypothesis that pre-treating GP with fibrolytic enzymes and/or PEG will improve physiological parameters and meat quality traits of Cobb 500 broiler chickens was tested.

2. Materials and Methods

Rearing and slaughter procedures of the research birds were approved by the Animal Research Ethics Committee of the North-West University (approval no. NWU-00239-18-A5), which conforms to the guidelines and use of research animals.

2.1. Study Site and Ingredient Sources

Red grape (*Vitis vinifera L.* var. Shiraz) pomace (GP) was sourced from Blaauwklippen Wine Estate (33.969° S; 18.844° E) (Stellenbosch, South Africa), the soil types of which ranged from dark alluvial to clay. In this Estate, daily temperature averages 16.4 °C, while annual rainfall averages 802 mm. Associated Chemical Enterprises (Johannesburg, South Africa) supplied PEG (Mr 4000), while the enzyme Viscozyme® L (a cellulolytic mixture of arabinase, cellulase, β-glucanase, hemicellulase and

xylanase enzymes, with an equivalent enzymatic activity of 100 fungal beta-glucanase per gram), were supplied by Sigma–Aldrich, Modderfontein, South Africa. The broiler feeding trial was conducted at Molelwane Research Farm (33.969° S; 18.844° E) during the winter season, when ambient temperatures ranged from −3 to 25 °C. The broiler house was fitted with semi-automatic curtains that were rolled down in the morning and closed in the evening. The temperature in the pens was monitored using a thermometer and light was supplied using fluorescent lights. Sunflower husks were used as bedding in all the pens.

2.2. Pre-Treatment of Red Grape Pomace with Polyethylene Glycol and Enzyme

Red grape pomace (5 kg per treatment) was pre-treated with aqueous solutions of PEG (5 g PEG/100 g milled GP), Viscozyme® (0.1 g enzyme/100 g milled GP), and a combination of PEG and enzyme. For the PEG treatment, 5 kg GP was sprayed and mixed with 5 L of distilled water in which 250 g of PEG had been dissolved. For the enzyme treatment, 5 kg of GP was sprayed and mixed with 5 L of distilled water in which 4.2 mL of Viscozyme® (density: 1.2 g/mL) had been added. For the combined treatment of PEG and Viscozyme®, 250 g of PEG and 4.2 mL of Viscozyme® were both dissolved in 5 L of distilled water, which was then sprayed on 5 kg GP. The untreated GP (5 kg) was sprayed with 5 L of distilled water only. The amount of distilled water used to dissolve both the PEG and enzyme was determined through an iterative process, with the objective of avoiding excess run-off liquid that would have leached the GP. Treated and untreated GP were stored for a period of 24 h under room temperature to allow time for PEG and Viscozyme® to react with GP tannins and fiber, respectively. At the end of this incubation period, treated and untreated GP were oven-dried at 50 °C and then crushed to break up lumps before being incorporated into commercial grower and finishing diets.

2.3. Diet Formulation

Five isonitrogenous and isoenergetic experimental diets were formulated to meet the daily nutritional requirements of growing and finishing chickens according to National Research Council [15] guidelines. The diets, for grower and finisher phases, were formulated by including treated or untreated GP at 100 g/kg as follows: (1) Commercial chicken diet without red grape pomace (CON); (2) Commercial chicken diet containing 100 g/kg untreated red grape pomace (dGP); (3) Commercial chicken diet containing 100 g/kg red grape pomace pre-treated with PEG (50 g/kg) (dPEG); (4) Commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme® (1 g/kg) (ENZ); and (5) Commercial chicken diet containing 100 g/kg GP pre-treated with PEG (50 g/kg) and Viscozyme® (1 g/kg) (PENZ). The ingredient composition of the five diets is presented in Table 1.

2.4. Chemical Analyses

Samples of diets were milled and analyzed using the Official Analytical Chemists International methods [16]: 930.15 for dry matter, 924.05 for organic matter, 984.13 for crude protein, 978.10 for crude fiber and 920.39 for crude fat (Table 2). Near infrared reflectance spectroscopy models were used to predict the metabolizable energy content of the diets. Minerals were analyzed following the Agricultural Laboratory Association of Southern Africa [17] guidelines.

Table 1. Ingredient composition (g/kg dry matter, unless otherwise stated) of the experimental diets.

Ingredients	Diets [1]									
	Grower					Finisher				
	CON	dGP	dPEG	ENZ	PENZ	CON	dGP	dPEG	ENZ	PENZ
Polyethylene glycol	0	0	5	0	5	0	0	5	0	5
Viscozyme®-L	0	0	0	0.1	0.1	0	0	0	0.1	0.1
Grape pomace	0	100	100	100	100	0	100	100	100	100
Soy oilcake	245	12	12	12	12	168	0	0	0	0
Fullfat soya	10	229	229	229	229	55	262	262	262	262
Gluten 60	5	38	38	38	38	0	0	0	0	0
Sint lysine	1.39	2.71	2.71	2.71	2.71	1.93	1.52	1.52	1.52	1.52
Methionine	1.42	0.8	0.8	0.8	0.8	1.51	0.97	0.97	0.97	0.97
Threonine	0	0.01	0.01	0.01	0.01	0.1	0	0	0	0
Yellow maize	709	589	589	589	589	751	601	601	601	601
Feed lime	14.6	13	13	13	13	12.5	11.3	11.3	11.3	11.3
Monocalcium phosphate	7	7.9	7.9	7.9	7.9	2.2	2.2	2.2	2.2	2.2
Salt—fine	3.29	3.35	3.35	3.35	3.35	2.78	3.11	3.11	3.11	3.11
Sodium bicarbonate	1.59	1.45	1.45	1.45	1.45	1.91	1.28	1.28	1.28	1.28
Axtra phytase	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Choline	0.8	0.8	0.8	0.8	0.8	0	0	0	0	0
Salinomycin	0.5	0.5	0.5	0.5	0.5	0	0	0	0	0
Olaquindox	0.4	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.2
Premix	0.5	0.5	0.5	0.5	0.5	2.5	2.5	2.5	2.5	2.5
Zinc bacitracin	0	0	0	0	0	0.5	0.5	0.5	0.5	0.5
Oil crude soya	0	0	0	0	0	0	13.17	13.17	13.17	13.17

[1] Diets: CON = commercial chicken diet without red grape pomace; dGP = commercial chicken diet containing 100 g/kg untreated red grape pomace; dPEG = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg); ENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme®-L (1 g/kg); PENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg) and Viscozyme®-L (1 g/kg).

Table 2. Chemical composition (g/kg dry matter, unless otherwise stated) of the experimental diets.

Parameters	Diets [1]									
	Grower					Finisher				
	CON	dGP	dPEG	ENZ	PENZ	CON	dGP	dPEG	ENZ	PENZ
Dry matter (g/kg)	893.7	906.7	906.7	906.7	906.7	888.6	904.2	904.2	904.2	904.2
[2] ME (MJ/kg)	11.9	11.9	11.9	11.9	11.9	12.2	12.2	12.2	12.2	12.2
Crude protein	177.1	170.1	170.1	170.1	170.1	160.0	160.0	160.0	160.0	160.0
Crude fat	33.5	72.6	72.6	72.6	72.6	42.65	89.8	89.8	89.8	89.8
Crude fiber	25.0	74.6	74.6	74.6	74.6	35.2	80.7	80.7	80.7	80.7
Organic matter	844.1	857.3	857.3	857.3	857.3	849	862.5	862.5	862.5	862.5
Calcium	8.2	8.2	8.2	8.2	8.2	6.5	6.5	6.5	6.5	6.5
Phosphorus	5.0	4.7	4.7	4.7	4.7	3.4	3.28	3.28	3.28	3.28
Chloride	2.8	3.0	3.0	3.0	3.0	2.5	2.5	2.5	2.5	2.5

[1] Diets: CON = commercial chicken diet without red grape pomace; dGP = commercial chicken diet containing 100 g/kg untreated red grape pomace; dPEG = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg); ENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme®-L (1 g/kg); PENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg) and Viscozyme®-L (1 g/kg). [2] ME = metabolizable energy.

2.5. Feeding Trial

Four hundred, day-old mixed-gender Cobb 500 broiler chicks (OptiChicks, South Africa) were fed a starter mash diet supplied by Nutri-Feeds (South Africa) for a period of 10 days. The chicks were allocated to 40 pens (3.5 m long × 1.0 m wide × 1.85 m high), with each pen (experimental unit) carrying 10 birds. The five experimental diets were randomly allocated to the pens (8 replicates per diet). Days 11 to 13 were used as an adaptation period such that measurements were only taken from day 14 to day 42. Clean fresh water was provided at all times and rearing was done under natural lighting (10 h of daylight).

2.6. Feed Intake, Growth Performance and Blood Analyses

Average weekly feed intake (AWFI), average weekly body weight gain (ABWG) and the feed conversion ratio (FCR) were determined as described by Kumanda et al. [2]. At 40 days of age, two broiler chickens, randomly selected from each pen, were used to collect blood from the brachial vein. Whole blood and sera blood were analyzed using an automated IDEXX LaserCyte Haematology Analyser and an automated IDEXX Catalyst One Chemistry Analyser (IDEXX Laboratories Inc., Maine, US), respectively.

2.7. Carcass Characteristics and the Size of Internal Organs

On day 42, feed was withheld for 13 h before the birds were weighed (slaughter body weight) and transported to an abattoir. All 10 birds from each replicate pen were electrically stunned, slaughtered, bled and defeathered. Carcass weights (Explorer EX224, 0.01 g readability (2 decimal places), supplied by OHAUS Corp, Parsippany, NJ, USA) were taken immediately after slaughter (hot carcass weight (HCW)) and also after chilling at 4 °C for 24 h (cold carcass weight, CCW). The dressing out percentage was determined as a proportion of HCW to slaughter body weight. The size of internal organs (liver, gizzard, heart, proventriculus, spleen, pancreas, duodenum, ileum, jejunum, large intestine, caeca and lungs) were measured and expressed as a proportion of HCW.

2.8. Meat Quality Traits

All carcasses from each replicate pen and treatment were used to measure meat quality parameters. Breast meat pH and temperature were recorded 24 h post-mortem using a Corning Model 4 pH/temperature meter (Corning Glass Works, Medfield, MA). Meat color indices (L^* = lightness, a^* = redness and b^* = yellowness) were determined on the inner surface of raw thigh muscle, 24 h post-mortem using a Minolta color guide (BYK-Gardener GmbH, Geretsried, Germany) according to Commission Internationale de l'Eclairage [18]. Hue angle and chroma were calculated according to Priolo et al. [19]. The water-holding capacity (WHC) of the pectoralis major muscle (8–16 g) was determined as described by Mulaudzi et al. [20]. Drip loss [21] and cooking loss [22] were determined using breast meat samples. The samples used for cooking loss determination were then used for shear force determination with a Texture Analyser (TA XT plus, Stable Micro Systems, Surrey, UK).

2.9. Statistical Analysis

Data for each parameter collected per replicate pen were averaged first before statistical analysis. The NORMAL option in the Proc Univariate statement was used to test for normality of measured parameters. Repeated measures procedures of Statistical Analysis System (SAS) [23] were used to analyze data for parameters measured weekly (feed intake, body weight gain and feed conversion ratio). Overall growth performance parameters as well as blood parameters, carcass characteristics and meat quality data were analyzed using the general linear model procedure of SAS [23] according to the following linear statistical model:

$$Y_{ik} = \mu + D_i + E_{ik} \quad (1)$$

where Y_{ik} = response variable, μ = overall mean, D_i = dietary effect and E_{ik} = random error associated with observation ik, assumed to be normally and independently distributed. For all statistical tests, significance was declared at $p < 0.05$.

3. Results

3.1. Feed Intake, Growth Performance and Blood Parameters

There were no significant ($p > 0.05$) week × diet interaction effects on AWFI, ABWG and FCR. Table 3 shows that there were no significant ($p > 0.05$) dietary influences on overall feed intake (g/bird), initial body weight (g/bird) and overall FCR. The CON diet promoted the highest overall BWG (1351.4 g/bird) and final BW (1653.2 g/bird), which did not differ ($p > 0.05$) from dPEG, ENZ and PENZ diets. However, birds on untreated GP had the lowest body weight gain (1188.9 g/bird) and final BW (1468.4 g/bird).

Table 3. Effect of treating red grape pomace with polyethylene glycol and a cellulolytic enzyme mixture on overall feed intake (14–42 d), initial body weight (14 d), final body weight (42 d), and overall body weight gain (14–42 d) and the overall feed conversion ratio (14–42 d) of broiler chickens.

[2] Parameters	Diets [1]					[3] SEM	*p* Value
	CON	dGP	dPEG	ENZ	PENZ		
Overall FI (g/bird)	2957.5	2844.9	2931.6	2930.8	2913.6	40.52	0.371
Initial BW (g/bird)	301.8	279.4	295.5	288.0	293.6	7.13	0.255
Final BW (g/bird)	1653.2 [b]	1468.4 [a]	1604.4 [a,b]	1523.0 [a,b]	1564.9 [a,b]	35.53	0.009
Overall BWG (g/bird)	1351.4 [b]	1188.9 [a]	1308.9 [a,b]	1234.9 [a,b]	1271.3 [a,b]	35.69	0.027
[3] Overall FCR	2.20	2.40	2.24	2.24	2.29	0.061	0.057

[1] Diets: CON = commercial chicken diet without red grape pomace; dGP = commercial chicken diet containing 100 g/kg untreated red grape pomace; dPEG = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg); ENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme®-L (1 g/kg); PENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg) and Viscozyme®-L (1 g/kg). [2] Parameters: Overall FI = feed intake from 14 to 42 days of age; Initial BW = initial body weight at 14 days of age; Final BW = final body weight at 42 days of age; Overall WG = body weight gain from 14 to 42 days of age; Overall FCR = feed conversion ratio from 14 to 42 days of age; [3] SEM: Standard error of the mean. [a,b] In a row, dietary treatment means with common superscripts do not differ ($p > 0.05$).

With the exception of mean corpuscular volume (MCV), all hematological parameters were not ($p > 0.05$) influenced by dietary treatments (Table 4). Broilers fed ENZ had a higher MCV (34.87 fL) when compared to those on CON (27. 66 fL), dGP (27.70 fL) and dPEG (27.89 fL) diets.

Table 4. Effect of treating red grape pomace with polyethylene glycol and cellulolytic enzyme on hematological parameters of broiler chickens.

[2] Parameters	[1] Diets					[3] SEM	*p* Value
	CON	dGP	dPEG	ENZ	PENZ		
Erythrocytes ($\times 10^{12}$/L)	1.26	1.47	1.33	1.05	1.23	0.309	0.909
Hematocrits (L/L)	5.48	5.73	5.41	6.19	5.47	0.565	0.859
Hemoglobin (g/dL)	9.41	9.89	10.08	9.74	9.52	0.267	0.413
MCV (fL)	27.66 [a]	27.70 [a]	27.89 [a,b]	34.87[c]	33.48[bc]	1.432	0.001
MCH (pg)	49.17	51.98	53.31	52.52	59.09	6.195	0.846
RDW ($\times 10^9$/L)	40.81	40.69	39.58	38.74	36.73	1.106	0.078
Reticulocytes (K/µL)	235.2	186.1	178.3	86.0	86.2	50.87	0.176
Lymphocytes ($\times 10^9$/L)	19.85	27.56	93.92	52.11	50.71	26.141	0.277
Neutrophils ($\times 10^9$/L)	3.54	3.97	3.73	4.15	4.56	0.494	0.640
Monocytes ($\times 10^9$/L)	1.91	2.01	2.36	2.48	1.85	0.356	0.649
Eosinophils ($\times 10^9$/L)	0.55	0.66	0.82	0.84	0.79	0.134	0.508
Basophils ($\times 10^9$/L)	0.11	0.12	0.14	0.16	0.16	0.020	0.294

[1] Diets: CON = commercial chicken diet without red grape pomace; dGP = commercial chicken diet containing 100 g/kg untreated red grape pomace; dPEG = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg); ENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme®-L (1 g/kg); PENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg) and Viscozyme®-L (1 g/kg). [2] Parameters: MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; RDW = red cell distribution width; [3] SEM: Standard error of the mean. [a,b,c] In a row, dietary treatment means with common superscripts do not differ ($p > 0.05$).

With the exception of phosphorus, all serum biochemical parameters were not significantly affected by dietary treatments (Table 5). Broilers on dGP had the lowest serum phosphorus level, which did not differ ($p > 0.05$) with those on CON and PENZ diets. Broilers on ENZ had the highest serum phosphorus level (4.74 mmol/L), which did not differ from dPEG and CON diets.

Table 5. Effect of treating red grape pomace with polyethylene glycol and a cellulolytic enzyme mixture on serum biochemical parameters of broiler chickens.

[2] Parameters	[1] Diets					[3] SEM	*p* Value
	CON	dGP	dPEG	ENZ	PENZ		
Glucose (mmol/L)	8.06	6.70	8.54	8.54	8.06	1.241	0.827
Creatinine (μmol/L)	13.81	10.94	18.00	15.75	15.38	2.416	0.340
Urea (mmol/L)	0.66	0.66	0.71	0.69	0.70	0.021	0.354
Phosphorus (mmol/L)	3.88 [a,b]	3.30 [a]	4.54 [a,b]	4.74 [b]	3.64 [a,b]	0.300	0.008
Calcium (mmol/L)	2.24	2.18	2.20	2.55	2.56	0.241	0.644
Total protein (g/L)	51.38	51.88	55.11	59.38	62.06	3.776	0.215
Albumin (g/L)	19.25	18.88	21.38	23.88	22.19	1.990	0.375
Globulin (g/L)	33.44	33.13	31.50	35.44	39.31	2.465	0.228
ALT (U/L)	53.81	55.13	50.00	71.50	64.44	6.913	0.192
ALKP (U/L)	696.1	508.1	566.7	640.6	676.9	94.42	0.600
GGT (U/L)	17.06	15.81	18.88	15.75	12.94	1.762	0.219
Total bilirubin (μmol/L)	11.81	15.38	14.19	20.94	18.50	2.870	0.204
Amylase (U/L)	547.9	461.4	472.3	564.3	516.6	42.81	0.364
Lipase (U/L)	317.3	298.6	371.3	465.3	445.6	60.82	0.223
Cholesterol (mmol/L)	6.24	6.14	6.04	6.57	6.68	0.450	0.818

[1] Diets: CON = commercial chicken diet without red grape pomace; dGP = commercial chicken diet containing 100 g/kg untreated red grape pomace; dPEG = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg); ENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme®-L (1 g/kg); PENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg) and Viscozyme®-L (1 g/kg). [2] Parameters: ALT = alanine transaminase; ALKP = alkaline phosphate; GGT = gamma glutamyl transferase; [3] SEM: Standard error of the mean. [a,b] In a row, dietary treatment means with common superscripts do not differ ($p > 0.05$).

3.2. Carcass Characteristics, Internal Organs and Meat Quality Parameters

Table 6 shows that there were significant dietary effects on slaughter BW, HCW and CCW of broiler chickens ($p < 0.05$). The slaughter body weights of CON, dPEG, ENZ and PENZ chickens did not differ ($p > 0.05$). However, dGP diet promoted the lowest slaughter body weight (1468.4 g) in chickens. Broiler chickens on CON (1276.5 g) and dPEG (1243.6 g) diets had a higher HCW, which did not differ. However, dGP promoted the lowest (1120.6 g) HCW while the HCW of dPEG, ENZ and PENZ chickens did not differ ($p > 0.05$). Broilers on the CON (1227.4 g) and dPEG (1210.0 g) diets had a higher CCW compared to those fed dGP, ENZ and PENZ diets, whose CCW did not differ.

There were no dietary effects ($p > 0.05$) on the size of the liver (2.2–2.3 g/100 g HCW), gizzard (2.3–2.5 g/100 g HCW), heart (0.6–0.7 g/100 g HCW), proventriculus (0.5–0.6 g/100 g HCW), spleen (0.1–0.2 g/100 g HCW), pancreas (0.2–0.3 g/100 g HCW), large intestine (0.2–0.7 g/100 g HCW) and lung (0.6–0.7 g/100 g HCW) of broiler chickens. However, broilers on the CON diet had the lightest duodenum (0.7 g/100 g HCW), while those on dGP, dPEG ENZ and PENZ diets had the heaviest duodenum. Chickens on the CON diet had the lightest ileum (1.4 g/100 g HCW), which did not differ from those fed dPEG, ENZ and PENZ diets. Birds on the dGP diet had the heaviest ileum (1.7 g/100 g HCW), which did not differ from those fed dPEG, ENZ and PENZ diets. The CON experimental diet promoted a lighter ($p < 0.05$) jejunum (1.4 g/100 g HCW) compared to dGP, dPEG, ENZ and PENZ diets, which did not differ. The broilers on CON (0.8 g/100 g HCW) and dPEG (1.0 g/100 g HCW) diets had the lightest caeca, while birds on dGP and PENZ diets had the heaviest caeca.

Table 6. Effect of treating dietary red grape pomace with polyethylene glycol and a cellulolytic enzyme mixture on internal organs and carcass characteristics (g/100 g HCW, unless otherwise stated) of broiler chickens.

[2] Parameters	[1] Diets					[3] SEM	*p* Value
	CON	dGP	dPEG	ENZ	PENZ		
Slaughter body weight (g)	1653.2 [b]	1468.4 [a]	1604.4 [a,b]	1523.0 [a,b]	1564.9 [a,b]	35.53	0.009
HCW (g)	1276.5 [c]	1120.6 [a]	1243.6 [b,c]	1177.9 [a,b]	1181.4 [a,b]	18.38	0.0001
CCW (g)	1227.4 [c]	1075.8 [a]	1210.0 [b,c]	1133.2 [a]	1141.1 [a,b]	18.53	0.0001
Dressing percentage	77.2	76.5	77.7	77.7	75.6	1.499	0.840
Liver	2.31	2.22	2.22	2.28	2.23	0.043	0.565
Gizzard	2.30	2.47	2.34	2.33	2.45	0.072	0.381
Heart	0.64	0.69	0.69	0.74	0.69	0.022	0.070
Proventriculus	0.51	0.54	0.55	0.55	0.54	0.017	0.540
Spleen	0.11	0.16	0.12	0.13	0.12	0.013	0.265
Pancreas	0.26	0.25	0.26	0.28	0.24	0.014	0.304
Duodenum	0.66 [a]	0.84 [b]	0.83 [b]	0.82 [b]	0.80 [b]	0.027	0.0003
Ileum	1.36 [a]	1.54 [a,b]	1.66 [b]	1.48 [a,b]	1.53 [a,b]	0.050	0.004
Jejunum	1.38 [a]	1.61 [b]	1.72 [b]	1.59 [b]	1.68 [b]	0.045	0.0001
Large intestine	0.71	0.37	0.38	0.22	0.39	0.112	0.057
Ceaca	0.82[a]	1.14 [b]	1.40 [c]	1.04 [a,b]	1.23 [b,c]	0.056	0.0001
Lungs	0.65	0.65	0.68	0.73	0.67	0.031	0.380

[1] Diets: CON = commercial chicken diet without red grape pomace; dGP = commercial chicken diet containing 100 g/kg untreated red grape pomace; dPEG = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg); ENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme®-L (1 g/kg); PENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg) and Viscozyme®-L (1 g/kg). [2] Parameters: HCW = hot carcass weight; CCW = water cold carcass weight; [3] SEM: Standard error of the mean; [a,b,c] In a row, dietary treatment means with common superscripts do not differ ($p > 0.05$). [a,b,c] In a row, dietary treatment means with common superscripts do not differ ($p > 0.05$).

Table 7 shows that diets influenced the WHC of breast meat with PENZ promoting the highest WHC (8.32%) and dGP promoting the lowest (5.22%). Experimental diets had no effect on meat temperature 24 h after slaughter, meat pH, *L* *, *a* *, *b* *, chroma and hue angle of broiler chickens.

Table 7. Effect of grape pomace-containing diets treated with polyethylene glycol and a cellulolytic enzyme mixture on meat quality parameters of broiler chickens.

Parameters	[1] Diets					[3] SEM	*p* Value
	CON	ENZ	dGP	dPEG	PENZ		
Cooking loss (%)	19.19	22.72	21.32	22.91	23.31	1.060	0.058
Shear force (N)	4.84	4.40	4.86	5.65	4.89	0.366	0.225
[2] WHC (%)	7.82 [a,b]	7.58 [a,b]	5.22 [a]	5.65 [a,b]	8.32 [b]	0.763	0.020
Temperature (°C)	14.58	16.55	14.83	15.68	15.78	0.482	0.070
Meat pH	6.95	6.76	6.92	6.91	6.82	0.086	0.500
Lightness (*L* *)	48.59	49.85	49.76	48.52	49.50	0.614	0.393
Redness (*a* *)	1.47	1.48	1.51	1.49	1.50	0.014	0.293
Yellowness (*b* *)	13.75	12.23	14.50	13.45	13.81	0.571	0.128
Chroma	13.85	12.30	14.55	13.54	13.87	0.578	0.141
Hue angle	1.28	1.05	0.84	1.12	1.00	0.180	0.554

[1] Diets: CON = commercial chicken diet without red grape pomace; dGP = commercial chicken diet containing 100 g/kg untreated red grape pomace; dPEG = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (50 g/kg); ENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with Viscozyme®-L (1 g/kg); PENZ = commercial chicken diet containing 100 g/kg red grape pomace pre-treated with polyethylene glycol (5 g/kg) and Viscozyme®-L (1 g/kg); [2] WHC = water-holding capacity. [3] SEM: Standard error of the mean; [a,b] In a row, dietary treatment means with common superscripts do not differ ($p > 0.05$). [a,b] In a row, dietary treatment means with common superscripts do not differ ($p > 0.05$).

4. Discussion

Red grape pomace contains high levels of fiber and polymeric polyphenols such as proanthocyanidins that reduce the digestion and absorption of nutrients and other dietary compounds. As such, the incorporation of high levels of GP in chicken diets might impair nutrient digestion and growth. The application of PEG to ameliorate the anti-nutritional effects of condensed tannins in animal diets has been widely practiced; however, there are no documented studies of its application to improve the utilization of dietary GP in broiler chickens. In addition, possible additive effects of PEG and cell wall-degrading enzymes on physiological and meat quality parameters of broiler chickens have not been investigated. This is despite the possibility that a combined treatment of PEG and Viscozyme® may allow for higher inclusion levels of GP in broiler diets, resulting in reduced feed costs, improved broiler meat quality and good environmental stewardship.

Results from this study suggest that the inclusion of PEG and/or fibrolytic enzyme-treated GP in chicken diets at 100 g/kg did not depress overall feed intake. Indeed, the feed intake in chickens receiving treated GP was similar to that of birds on the commercial control. These results are in agreement with Chamorro et al. [24], who found no effect of carbohydrases and tannase enzymes on the feed intake of male broiler Cobb chicks when reared on diets containing GP. In this study, it was expected that broilers fed a commercial diet containing GP pre-treated with a combination of PEG and Viscozyme® (PENZ) would have the highest overall feed intake. This is because Viscozyme® has the capacity to hydrolyze complex plant cell walls, while PEG binds to tannins, thereby neutralizing their anti-nutritional effects. It was, therefore, anticipated that GP intake would be enhanced through the additive effects of these two treatments. However, the results did not support this hypothesis and no differences in feed intake were observed across all the experimental diets.

The application of PEG and Viscozyme® to GP affected the overall body weight gain of broiler chickens. The pre-treated GP promoted similar overall WG as the commercial control diet, which was higher than for untreated GP, suggesting that the anti-nutritional effects of tannins and fiber were successfully ameliorated, leading to enhanced nutrient bioavailability. These findings contradict those of Chamorro et al. [11], who investigated the effect of an exogenous enzyme, tannase, on the utilization of polyphenolics as well as the oxidation of meat lipids in chicks fed GP. This could be because Chamorro et al. [11] only used cell wall-degrading enzymes but did not attempt to ameliorate the negative nutritional effects of condensed tannins. However, a recent study by Ebrahimzadeh et al. [25] showed that treating GP (included at 100 g/kg level) with a tannin-degrading enzyme, tannase, did not affect the chick growth performance. The fact that PEG treatment improved the body weight gain of chickens in this study shows that this tannin-binding compound may be a better alternative to the enzyme tannase as a strategy to reduce the anti-nutritional effects of tannins. The effect of treated GP-containing diets on growth performance is an indication that PEG and Viscozyme® treatment increased the amount of nutrients released in the intestines. Generally, high tannin diets reduce growth performance as shown by the reduction in the overall WG of broilers reared on the commercial diet containing untreated GP.

In a study conducted by Lichovnikova et al. [26], feeding broiler chickens with a 1.5% diet of red GP had a positive effect on the antioxidant activity of the blood, indicating its potential as a biological antioxidant. In this study, most of the hematological and serum biochemical parameters were not influenced by dietary treatments and fell within the normal range for chickens. Nonetheless, mean corpuscular volume (MCV), an anemia diagnostic parameter [27] and phosphorus levels were higher on Viscozyme®-treated diets indicating that treating GP with Viscozyme® did not compromise the health status of the birds. In contrast to the current findings, Aditya et al. [28] reported lower serum total cholesterol levels in broilers fed GP. However, Kara et al. [29] reported no change in serum triglyceride and total cholesterol levels when laying hens were supplemented with GP.

Theoretically, the consumption of high fiber diets is expected to induce changes in the size of intestines and gizzards in birds as an adaptation mechanism [30]. However, the relative weights of livers, intestines, gizzards and pancreas were not affected by the diets. Although these results were not

expected, they are in agreement with Brenes et al. [31] who reported that broilers fed diets containing GP showed no significant differences in the relative weight of internal organs when compared with the control groups. The expectation was that untreated and enzyme-treated GP would still contain phenolics that require detoxification by the liver upon absorption from the digestive tract, leading to atrophy of the organ. However, it is quite possible that at a 100 g/kg inclusion rate, GP did not supply enough phenolics to overwhelm the chicken's detoxifying mechanisms. For untreated and PEG-treated GP, no reduction in fiber levels was expected, which should have resulted in an increase in the size of the gizzard compared to the control diet.

Chickens reared on the diet containing untreated GP had a lower slaughter body weight compared to those on diets containing treated GP. Treating GP with PEG and Viscozyme® resulted in bigger carcasses due to amelioration of the anti-nutritional effects of tannins and fiber. However, these findings contradict those of Brenes et al. [31], who reported that graded levels of GP and vitamin E had no effect on carcass weight in chickens. The lack of dietary influence on meat color in this study was surprising given that GP contains anthocyanins that cause meat pigmentation. Indeed, a study by Kasapidou et al. [32] on the effect of grape pomace supplementation on broiler meat quality characteristics confirms that dietary GP affects meat redness.

5. Conclusions

The dietary inclusion of untreated red grape pomace at 100 g/kg reduced body weight gain in broilers. However, when red grape pomace was pre-treated with polyethylene glycol, broilers had a similar body weight gain and hot carcass weight as those on the commercial control diet. It was concluded that enzyme and polyethylene glycol pre-treatments promoted a similar body weight gain as the conventional commercial diet, suggesting that the anti-nutritional effects of tannins and fiber were successfully ameliorated.

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Soybean Lecithin High in Free Fatty Acids for Broiler Chicken Diets: Impact on Performance, Fatty Acid Digestibility and Saturation Degree of Adipose Tissue

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Simple Summary: The search of alternatives for soybean oil, as a dietary energy source, has generated a lot of interest in broiler feeding due to economic and supply reasons. Soybean lecithin, as a co-product derived from the soybean oil degumming process, and its blending with other by-products derived from the vegetable oil refining process such as acid oils, may represent an alternative energy source for broiler chicken diets formulation. The current study has demonstrated that soybean lecithin high in free fatty acids can be included in grower–finisher diets, as a partial replacer of soybean oil or in combination with an acid oil, without impairing performance or fatty acid digestibility and causing minor changes in the fatty acid composition of the abdominal fat pad.

Abstract: Two experiments were conducted to evaluate the inclusion of soybean lecithin with a high free fatty acid content (L) in starter and grower–finisher broiler diets, as well as its influence on performance, energy and fatty acid (FA) utilization and the FA profile of the abdominal fat pad (AFP). A basal diet was supplemented with soybean oil (S; Experiment 1) or acid oil (AO; Experiment 2) at 3%, and increasing amounts of L (1%, 2% and 3%) were included in replacement. The inclusion of L did not modify performance parameters ($p > 0.05$). The S replacement by L reduced energy and total FA utilization ($p \leq 0.05$) in starter diets; however, in grower–finisher diets, a replacement up to 2% did not modify energy and FA utilization ($p > 0.05$). The AO substitution by L produced no modifications on energy and FA utilization ($p > 0.05$) during the starter phase, while the blend of 1% of AO and 2% of L resulted in the best combination in terms of the FA digestibility. The FA profile of the AFP reflected the FA composition of diets. The addition of L could replace, up to 2% or be blended with AO in broiler grower–finisher diets as an energy source.

Keywords: broiler chickens; alternative energy source; soybean lecithin; phospholipids; vegetable acid oil; digestibility balance; free fatty acids; triacylglycerols

1. Introduction

Co-products and by-products derived from the vegetable oil refining process may represent an interesting and economic alternative to conventional fat sources used in broiler feeding, such as soybean oil. During degumming, most phospholipids (PL) present in crude soybean oil are extracted, generating a co-product known as crude soybean lecithin. Lecithins are defined as a lipid mixture highly composed of PL, but they are also rich in glycolipids, carbohydrates and neutral lipids, such as triacylglycerols [1]. Soybean lecithin is an available low-cost energetic source [2] with a similar fatty acid (FA) profile to soybean oil [3,4]. In addition, its elevated surface-active PL content of soybean lecithin represents an added value as an emulsifier; hence, its dietary inclusion may improve fat

absorption [5,6]. However, soybean lecithin has a high viscosity that hampers its inclusion during feed manufacturing. For this reason, in order to facilitate its homogeneous blending in feed, mixing lecithin at different ratios with acid or crude oils is a common practice [7]. On the other hand, vegetable acid oils derived from the chemical refining process of crude oils are normally composed of a large quantity of free fatty acids (FFA; 40%–60%) and represent an important source of energy [8,9]. Nevertheless, it has been observed that a high dietary FFA concentration may reduce energy utilization by impairing dietary fat solubilization in the gastrointestinal tract [5].

We hypothesized that soybean lecithin could be considered as an alternative energy source for broiler chicken diets, in replacement or combined with other fats, with no negative effects on the performance, nutrient digestibility, and FA composition of adipose tissue. Therefore, a total of two experiments were conducted to assess the potential use of a soybean lecithin high in FFA (L) as an alternative energy source in broiler feeding when combined with soybean oil (S; Experiment 1) or a monounsaturated vegetable acid oil (AO; Experiment 2). The evaluation was based on the study of the influence of L inclusion on performance, feed energetic content, FA digestibility, and, thus, the effect on the FA profile of the abdominal fat pad (AFP) of the broiler carcass.

2. Materials and Methods

2.1. Experimental Design and Diets

The experiments were performed at Servei de Granges i Camps Experimentals (Universitat Autònoma de Barcelona, Bellaterra, Spain), were in accordance with the European Union Guidelines (2010/63/EU), and were approved by the Animal Ethics Committee (CEEAH) of the same institution (number code: 4006). Two different trials of 38 days (d) each were performed with a feeding program in two phases: Starter (from 0 to 21 d) and grower–finisher (from 22 to 38 d). Experimental diets (Table 1) were based on wheat and soybean meal, presented in mash form, and were formulated to meet or exceed FEDNA (Fundación Española para el Desarrollo de la Nutrición Animal) requirements [10]. Furthermore, titanium dioxide (TiO_2) was used as an inert marker at 5 g/kg in order to perform digestibility balances.

Table 1. Ingredient composition of the starter and grower–finisher broiler chicken diets on an as-fed basis (Experiments 1 and 2).

Ingredients (%)	Experiment 1		Experiment 2	
	Starter Diet (0–21 d)	Grower–Finisher Diet (22–38 d)	Starter Diet (0–21 d)	Grower–Finisher Diet (22–38 d)
Wheat	36.55	46.84	36.64	45.92
Soybean meal 47%	29.43	21.09	30.46	24.25
Corn	9.71	-	9.71	-
Barley	9.71	15.58	8.33	15.76
Extruded full-fat soybean	4.76	-	4.73	-
Added fats [1]	3.00	3.00	3.00	3.00
Rapeseed meal 00	-	3.42	-	3.41
Sunflower meal 28%	-	2.44	-	-
Sepiolite	1.93	1.90	2.03	2.03
Palm oil	-	1.50	-	1.51
Calcium carbonate	1.19	1.08	1.16	1.00
Monocalcium phosphate	0.97	0.57	0.93	0.48
Trace minerals/vitamin premix [2]	1.15	1.01	1.44	1.17
Titanium dioxide	0.50	0.50	0.50	0.50
Salt	0.30	0.23	0.30	0.23
L-lysine	0.30	0.35	0.28	0.28
DL-methionine	0.28	0.21	0.28	0.22
L-threonine	0.08	0.09	0.07	0.07
Sodic bicarbonate	0.07	0.12	0.07	0.11
Clorure choline 75%	0.07	0.07	0.07	0.06

[1] Soybean oil and soybean lecithin high in free fatty acids and monounsaturated acid oil in different blending proportions. [2] Provides per kg feed: Vitamin A (from retinol), 13,500 IU; vitamin D3 (from cholecalciferol), 4800 IU; vitamin E (from alfa-tocopherol), 49.5 IU; vitamin B1, 3 mg; vitamin B2, 9 mg; vitamin B6, 4.5 mg; vitamin B12, 16.5 µg; vitamin K3, 3 mg; calcium pantothenate, 16.5 mg; nicotinic acid, 51 mg; folic acid, 1.8 mg; biotin, 30 µg; Fe (from $FeSO_4 \cdot 7\ H_2O$), 54 mg; I [from $Ca(I_2O_3)_2$], 1.2 mg; Co (from 2 $CoCO_3 \cdot 3\ Co(OH)_2 \cdot H_2O$), 0.6 mg; Cu (from $CuSO_4 \cdot 5\ H_2O$), 12 mg; Mn (from MnO), 90 mg; Zn (from ZnO), 66 mg; Se (from Na_2SeO_3), 0.18 mg; Mo [from $(NH_4)_6Mo_7O_{24}$], 1.2 mg; organic acids (starter diets at 4 g/kg; grower–finisher diets at 3 g/kg); β-glucanase 350 IU; xylanase 1125 IU.

Experiment 1: A total of 96 Ross 308 newly hatched female broiler chickens were randomly assigned to one of four experimental treatments (six replicates/treatment) and allocated in cages (four birds/cage). A control basal diet was supplemented with S at 3% (S3), and increasing amounts of L (soybean lecithin blended with soybean acid oil in a 5:1 proportion) were included in replacement of S as added fat: 1% (S2–L1), 2% (S1–L2) and 3% (L3).

Experiment 2: A total of 120 Ross 308 newly hatched female broiler chickens were randomly assigned to one of five experimental treatments (six replicates/treatment) and allocated in cages (four birds/cage). A control basal diet was supplemented with AO (a 1:1 blend of olive pomace acid oil and sunflower acid oil) at 3% (AO3), and increasing amounts of L were included in replacement of AO: 1% (AO2–L1), 2% (AO1–L2) and 3% (L3). The S3 diet was included as a reference treatment.

2.2. Animal Husbandry and Controls

The animals were obtained from a local hatchery (Pondex S.A.U., Juneda, Spain), weighed, wing-tagged and randomly distributed in cages with a grid floor and a tray for excreta collection. The temperature and light program used was consistent with the specifications in the Ross 308 lineage management handbook [11], and the animals were allowed to consume feed and water ad libitum. Broiler body weight (BW) was recorded individually at 21 and 38 d post-hatch, whereas feed intake was measured by cage at 21 and 38 d post-hatch. The data were used to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) from both phases and the overall results of each experiment. Mortality was recorded daily to adjust ADG and ADFI. Two nutritional balances were performed for each experiment between d 9 and 11 (starter period) and d 36 and 37 (grower–finisher period), where excreta samples (free of contaminants) were taken on each day of the digestibility balance (once per d), homogenized, freeze-dried, ground, and kept at 4 °C until further analysis. At the end of each experiment, all the animals used in both experiments were slaughtered in a commercial abattoir, and carcasses were recovered.

Carcasses (total BW excluding blood and feathers) were weighed, and the AFP (from the proventriculus surrounding the gizzard down to the cloaca) of each bird was removed and weighed in order to calculate the AFP carcass percentage. Furthermore, representative sample of the AFP of each bird was taken, pooled by replicate, and frozen at −20 °C for further analysis.

2.3. Laboratory Analyses

Experimental oil samples (S, L and AO) were chemically characterized, as shown in Table 2.

The FA composition was analyzed by gas chromatography following the methodology described by Guardiola et al. [12]. The acid value was determined according to International Organization for Standardization (ISO) 660 [13], and the acidity was expressed as the FFA percentage of oleic acid. In the case of the soybean lecithin high in FFA, the acetone insoluble matter was analyzed using the Ja 4–46 method from the American Oil Chemists' Society (AOCS) [14], and the PL composition was determined by HPLC (D450 MT1, Kontron; Eching, Germany) according to the method described by Helmerich and Koehler [15].

Regarding the experimental feed samples, the proximate analysis was performed following AOAC methodology [16]: Ether extract (Method 920.39), crude protein (Method 968.06), ash (Method 942.05), dry matter (Method 934.01), and crude fiber (Method 962.09). The gross energy content was determined for oil, feed and excreta samples by an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000; Staufen, Germany). Titanium dioxide was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 3200 RL, Perkin Elmer; Waltham, MA, USA) in Experiment 1, while it was determined in Experiment 2 by the method described by Short et al. [17].

The FA profile of the feed and excreta was analyzed by adding nonadecanoic acid (C19:0, Sigma-Aldrich Chemical Co.; St. Louis, MO) as an internal standard and following the method described by Sukhija and Palmquist [18], whereas in the case of the AFP, the method described by Carrapiso et al. [19] was used. The final extract obtained was injected into a gas chromatograph

(HP6890, Agilent Technologies; Waldbronn, Germany) following the method conditions described by Cortinas et al. [20].

Table 2. Chemical analysis of the experimental added oils.

Item	Experimental Fats [1]				
	Experiment 1		Experiment 2		
	S	L	S	AO	L
Fatty Acid Profile (%) [2]					
SFA	16.5	20.4	16.0	15.1	21.9
C16:0	11.7	15.7	10.6	9.97	16.1
C18:0	3.55	4.68	4.26	3.84	5.86
MUFA	24.3	19.4	23.5	54.2	19.6
C18:1 ω-9	22.3	19.4	21.8	51.3	19.6
PUFA	59.2	60.2	60.5	30.7	58.5
C18:2 ω-6	53.4	54.2	52.8	29.2	52.6
C18:3 ω-3	5.76	6.09	7.67	1.55	5.90
Minor FA	3.32	N.D.	2.85	4.21	N.D.
UFA:SFA	5.06	3.90	5.25	5.62	3.57
PUFA:SFA	3.59	2.95	3.78	2.03	2.67
Acidity (%) [2]					
FFA	2.41	22.6	1.49	52.9	25.5
Phospholipids (%) [2]					
AI	N.D.	48.7	N.D.	N.D.	46.8
Total PL	N.D.	24.6	N.D.	N.D.	27.8
PC	N.D.	9.42	N.D.	N.D.	9.96
PI	N.D.	5.80	N.D.	N.D.	7.38
PE	N.D.	4.62	N.D.	N.D.	5.56
AP	N.D.	2.11	N.D.	N.D.	3.58
LPC	N.D.	2.68	N.D.	N.D.	1.31
Energy Content (MJ/kg)					
GE	39.3	34.0	40.3	39.5	34.7

SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; UFA:SFA: Unsaturated-to-saturated fatty acid ratio; PUFA:SFA: Polyunsaturated-to-saturated fatty acid ratio; FFA: Free fatty acid; AI: Acetone insoluble matter; Total PL: Total phospholipids; PC: Phosphatidylcholine; PI: Phosphatidylinositol; PE: Phosphatidylethanolamine; AP: Phosphatidic acid; LPC: Lysophosphatidylcholine; GE: Gross energy; N.D.: Not determined. [1] S: Soybean oil; L: Soybean lecithin high in free fatty acids; AO: Monounsaturated acid oil. [2] Percentage of total product.

2.4. Calculations and Statistical Analysis

The apparent digestibility of FA (%) was calculated using the following equation:

$$\text{The apparent digestibility of FA} = 1 - \{(TiO_2 \text{ in feed/FA concentration in feed})/(TiO_2 \text{ concentration in excreta/FA concentration in excreta})\}.$$

The apparent metabolizable energy (AME) of the diets was calculated multiplying the apparent absorption of the gross energy by its corresponding diet gross energy. Both calculation formulas were in accordance with Rodriguez-Sanchez et al. [21].

Cage means were used as the experimental unit (six replicates/treatment) in performance (except BW), FA digestibility, the FA profile of the AFP, and the AME values of the diets. A Shapiro–Wilk test indicated a normal distribution of the data. In Experiment 1, data were analyzed by a one-way ANOVA using R Statistics (Version 3.3.1; R Core Team, Vienna, Austria), with treatment as the main factor. In Experiment 2, soybean oil treatment (S3) was compared against the AO3 treatment separately with a one-way ANOVA (S3 vs. AO3), whereas diets containing co-products and by-products were compared with a one-way ANOVA (AO3 vs. AO2–L1 vs. AO1–L2 vs. L3). Tukey's multiple-range test was performed to determine whether means were significantly different ($p \leq 0.05$). The linear model used was: $Y_{ij} = \mu + \alpha_i + \varepsilon_j$, where μ is the global mean, α is the treatment effect, and ε is the residual error.

3. Results

3.1. Experimental Fats and Diets Composition

The FA profiles of S and L (Table 2) were similar regarding polyunsaturated FA (PUFA) content; nevertheless, L presented a higher content in saturated FA (SFA) and a lower content in monounsaturated FA (MUFA) than S. In the case of AO, oleic acid was the most abundant FA, followed by linoleic acid. Furthermore, the three added fats differed in their average unsaturated-to-saturated FA ratio (UFA:SFA), where S and AO presented higher average values (5.14 and 5.60, respectively) than L (3.74); the three fats also differed in their average polyunsaturated-to-saturated FA ratio (PUFA:SFA), where AO presented the lowest value (2.04), followed by L (2.82) and S (3.67). Concerning FFA content, AO presented the highest value, representing its main lipid molecular structure (52.9%), whereas L showed a medium average content (24.1%), and S showed the average lowest value (1.95%). Additionally, both S and AO presented higher average values of gross energy (39.8 and 39.5 MJ/kg, respectively) than L (34.4 MJ/kg).

The proximate analysis results and the FA profile of the experimental diets are shown in Table 3 (Experiment 1) and Table 4 (Experiment 2). The experimental treatments showed a similar macronutrient content, and their main differences were related to the FA profile and the energetic content. In Experiment 1, the replacement of S by L increased dietary SFA in starter (9.4%) and grower–finisher diets (11.9%), whereas a decrease in MUFA was observed (11.3% and 7.1% for starter and grower–finisher diets, respectively), causing a reduction in dietary the UFA:SFA. In Experiment 2, the replacement of AO by L increased dietary SFA (9.0% and 7.2%, for starter and grower–finisher diets, respectively) and dietary PUFA (28.4% and 36.8% for starter and grower–finisher diets, respectively). On the contrary, this replacement reduced the MUFA content (42.8% and 36.7% for starter and grower–finisher diets, respectively). The replacement of AO by L reduced the UFA:SFA, whereas it increased the PUFA:SFA.

Table 3. Analyzed gross energy, macronutrient content and fatty acid composition of starter and grower–finisher broiler chicken diets (Experiment 1).

Item [2]	Dietary Treatments [1]							
	Starter (0–21 d)				Grower–Finisher (22–38 d)			
	S3	S2–L1	S1–L2	L3	S3	S2–L1	S1–L2	L3
Macronutrient Content (%)								
Dry Matter	91.7	91.4	91.2	91.3	90.6	90.2	90.7	90.3
Crude Protein	23.7	23.1	22.7	23.2	21.5	20.8	21.4	20.7
Crude Fat	5.37	5.33	4.91	5.38	6.24	6.03	5.82	5.75
Crude Fiber	3.94	4.20	3.50	3.95	4.88	3.75	4.86	3.80
Ash	8.54	8.86	8.51	8.97	8.71	8.53	8.22	8.87
Fatty Acid Profile (%)								
SFA	18.2	18.7	19.1	19.9	25.0	25.9	27.0	28.0
C16:0	13.9	14.4	15.0	15.6	20.6	21.5	22.5	23.2
C18:0	3.55	3.58	3.70	3.88	3.44	3.51	3.55	3.80
MUFA	21.1	20.4	19.6	18.7	26.3	25.8	25.4	24.4
C18:1 ω-9	19.4	18.8	18.1	17.2	24.4	24.0	23.7	22.9
PUFA	60.7	60.9	61.3	61.4	48.7	48.3	47.6	47.6
C18:2 ω-6	54.6	54.7	54.7	54.7	44.2	43.8	43.1	43.0
C18:3 ω-3	6.16	6.18	6.23	6.39	4.80	4.68	4.64	4.75
Minor fatty acids	2.39	2.34	2.27	2.23	2.56	2.51	2.51	2.35
UFA:SFA	4.49	4.35	4.24	4.03	3.00	2.86	2.70	2.55
PUFA:SFA	3.34	3.26	3.21	3.09	1.95	1.86	1.76	1.70
Energy Content (MJ/kg)								
GE	17.4	17.3	17.1	17.1	17.5	17.4	17.4	17.2

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; UFA:SFA: Unsaturated-to-saturated fatty acid ratio; PUFA:SFA: Polyunsaturated-to-saturated fatty acid ratio; GE: Gross energy. [1] S3: Soybean oil (S) at 3.00%; S2–L1: S at 2.00% and soybean lecithin high in free fatty acids (L) at 1.00%; S1–L2: S at 1.00% and L at 2.00%; L3: L at 3.00%. [2] Samples were analyzed twice.

Table 4. Analyzed gross energy, macronutrient content, and fatty acid composition of starter and grower–finisher broiler chicken diets (Experiment 2).

Item [2]	Dietary Treatments [1]									
	Starter (0–21 d)					Grower–Finisher (22–38 d)				
	S3	AO3	AO2–L1	AO1–L2	L3	S3	AO3	AO2–L1	AO1–L2	L3
Macronutrient Content (%)										
Dry Matter	91.8	91.8	91.4	92.1	91.0	90.9	90.9	90.9	90.7	91.0
Crude Protein	22.3	22.2	23.1	22.5	22.5	22.1	21.3	20.6	20.6	20.8
Crude Fat	5.46	5.33	5.58	5.30	5.10	6.60	6.60	6.37	6.15	6.23
Crude Fiber	4.38	3.84	4.04	3.98	4.13	4.62	4.17	3.81	4.02	4.20
Ash	8.50	8.36	8.73	8.53	8.65	9.86	9.21	10.2	10.5	9.53
Fatty Acid Profile (%)										
SFA	17.2	17.9	18.2	18.8	19.6	23.6	24.7	25.4	25.9	26.5
C16:0	13.5	14.3	14.6	15.1	15.7	19.4	20.5	21.1	21.6	22.2
C18:0	3.70	3.66	3.63	3.68	3.87	3.96	3.95	3.98	3.99	3.99
MUFA	22.1	35.0	30.5	25.5	19.9	27.1	40.1	35.1	30.5	25.4
C18:1 ω-9	20.7	33.5	29.1	24.1	18.7	25.6	38.3	33.4	29.0	24.0
PUFA	60.7	47.1	51.3	55.7	60.5	49.3	35.2	39.5	43.6	48.1
C18:2 ω-6	54.1	43.4	46.8	50.6	54.6	43.7	32.5	36.0	39.6	43.3
C18:3 ω-3	6.61	3.74	4.45	5.14	5.90	5.56	2.73	3.46	4.07	4.81
Minor fatty acids	1.39	1.40	1.42	1.38	1.23	1.78	2.02	2.06	1.74	1.70
UFA:SFA	4.81	4.59	4.49	4.32	4.10	3.24	3.05	2.94	2.86	2.77
PUFA:SFA	3.53	2.63	2.82	2.96	3.09	2.09	1.43	1.56	1.68	1.82
Energy Content (MJ/kg)										
GE	17.3	17.3	17.1	17.1	16.9	17.6	17.6	17.5	17.5	17.4

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; UFA:SFA: Unsaturated-to-saturated fatty acid ratio; PUFA:SFA: Polyunsaturated-to-saturated fatty acid ratio; GE: Gross energy. [1] S3: Soybean (S) oil at 3.00%; AO3: Acid oil (AO) at 3.00%; AO2–L1: AO at 2.00% and soybean lecithin high in free fatty acids (L) at 1.00%; AO1–L2: AO at 1.00% and L at 2.00%; L3: L at 3.00%. [2] Samples were analyzed twice.

3.2. Growth Performance and Abdominal Fat Deposition

The growth performance and abdominal fat deposition parameters of Experiments 1 and 2 are shown in Tables 5 and 6, respectively.

Table 5. Growth performance and abdominal fat pad deposition of broiler chickens according to different dietary added fats (Experiment 1).

Item	Dietary Treatments [1]					
	S3	S2–L1	S1–L2	L3	RSE	*p*-Value
From 0 to 21 d						
BW at 0 d (g)	43.0	42.9	42.9	43.1	2.64	0.996
BW at 21 d (g)	825	816	836	825	85.6	0.891
ADFI (g/bird/d)	54.9	55.7	52.5	54.3	3.03	0.338
ADG (g/bird/d)	37.1	37.7	38.3	36.6	2.19	0.618
FCR (g/g)	1.45	1.41	1.40	1.44	0.039	0.170
From 22 to 38 d						
BW at 38 d (g)	2408	2461	2500	2428	186.8	0.509
ADFI (g/bird/d)	167.1	172.0	170.6	171.1	10.14	0.855
ADG (g/bird/d)	93.8	94.5	95.6	91.9	5.75	0.724
FCR (g/g)	1.78	1.79	1.82	1.86	0.075	0.287
From 0 to 38 d						
ADFI (g/bird/d)	105.1	107.8	105.3	105.9	6.00	0.885
ADG (g/bird/d)	62.3	61.8	63.9	62.2	3.70	0.755
FCR (g/g)	1.69	1.71	1.68	1.70	0.054	0.780
Carcass weight (g)	2147	2224	2241	2173	109.1	0.463
Abdominal Fat Depot						
g	40.01	35.86	33.78	39.45	3.835	0.062
(%)	1.93	1.61	1.64	1.82	0.251	0.134

BW: Body weight; ADFI: Average daily feed intake; ADG: Average daily gain; FCR: Feed conversion ratio; RSE: Residual standard error. [1] S3: Soybean oil (S) at 3.00%; S2–L1: S at 2.00% and soybean lecithin high in free fatty acids (L) at 1.00%; S1–L2: S at 1.00% and L at 2.00%; L3: L at 3.00%.

In both experiments, performance parameters were not affected by the replacement of the added fats (S and AO) by L in any phase, nor were the overall parameters of the experiments ($p > 0.05$). Nevertheless, in Experiment 2, the S replacement by AO impaired the feed conversion ratio in the grower–finisher phase and the global period of the experiment ($p \leq 0.05$); the AO replacement by L tended to improve the feed conversion ratio ($p = 0.055$). Concerning the effect of dietary added fats on abdominal fat deposition, no significant differences were observed between experimental treatments ($p > 0.05$).

Table 6. Growth performance and abdominal fat pad deposition of broiler chickens according to different dietary added fats (Experiment 2).

	Dietary Treatments [1]						
Item	**S3** [2]	**AO3**	**AO2–L1**	**AO1–L2**	**L3**	**RSE**	***p*-Value**
From 0 to 21 d							
BW at 0 d (g)	45.1	45.2	45.1	45.1	45.1	2.44	0.999
BW at 21 d (g)	876	878	870	864	876	89.6	0.943
ADFI (g/bird/d)	56.2	57.1	57.7	55.6	57.6	2.35	0.400
ADG (g/bird/d)	39.6	39.7	39.3	39.0	40.7	2.06	0.561
FCR (g/g)	1.40	1.44	1.47	1.41	1.45	0.052	0.943
From 22 to 38 d							
BW at 38 d (g)	2469	2395	2430	2418	2469	186.1	0.927
ADFI (g/bird/d)	163.5	160.9	165.4	159.8	164.7	9.90	0.706
ADG (g/bird/d)	91.7	87.8	90.3	89.8	90.0	6.36	0.897
FCR (g/g)	1.78 [x]	1.86 [y]	1.83	1.80	1.81	0.033	0.171
From 0 to 38 d							
ADFI (g/bird/d)	104.2	103.5	105.9	102.2	104.6	5.41	0.679
ADG (g/bird/d)	62.7	61.2	62.1	62.5	61.8	4.09	0.954
FCR (g/g)	1.66 [x]	1.71 [y]	1.71	1.66	1.67	0.032	0.055
Carcass weight (g)	2229	2183	2193	2172	2193	141.3	0.999
Abdominal Fat Depot							
g	43.86	40.61	45.04	39.34	45.31	5.528	0.185
(%)	1.97	1.88	2.05	1.79	2.06	0.175	0.064

[1] S3: Soybean oil (S) at 3.00%; AO3: Acid oil (AO) at 3.00%; AO2–L1: AO at 2.00% and L at 1.00%; AO1–L2: AO at 1.00% and L at 2.00%. L3: L at 3.00%. [2] S3 was not included in the statistical analysis against diets containing co- and by-products. [x,y] ANOVA AO3 vs S3: Values within the same row with no common superscripts are significantly different, $p \leq 0.05$. BW: Body weight; ADFI: Average daily feed intake; ADG: Average daily gain; FCR: Feed conversion ratio; RSE: Residual standard error.

3.3. Digestibility Balances

The influence of the added fats on the dietary feed AME and the FA digestibility in both feeding periods can be seen in Table 7 (Experiment 1) and Table 8 (Experiment 2).

The digestibility balance of Experiment 1 indicated, in starter diets, that the partial and total replacement of S by L (S2–L1, S1–L2, and L3) negatively affected the feed AME value ($p < 0.001$) and the FA digestibility. Animals fed diets with 2% and 3% of L (S1–L2 and L3) showed a lower total fatty acid (TFA; $p = 0.017$), MUFA ($p = 0.026$) and PUFA ($p = 0.004$) digestibility, and they tended to absorb SFA worse than animals fed S3 ($p = 0.055$). In the case of grower–finisher diets, animals fed L3 presented a lower feed AME ($p < 0.001$), and a lower TFA ($p = 0.020$), oleic acid ($p < 0.001$) and PUFA ($p = 0.003$) digestibility as compared to animals fed S3. However, no differences were observed between S3 and treatments with partial replacement by L (S2–L1 and S1–L2).

Results from Experiment 2 showed that the S3 treatment presented a higher dietary AME and TFA digestibility than AO3 in both periods ($p \leq 0.05$). Regarding the use of co-products (AO and L) as added fats, in the starter period, replacing AO by L led to no observable differences in the feed AME

and the digestibility of TFA, SFA and MUFA ($p > 0.05$). Nevertheless, L3 presented a higher digestibility of linolenic acid ($p = 0.011$) in contrast to AO3. On the other hand, grower–finisher diets showed differences between treatments in the SFA, MUFA and PUFA digestibility. The total replacement of AO by L (L3) did not modify the dietary AME or the digestibility of TFA and SFA, but it caused a lower MUFA ($p < 0.001$) and a higher linolenic acid ($p = 0.006$) digestibility. The lowest feed AME value was observed in AO2–L1 ($p < 0.001$), which was consistent with the FA digestibility. The AO2–L1 treatment presented a lower TFA and MUFA digestibility than AO3 ($p \leq 0.05$), and it presented a lower SFA digestibility than AO1–L2 and L3 ($p < 0.01$). Nonetheless, animals fed AO1–L2 did not show differences with the AO3 treatment and presented a higher MUFA digestibility in comparison to L3 ($p < 0.001$).

Table 7. Feed apparent metabolizable energy value and fatty acid digestibility of starter and grower–finisher broiler chicken diets according to added fat sources (Experiment 1).

	Dietary Treatments [1]					
Item	**S3**	**S2–L1**	**S1–L2**	**L3**	**RSE**	***p*-Value**
From 9 to 11 d						
AME (MJ/kg)	12.9 [a]	11.6 [b]	11.6 [b]	11.4 [b]	0.36	<0.001
Fatty Acid Digestibility (%)						
TFA	81.5 [a]	77.5 [a,b]	71.1 [b]	70.9 [b]	5.91	0.017
SFA	62.3	56.6	48.9	49.7	7.70	0.055
C16:0	69.5	65.0	58.4	60.4	6.83	0.098
C18:0	50.2	50.1	38.7	37.9	11.07	0.153
MUFA	79.2 [a]	75.1 [a,b]	67.9 [b]	68.8 [b]	6.50	0.026
C18:1 ω-9	80.3 [a]	78.0 [a]	69.8 [b]	69.8 [b]	3.98	<0.001
PUFA	88.0 [a]	83.6 [a,b]	75.6 [b]	78.0 [b]	5.45	0.004
C18:2 ω-6	87.7 [a]	83.2 [a,b]	74.9 [b]	77.3 [b]	5.60	0.003
C18:3 ω-3	90.6 [a]	87.4 [a,b]	80.6 [b]	83.0 [b]	4.31	0.003
From 36 to 37 d						
AME (MJ/kg)	13.0 [a]	12.8 [a]	12.9 [a]	11.8 [b]	0.39	<0.001
Fatty acid digestibility (%)						
TFA	85.0 [a]	83.5 [ab]	83.0 [a,b]	79.0 [b]	2.96	0.020
SFA	80.7	81.6	81.4	79.0	2.96	0.446
C16:0	82.3	83.3	83.3	81.0	2.84	0.480
C18:0	80.9	81.1	81.7	79.9	3.21	0.807
MUFA	84.8	83.8	81.8	78.7	4.25	0.141
C18:1 ω-9	88.5 [a]	86.6 [a]	87.9 [a]	83.9 [b]	1.46	<0.001
PUFA	85.3 [a]	82.2 [a,b]	84.7 [a]	79.9 [b]	2.29	0.003
C18:2 ω-6	85.1 [a]	82.1 [a,b]	84.5 [a]	79.7 [b]	2.33	0.003
C18:3 ω-3	86.4 [a]	83.4 [a,b]	85.7 [a]	81.7 [b]	2.00	0.003

[a–c] Values within the same row with no common superscripts are significantly different, $p \leq 0.05$. AME: Apparent metabolizable energy; TFA: Total fatty acid; SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; RSE: Residual standard error. [1] S3: Soybean oil (S) at 3.00%; S2–L1: S at 2.00% and soybean lecithin high in free fatty acids (L) at 1.00%; S1–L2: S at 1.00% and L at 2.00%; L3: L at 3.00%.

Table 8. Feed apparent metabolizable energy value and fatty acid digestibility of starter and grower–finisher broiler chicken diets according to added fat sources (Experiment 2).

Item	Dietary Treatments [1]						
	S3 [2]	AO3	AO2–L1	AO1–L2	L3	RSE	*p*-Value
From 9 to 11 d							
AME (MJ/kg)	12.5 ^{x}	12.0 ^{y}	11.7	12.1	11.9	0.41	0.252
Fatty Acid Digestibility (%)							
TFA	79.6 ^{x}	65.9 ^{y}	66.1	71.4	70.1	7.06	0.478
SFA	68.1 ^{x}	51.7 ^{y}	53.4	59.9	60.6	9.62	0.344
C16:0	73.1 ^{x}	61.5 ^{y}	61.6	68.1	66.2	7.94	0.428
C18:0	70.3 ^{x}	47.6 ^{y}	58.8	62.6	62.3	12.69	0.186
MUFA	80.3 ^{x}	70.5 ^{y}	70.6	75.1	70.5	7.60	0.678
C18:1 ω-9	80.8	71.9	71.5	76.3	71.9	7.26	0.649
PUFA	82.6 ^{x}	67.9 ^{y}	68.0	75.4	73.8	5.67	0.097
C18:2 ω-6	82.1 ^{x}	67.6 ^{y}	67.7	73.3	73.3	6.13	0.234
C18:3 ω-3	86.7 ^{x}	70.8 ^{y,b}	71.3 ^{b}	77.3 ^{ab}	78.2 ^{a}	4.12	0.011
From 36 to 37 d							
AME (MJ/kg)	12.7 ^{x}	12.3 ^{y,a}	11.7 ^{b}	12.5 ^{a}	12.4 ^{a}	0.25	<0.001
Fatty Acid Digestibility (%)							
TFA	87.0 ^{x}	84.3 ^{y,a}	81.4 ^{b}	84.6 ^{a}	83.3 ^{a,b}	1.73	0.022
SFA	83.3	81.1 ^{a,b}	78.6 ^{b}	82.8 ^{a}	82.6 ^{a}	1.80	0.002
C16:0	86.1	84.1 ^{a}	81.4 ^{b}	85.4 ^{a}	85.0 ^{a}	1.58	0.001
C18:0	88.1 ^{x}	84.4 ^{y,a,b}	84.0 ^{b}	86.2 ^{a}	86.2 ^{a}	1.51	0.041
MUFA	88.2	88.6 ^{a}	85.5 ^{bc}	87.5 ^{a,b}	84.7 ^{c}	1.24	<0.001
C18:1 ω-9	90.1	90.0 ^{a}	87.5 ^{bc}	89.1 ^{a,b}	87.1 ^{c}	1.18	0.001
PUFA	87.9 ^{x}	81.5 ^{y}	79.3	82.9	82.9	2.52	0.071
C18:2 ω-6	87.5 ^{x}	81.6 ^{y}	79.2	82.8	82.6	2.52	0.080
C18:3 ω-3	90.5 ^{x}	80.1 ^{y,b}	80.1 ^{b}	83.9 ^{a,b}	84.9 ^{a}	2.63	0.006

[1] S3: Soybean oil at 3.00%; AO3: Acid oil (AO) at 3.00%; AO–L1: AO at 2.00% and soybean lecithin high in free fatty acids (L) at 1.00%; AO–L2: AO at 1.00% and L at 2.00%; L3: L at 3.00%. [2] S3 was not included in the statistical analysis against diets containing co-products. $^{a–c}$ Values within the same row with no common superscripts are significantly different, $p \leq 0.05$; ^{x,y} ANOVA S3 vs. AO3: Values within the same row with no common superscripts are significantly different, $p \leq 0.05$. AME: Apparent metabolizable energy; FA: Fatty acid; SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; RSE: Residual standard error.

3.4. Fatty Acid Composition of Abdominal Fat Adipose Tissue

The effect of dietary added fat on the FA composition of the AFP can be seen in Table 9. Total replacement of S by L increased SFA, in particular palmitic acid concentration ($p < 0.01$), whereas it reduced the UFA:SFA and the PUFA:SFA ($p < 0.01$). Furthermore, a tendency for a reduction of linoleic acid concentration ($p = 0.069$) was observed. In contrast to S3, animals feed AO3 presented an AFP with a higher MUFA concentration, concretely oleic acid ($p \leq 0.05$), and a lower PUFA content, concretely linoleic and linolenic acid ($p \leq 0.05$), thus reducing the PUFA:SFA ($p \leq 0.05$). Finally, the use of L as a substitute for AO caused an increase in PUFA, specifically linoleic and linolenic acid ($p < 0.01$), and a reduction in the MUFA content ($p < 0.01$). In this case, the PUFA:SFA increased as long as L replaced AO ($p = 0.014$).

Table 9. Fatty acid composition of abdominal fat pad of broiler chickens according to different fat sources [1] in diet (Experiments 1 and 2).

Item	Experiment [1]						Experiment [2]						
	Dietary Treatments				RSE	*p*-Value	Dietary Treatments					RSE	*p*-Value
	S3	S2–L1	S1–L2	L3			S3 [2]	AO3	AO2–L1	AO1–L2	L3		
	Fatty Acid Profile (%)												
SFA	29.8 ^{b}	30.0 ^{b}	30.3 ^{b}	32.1 ^{a}	1.06	0.005	29.2	29.8	30.8	30.3	31.1	1.14	0.287
C16:0	23.6 ^{b}	24.0 ^{b}	24.3 ^{b}	25.7 ^{a}	0.76	<0.001	23.1	23.6	24.2	23.9	24.5	0.98	0.463
C18:0	5.31	5.46	5.17	5.55	0.64	0.779	5.37	5.34	5.73	5.39	5.44	0.40	0.364
MUFA	44.9	46.4	46.4	46.9	2.81	0.661	46.8 ^{y}	53.6 ^{x,a}	50.8 ^{a,b}	48.5 ^{b}	46.7 ^{b}	2.76	0.002
C18:1 ω-9	37.4	38.5	37.3	38.3	1.45	0.468	39.0 ^{y}	45.4 ^{x,a}	42.8 ^{a,b}	40.6 ^{bc}	38.6 ^{c}	2.06	<0.001
PUFA	25.3	23.7	23.3	21.0	2.85	0.107	24.5 ^{x}	16.6 ^{y,b}	17.8 ^{a,b}	21.4 ^{a}	22.5 ^{a}	2.75	0.004
C18:2 ω-6	22.4	20.9	19.7	18.5	2.40	0.069	21.3 ^{x}	14.9 ^{y,b}	16.0 ^{a,b}	19.0 ^{a}	19.9 ^{a}	2.41	0.006
C18:3 ω-3	2.19	2.06	2.03	1.88	0.27	0.293	2.47 ^{x}	1.06 ^{y,c}	1.42 ^{b,c}	1.81 ^{a,b}	2.02 ^{a}	0.26	<0.001
UFA:SFA	2.37 ^{a}	2.34 ^{a}	2.30 ^{a,b}	2.11 ^{b}	0.12	0.007	2.43	2.36	2.25	2.32	2.22	0.12	0.223
PUFA:SFA	0.85 ^{a}	0.79 ^{ab}	0.79 ^{a,b}	0.65 ^{b}	0.09	0.009	0.84 ^{x}	0.55 ^{y,b}	0.60 ^{a,b}	0.74 ^{a}	0.73 ^{a}	0.10	0.014

[1] S3: Soybean oil (S) at 3.00%; S2–L1: S at 2.00% and soybean lecithin high in free fatty acids (L) at 1.00%; S1–L2: S at 1.00% and L at 2.00%; AO3: Acid oil (AO) at 3.00%; AO2–L1: AO at 2.00% and L at 1.00%; AO1–L2: AO at 1.00% and L at 2.00%; L3: L at 3.00%. [2] S3 was not included in the statistical analysis against diets containing co-products. $^{a–c}$ Values within the same row with no common superscripts are significantly different, $p \leq 0.05$. ^{x,y} ANOVA S3 vs. AO3: Values within the same row with no common superscripts are significantly different, $p \leq 0.05$. SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; UFA:SFA: Unsaturated-to-saturated fatty acid ratio; PUFA:SFA: Polyunsaturated-to-saturated fatty acid ratio; RSE: Residual standard error.

4. Discussion

4.1. Chemical Composition of the Experimental Fats and Diets

The gross energy content of the added fats indicated that L resulted in being less energetic than S and AO. This fact is a direct consequence of PL releasing less energy than triacylglycerol and FFA. Furthermore, the L included in both experiments contained high levels of FFA (24.1%) because it was blended with soybean acid oil. The standard FFA content of soybean lecithin products is normally established at between 1.0% and 3.0% [1,22]. It is important to mention that available literature regarding the use of a soybean lecithin high in FFA in monogastric nutrition is scarce, and the literature review was based on studies that used a regular soybean lecithin with a lower FFA content. The chemical composition of the experimental diets reflected the FA profile of the added fats. The dietary UFA:SFA was reduced, as L was included in the replacement of S, which was also reported by Soares and Lopez-Bote [3].

4.2. Growth Performance and Abdominal Fat Deposition

The inclusion of L as a substitute for S did not lead to any negative effect on growth efficiency. Results agree with Azman and Cifti [23], who observed that a partial replacement (50%) of soybean oil by a soybean lecithin (4% and 6% of total added fats for starter and grower–finisher diets, respectively) did not modify final the BW or the global feed conversion ratio. However, the replacement of S by AO reduced feed conversion efficiency in the grower–finisher phase and the global period of Experiment 2. Some authors have stated that acid oils present a lower nutritive value than native oils due to their main lipid molecular structure being FFA, negatively affecting FA absorption and energy utilization [6,24].

Regarding abdominal fat deposition, results indicated that the different added fats included had no influence. It was demonstrated by Ferrini et al. [25] that animals fed a diet high in SFA content (PUFA:SFA = 0.25) presented a higher AFP deposition than animals fed diets rich in PUFA (PUFA:SFA = 6.72). The lack of differences observed in fat deposition in the present studies could be related to the slight changes in saturation degree between treatments (grower–finisher S3 and L3 PUFA:SFA = 1.95 and 1.70, respectively).

4.3. Digestibility Balances

Results extracted from Experiment 1 showed that, in terms of FA and energy utilization, the substitution of S by L at any level in starter diets is not recommended. However, the results in adult broilers suggest that L can partially replace S up to 2%. In accordance with our results, Huang et al. [4] observed, in young broiler chickens, that the partial (1%) and total replacement (2%) of soybean oil by soybean lecithin reduced the feed AME content. In the case of adult broilers, they reported that the partial (0.5% and 1%) and total (2%), replacement of soybean oil by soybean lecithin did not affect the feed AME value or ether extract utilization. Tancharoenrat et al. [26] indicated that young chicks present a limited capacity to digest and absorb fats; however, this capacity is improved from two weeks of life. Our results are consistent with this fact due to the fact that grower–finisher broilers showed a better utilization of L than starter broilers.

In Experiment 2, the comparison between S3 and AO3 demonstrated the lowering effect of the high FFA content on the FA digestibility and the feed AME, as other authors have previously stated [27,28]. It has been established that the presence of monoacylglycerols is essential for a correct solubilization of the products derived from the lipolysis into mixed-micelles [24]. In addition, Sklan [24] also suggested a direct relationship between monoacylglycerol presence in the duodenum and bile secretion, justifying the lower FA absorption rate of acid oils in comparison to crude oil. These facts were confirmed by Rodriguez-Sanchez et al. [9], who observed that a high presence of FFA was related to an insufficient solubilization and absorption of lipolysis products, and, in particular, this fact was more pronounced with unsaturated diets than saturated ones. The blending of AO and L in starter diets did not modify the FA digestibility except for linolenic acid, which was enhanced by the L inclusion. However, in grower–finisher diets, the blending of 1% of AO and 2% of L resulted in the best option in terms of FA utilization. Some authors have suggested that soybean lecithin, as an emulsifier, may enhance lipid absorption—in particular, SFA and long-chain FA—by facilitating FA incorporation inside the micelles [5,6]. However, in accordance with Soares and Lopez-Bote results [3], no improvement of the SFA digestibility related to L inclusion was demonstrated in the present experiments. This lack of effect could be related to the highly unsaturated degree of the experimental diets used in the present study. On the other hand, in the grower–finisher phase, the AO1–L2 treatment resulted in the best option, thanks to an improvement in linolenic acid, along with a tendency for a growth of the PUFA digestibility (p = 0.071), which suggests an emulsifying effect. It is well known that blending fats with a complementary FA profile and different lipid molecular structures (triacylglycerols, FFA and PL) produces positive interactions in terms of the AME content and the FA digestibility [2,27,28]. The synergic effect observed between 1% of AO and 2% of L can be explained because it might have been an adequate proportion of PL capable of better solubilizing FFA in the mixed micelle, facilitating its absorption. On the other hand, it is important to comment on the grower–finisher results shown in the AO2–L1 treatment, which was also a blending treatment but showed the lowest feed AME value and the lowest TFA digestibility. Results may suggest that replacing an acidic oil by a less energetic oil with a high acidity, such as L (Table 2), caused an elevated proportion of the FFA:PL, thus leading to an insufficient presence of PL capable of solubilizing the FFA into the mixed micelles. As a consequence, a chemical characterization of the different fats and oils used as energy sources can provide important information about the possible interactions between different lipid molecular structures, as Roll et al. [28] have previously stated.

4.4. Fatty acid Composition of Abdominal Fat Adipose Tissue

The FA profile of the AFP reflected the FA profile of the diets, in accordance with most of the published data [25,29]. Though some authors have reported that the presence of different dietary lipid molecular structures, such as randomized FA, influences the FA profile of the AFP [29,30], our results demonstrate that the saturation degree of the AFP is more influenced by the dietary saturation degree rather than by the lipid molecular structures (triacylglycerols, FFA and PL) present in the feed.

5. Conclusions

In summary, the inclusion of soybean lecithin high in FFA is suitable in grower–finisher diets as a partial replacer of soybean oil up to 2% without impairing performance, FA and energy utilization. Regarding to the use of a combination of co-products as an energy source, the best strategy in grower–finisher diets is a blend of 2% of high FFA soybean lecithin and 1% of monounsaturated vegetable acid oil; this is due to synergistic interactions on FA and energy utilization. Finally, the FA profile of the diets has a stronger impact on the FA profile of the AFP rather than the different lipid molecular structures.

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Effects of Mannanoligosaccharide Supplementation on the Growth Performance, Immunity, and Oxidative Status of Partridge Shank Chickens

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Simple Summary: To keep animals healthy and maintain sustainability, modern poultry production industry uses functional feed additives such as mannanoligosaccharides to minimize the potential threat of disease and protect the intestinal mucosa against invading microorganisms. However, most of them are obtained by chemical synthesis that may cause environmental pollution. Thus, we found a way to produce mannanooligosaccharides by an enzyme called β-mannanase to avoid pollution. This enzyme is produced by the fungus species *Aspergillus niger*. In the present study, we evaluated such enzymatic mannanooligosaccharide and found it can improve oxidative status and immunity in broiler chickens.

Abstract: Mannanoligosaccharides (MOS) can be used in poultry production to modulate immunity and improve growth performance. So, we hypothesized that our enzymatic MOS could achieve the same effects in broilers. To investigate this, a total of 192 one-day-old Partridge Shank chickens were allocated to four dietary treatments consisting of six replicates with eight chicks per replicate, and they were fed a basal diet supplemented with 0, 0.5, 1 and 1.5 g MOS per kg of diet(g/kg) for42 days. Treatments did not affect the growth performance of chickens. Dietary MOS linearly increased the relative weight of the bursa of Fabricius and jejunal immunoglobulin M (IgM) and immunoglobulin G (IgG) content, whereas it linearly decreased cecal *Salmonella* colonies at 21 days ($p < 0.05$). The concentration of jejunal secretory immunoglobulin A (sIgA) and IgG at 42 days as well as ileal sIgA, IgG, and IgM at 21 and 42 days were quadratically enhanced by MOS supplementation ($p < 0.05$). Also, chickens fed MOS exhibited linear and quadratic reduction in jejunal malondialdehyde (MDA) accumulation ($p < 0.05$). In conclusion, this enzymatic MOS can improve the immune function and intestinal oxidative status of Partridge Shank chickens.

Keywords: mannanoligosaccharide; growth performance; immunity; oxidative status; Partridge Shank chickens

1. Introduction

Oligosaccharides, such as mannanoligosaccharides (MOS) are now widely used as functional feed additives in modern poultry production. MOS are indigestible to monogastric animals and can inhibit colonization of pathogenic microorganisms in the intestinal tract by binding pathogenic bacteria

that possess mannose-specific type-I fimbriae and by its prebiotic activity. At the other hand, MOS have been found to enhance the growth of some probiotics such as cecal *Lactobacillus* species and *Bifidobacterium* species.

Extensive reports have proved that dietary MOS supplementation can enhance immunity and intestinal health, resulting in better growth performance of animals under both normal and adverse conditions [1–6]. Additionally, some exciting findings on MOS research have currently been observed by Bozkurt et al. [7], Attia et al. [8] and Zheng et al. [9], who have shown that dietary MOS addition can act as a free radical scavenger to improve the body's antioxidant capacity through inhibiting lipid peroxidation and/or elevating antioxidant enzymes activities in laying hens, broilers, and sheep. Furthermore, Liu et al. [10] have reported that the inclusion of dietary MOS can relieve hepatic oxidative damage of fish under adverse conditions. It has been demonstrated that dietary MOS supplementation increases water-holding capacity and tenderness [8,11], whereas it decreases the fat content of muscle in animals [8,12]. In a published paper, Zhang et al. [13] illustrated that dietary yeast cell wall inclusion, a widely used MOS product, reduced the concentration of malondialdehyde (MDA), an end-product of lipid peroxidation, in raw and boiled muscles in broilers.

MOS originates from different sources, and it has been repeatedly reported that various mannanases from bacteria, fungi, and plants can hydrolyze different mannan-containing polysaccharides to yield MOS [14–22]; however, the supply of MOS is not adequate to meet the demand. So, an economically viable technique for producing MOS has yet to be identified and developed. *Amorphophallus konjac* K. Koch is an underutilized agricultural material with low commercial value in China where it is typically used as animal feed and as a gelling and thickening ingredient for human foods [23]. It has been recognized as a safe material according to the FDA (Food and Drug Administration) [24]. Almost 60% of konjac is glucomannan, a previously noted precursor to MOS. The glucomannan from *Amorphophallus konjac* (KGM) and MOS from glucomannan consist of a linear chain of β-1,4-d-glucose and d-mannose. Structural studies of MOS from KGM revealed that it contains only glucose and mannose at a molar ratio of 1:1.6 [23]. In addition, it was found that branching occurs at β-1,6- glucoses approximately three times for every 32 sugar residues [25]. Finally, it has been found that most MOS has a degree of polymerization (DP) between 2 and 6. Little is known about the effect of this MOS on broilers, especially Partridge Shank chickens, an important local chicken breed. We hypothesized that the MOS would exhibit a high bioavailability in vivo. The current study was therefore conducted to evaluate the effects of enzymatic MOS from KGM on the growth performance, immunity, and antioxidant status of Partridge Shank chickens.

2. Materials and Methods

The experimental procedures used in this study were approved by the Nanjing Agricultural University Institutional Animal Care and Use Committee. The ethical code is NJAU20171104.

2.1. Mannanoligosaccharide

Mannanoligosaccharide (MOS) was prepared from KGM produced by the laboratory using enzymatic hydrolysis. The KGM used in this experiment was prepared from *Amorphophallus konjac* bought from the local market of Yunnan Province of China. The enzyme used was β-mannanase produced from *Aspergillus niger* by the laboratory. Hydrolysis was performed for 2 h at pH 5.0 with an environmental temperature of 50 °C. Post hydrolysis, enzymatic hydrolysate was free flowing. The enzyme activity was inactivated by putting enzymatic hydrolysate in a beaker into boiling water for 10 min, then ultrafiltration was used to separate the impurities to get MOS. Finally, spray drying (BUCHI, Flawil, Switzerland) was used to prepare solid MOS.

2.2. Husbandry, Diets and Experimental Design

A total of one hundred and ninety-two one-day-old Partridge Shank chicks with similar initial weight obtained from a commercial hatchery were randomly allocated into four dietary treatments.

Each treatment included 48 chicks that consisted of six replicates (one cage per replicate). Birds in the four treatments were fed a basal diet supplemented with 0, 0.5, 1 and 1.5 g MOS per kg of diet for 42 days. Ingredient composition and nutrient content of the basal diets are presented in Table 1. Birds had free access to mash feed and water in three-level cages (120 cm × 60 cm × 50 cm; 0.09 m^2 per chick) in a temperature-controlled room with continuous lighting. The temperature of the room was maintained at 32 to 34 °C for the first 3 days and then reduced by 2–3 °C per week to a final temperature of 26 °C. At 21 days and 42 days of age, birds were weighed after feed deprivation for 12 h and feed intake was recorded by replicate (cage) to calculate average daily feed intake (ADFI), and average daily gain (ADG). Birds that died during the experiment were weighed, and the data were included in the calculation of feed conversion ratio (FCR).

Table 1. Composition and nutrient level of basal diet (g/kg, as-fed basis unless otherwise stated).

Items	1–21 Days	22–42 Days
Ingredients		
Corn	576.1	622.7
Soybean meal	310	230
Corn gluten meal	32.9	60
Soybean oil	31.1	40
Limestone	12	14
Dicalcium phosphate	20	16
L-Lysine·HCL	3.4	3.5
DL-Methionine	1.5	0.8
Sodium chlodire	3	3
Premix [1]	10	10
Calculated nutrient levels [2]		
Apparent metabolizable energy (MJ/kg)	12.56	13.19
Crude protein	211	196
Calcium	10.00	9.50
Available phosphorus	4.60	3.90
Lysine	12.00	10.50
Methionine	5.00	4.20
Methionine + cysteine	8.50	7.60
Analyzed composition [3]		
Crude protein	208	192
Ash	57.2	56.5

[1] Premix provided per kilogram of diet: vitamin A (transretinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 3000 IU; vitamin E (all-rac-α-tocopherol), 30 IU; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 600 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B12 (cobalamin), 0.013 mg; Fe (from ferrous sulphate), 80 mg; Cu (from copper sulphate), 8.0 mg; Mn (from manganese sulphate), 110 mg; Zn (from zinc oxide), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg; [2] the nutrient levels were as fed basis; [3] Values based on analysis of triplicate samples of diets.

2.3. Sample Collection

At 21 and 42 days, one bird (close to the average body weight of birds in each cage) from each replicate (48 birds in total) was selected and weighed after feed deprivation for 12 h. After that, blood samples (around 5 mL each) were taken from the wing vein and centrifuged at 4450× *g*, 15 min at 4 °C to separate serum, which was frozen at −20 °C until analysis. After blood collection, the chickens were euthanized by cervical dislocation and immediately necropsied. Following necropsy, the whole gastrointestinal tracts were quickly removed. Bursa of Fabricius, thymus, and spleen were then collected and weighed to calculate the relative organ weights using the following formula: relative weight of immune organ (g/kg) = immune organ weight (g)/body weight (kg). Jejunum (from the end of the pancreatic loop to the Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileocecal junction) were then excised free of the mesentery and placed on a chilled stainless-steel tray. The jejunal, and ileal mucosa were

scratched carefully using a sterile glass microscope slide, which were then rapidly frozen in liquid nitrogen and stored at −80 °C for further analysis. Then cecum samples were quickly removed aseptically, and cecal contents were cultured to determine the population of *Lactobacillus*, *Salmonella* and *Escherichia coli*.

2.4. Microflora Population Measurement

Approximately 0.2 g of aseptically removed cecal contents were diluted in 2 mL of sterilized saline (154 mmol/L), and then three 10-fold serial dilutions were made from the diluted cecal contents (10^{-3}, 10^{-4} and 10^{-5} for *Salmonella*; 10^{-4}, 10^{-5} and 10^{-6} for *Escherichia coli* and *Lactobacillus*). A 100 μL portion of the last three dilutions were then spread evenly onto plates. *Escherichia coli* colonies were enumerated on MacConkey agar (Qingdao Hope Bio-Technology Co. Ltd., Qingdao, Shandong, China) at 37 °C for 24 h. *Lactobacillus* were enumerated on MRS agar (Qingdao Hope Bio-Technology Co. Ltd., Qingdao, Shandong, China) medium at 37 °C for 48 h. *Salmonella* colonies were determined on Bismuth sulfite agar (Qingdao Hope Bio-Technology Co. Ltd., Qingdao, Shandong, China) and incubated at 37 °C for 24 h. All plates with countable colonies were enumerated and averaged to express log CFU (Colony-Forming Units) per gram of cecal content.

2.5. Determination of Mucosal Immune and Antioxidant Parameters

Approximately 0.3 g mucosal samples from jejunum and ileum were homogenized (1:9, wt/vol) with ice-cold 154 mmol/L sodium chloride solution using an Ultra-Turrax homogenizer (Tekmar Co., Cincinatti, OH, USA) and then centrifuged at 4450× *g* for 15 min at 4 °C. The supernatant was then collected and stored at −20 °C for subsequent analysis.

Total superoxide dismutase (T-SOD) activity, and malondialdehyde (MDA) content were analyzed using commercial diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. The activity of T-SOD was analyzed by the hydroxylamine method [26], and one unit of T-SOD was defined as the amount of enzyme per milliliter of mucosa required to produce 50% inhibition of the rate of nitrite production at 37°C. MDA concentration was measured by barbiturate thiosulfate assay [27], and was expressed as nanomole per milliliter of mucosa.

Concentrations of immunoglobulin M (IgM), immunoglobulin G (IgG), and secretory immunoglobulin A (sIgA) were measured in appropriately diluted mucosal samples by enzyme-linked immunosorbent assay (ELISA) using microtiter plates and chicken-specific IgM, IgG, sIgA ELISA quantitation kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). All results were normalized against total protein concentration in each sample for inter-sample comparison. Finally, total protein concentration was determined by using a total protein quantitation kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.6. Statistical Analysis

Data was analyzed by one-way analysis of variance (ANOVA) using SPSS statistical software (Ver. 19.0 for windows, SPSS Inc., Chicago, IL, USA). The replicate (cage) was defined as the experimental unit. Polynomial contrasts were used to test the linear and quadratic effects of MOS levels. The level of significance was $p < 0.05$ in all analyses. Results are presented as means alongside their pooled standard errors of means.

3. Results

3.1. Growth Performance

Chickens given basal diets supplemented (Table 2) with MOS exhibited similar growth performance compared with the control group during the 42-day study ($p > 0.05$).

Table 2. Growth performance of Partridge Shank chickens fed diets supplemented with or without mannanoligosaccharide (MOS).

Items	Control	0.5 g/kg MOS	1 g/kg MOS	1.5 g/kg MOS	SEM	*p*-Value	
						L	Q
ADG (g/days)							
1–21days	17.31	16.50	16.72	16.92	0.151	0.489	0.102
22–42days	43.81	43.43	43.63	43.37	0.340	0.732	0.939
1–42days	32.68	32.12	32.33	32.26	0.221	0.617	0.601
ADFI (g/days)							
1–21days	27.04	25.89	26.72	25.74	0.246	0.156	0.860
22–42days	101.42	93.65	102.35	98.52	0.944	0.999	0.158
1–42days	69.55	64.78	69.99	67.41	0.580	0.753	0.204
FCR (g:g)							
1–21days	1.56	1.57	1.60	1.52	0.011	0.246	0.052
22–42days	2.31	2.16	2.35	2.27	0.020	0.605	0.191
1–42days	2.13	2.02	2.17	2.09	0.015	0.646	0.438

MOS = mannanoligosaccharide; ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio; SEM = standard error of means (each treatment included 48 chickens and consisted of 6 replicates); L = linear; Q = quadratic.

3.2. Realtive Immune Organ Weights

As shown in Table 3, the inclusion of MOS quadratically increased the relative weight of bursa of Fabricius at 21 days ($p < 0.05$), but this effect was not observed at 42 days ($p > 0.05$). Also, the relative weights of the thymus and spleen were not altered by the MOS diet ($p > 0.05$).

Table 3. Immune organ weights from Partridge Shank chickens fed diets supplemented with or without MOS (g/kg).

Items	Control	0.5 g/kg MOS	1 g/kg MOS	1.5 g/kg MOS	SEM	*p*-Value	
						L	Q
Thymus							
Days 21	1.10	1.07	1.06	1.10	0.06	0.976	0.785
Days 42	2.48	3.78	3.27	2.51	0.26	0.856	0.055
Spleen							
Days 21	0.83	0.74	0.73	0.91	0.04	0.546	0.132
Days 42	4.13	3.14	5.13	4.62	0.30	0.180	0.677
Bursa of Fabricius							
Days 21	0.98	1.69	1.34	1.42	0.07	0.110	0.031
Days 42	1.57	1.71	1.31	1.62	0.14	0.854	0.768

MOS = mannanoligosaccharide; relative immune organ weight that was expressed relative to body weight; SEM = standard error of means (each treatment included 48 chickens and consisted of 6 replicates); L = linear; Q = quadratic.

3.3. Cecal Microflora Population

In Table 4, it can be seen that MOS had a linear effect on *Salmonella* colonies ($p < 0.05$) in the cecal content at 21 days. However, cecal *Escherichia coli* and *Lactobacillus* colonies were not affected by MOS supplementation during the whole experiment ($p > 0.05$).

Table 4. Microflora population in the cecal content of Partridge Shank chickens fed diets supplemented with or without MOS (log CFU/g content).

Items	Control	0.5 g/kg MOS	1 g/kg MOS	1.5 g/kg MOS	SEM	*p*-Value	
						L	Q
Escherichia coli							
Days 21	7.98	8.04	7.97	8.45	0.15	0.360	0.514
Days 42	6.89	6.94	6.68	6.04	0.20	0.946	0.820
Salmonella							
Days 21	8.41	7.73	7.10	7.44	0.18	0.028	0.126
Days 42	6.16	6.35	6.11	6.52	0.18	0.652	0.777
Lactobacillus							
Days 21	8.41	8.18	8.51	8.06	0.08	0.326	0.501
Days 42	7.45	7.98	7.81	7.62	0.10	0.764	0.105

MOS = mannooligosaccharide; SEM = standard error of means (each treatment included 48 chickens and consisted of 6 replicates); L = linear; Q = quadratic.

3.4. Intestinal Immunoglobulins Contents

Chickens exhibited similar content of sIgA in the jejunal mucosa among groups at 21 days (Table 5, $p > 0.05$). MOS linearly increased jejunal IgM and IgG contents ($p < 0.05$) at 21 days and quadratically increased jejunal sIgA and IgG levels at 42 days ($p < 0.05$). Simultaneously, ileal sIgA, IgM and IgG contents were quadratically increased in 42 days ($p < 0.05$).

Table 5. Intestinal immunoglobulins contents of Partridge Shank chickens fed diets supplemented with or without MOS (μg/mg protein).

Items	Control	0.5 g/kg MOS	1 g/kg MOS	1.5 g/kg MOS	SEM	*p*-Value	
						L	Q
			Jejunum sIgA				
Days 21	7.37	7.62	8.43	8.31	0.24	0.110	0.708
Days 42	8.78	10.15	9.12	7.93	0.29	0.227	0.039
			IgM				
Days 21	7.64	7.71	8.96	9.06	0.27	0.024	0.966
Days 42	11.41	12.14	11.06	9.38	0.42	0.053	0.140
			IgG				
Days 21	108.17	138.09	143.99	168.33	7.46	0.004	0.823
Days 42	145.96	191.79	179.32	142.54	7.54	0.702	0.005
			Ileum sIgA				
Days 21	8.25	9.35	10.41	8.34	0.33	0.914	0.022
Days 42	9.92	11.55	10.19	9.27	0.03	0.163	0.022
			IgM				
Days 21	9.11	10.24	11.58	8.82	0.35	0.843	0.002
Days 42	10.91	14.87	12.90	10.81	0.49	0.505	0.001
			IgG				
Days 21	133.93	145.24	175.10	122.59	7.14	0.940	0.018
Days 42	185.97	229.32	196.24	174.00	7.36	0.243	0.019

MOS = mannooligosaccharide; sIgA = secretory immunoglobulin A; IgM = immunoglobulin M; IgG = immunoglobulin G; SEM = standard error of means (each treatment included 48 chickens and consisted of 6 replicates); L = linear; Q = quadratic.

3.5. Intestinal Oxidative Status

As shown in Table 6, chickens fed MOS exhibited linear and quadratic reduction in jejunal MDA accumulation at 21 days ($p < 0.05$), and quadratic effect on ileal MDA content at 42 days ($p < 0.05$). However, intestinal SOD activity was similar among treatments ($p > 0.05$).

Table 6. Intestinal antioxidant status of Partridge Shank chickens fed diets supplemented with or without MOS.

Items	Control	0.5 g/kg MOS	1 g/kg MOS	1.5 g/kg MOS	SEM	*p*-Value	
						L	Q
			Jejunum T-SOD (U/mL)				
Days 21	1112	1179	1147	1195	26.42	0.345	0.175
Days 42	1180	1158	1155	1283	29.67	0.260	0.219
			MDA (nmol/ mL)				
Days 21	6.99	3.76	3.53	3.92	0.44	0.004	0.024
Days 42	7.42	9.40	7.07	6.04	0.46	0.112	0.095
			Ileum T-SOD (U/mL)				
Days 21	1040	1038	1185	1219	29.58	0.081	0.430
Days 42	1069	1071	1043	1162	19.89	0.698	0.196
			MDA (nmol/ mL)				
Days 21	6.34	6.25	8.20	5.28	0.49	0.770	0.146
Days 42	7.10	13.56	10.50	6.51	0.73	0.244	<0.001

MOS = mannooligosaccharide; MDA = malondialdehyde; T-SOD = total superoxide dismutase; SEM = standard error of means (each treatment included 48 chickens and consisted of 6 replicates); L = linear; Q = quadratic.

4. Discussion

4.1. Growth Performance

Sims et al. [28] and Attia et al. [29] demonstrated that dietary MOS supplementation can improve the growth performance of poultry under normal conditions. In broilers, Geier et al. [30] found that when broiler feed contained MOS, the growth performance of broilers was unchanged. This study demonstrated that MOS supplementation exerted no significant effect on the growth performance of broilers, and this was consistent with the findings of Munyaka et al. [31], who reported that dietary supplementation with yeast-derived MOS preparation did not alter growth performance and mortality in broilers. In contrast, Churchil et al. [32] observed that yeast-derived MOS inclusion increased the body weight of broilers. In addition, Gao et al. [33] demonstrated that the growth performance of broilers was optimized by adding the yeast-derived MOS. Therefore, the unchanged growth performance observed in this study may be associated with the source of MOS used as the dietary supplement; that is, the broilers may digest less nutrients from our MOS. Based on this result, further studies are needed to evaluate the influences of different sources of MOS on the growth performance of chickens, and to evaluate how to further process our MOS so that it can increase nutrient digestibility of chickens.

4.2. Relative Immune Organ Weights

Relative immune organ weights could partially reflect the development and growth of immune organs. The current study showed that MOS quadratically increased the relative weight of bursa of Fabricius at 21 days, which plays a vital role in development and maturation of B-lymphocytes and the diversification of specific antibodies [34]. Thus, MOS supplementation may increase the weight of bursa

by stimulating the proliferation of bursal lymphocytes. Also, digestive microbial antigen stimulation plays a vital role in the development of lymphoid organ tissue [35]. Li et al. [36] reported that the increased weight of bursa may be associated with possible changes to the intestinal microorganism population induced by yeast derived MOS supplementation. Dietary MOS supplementation, therefore, represents a nutritional strategy that could favor intestinal colonization of beneficial bacteria, thereby conferring intestinal health benefits to the host. Further study is required to verify this conjecture.

4.3. Cecal Microflora Population

MOS in this experiment is a plant-derived oligosaccharide, which can promote the growth of *Bifdobacteria*, which decreases colonization by enteric pathobionts like *Salmonella* and *Escherichia coli*, regulates immune signaling, and improves mucosal integrity [37,38]. It is well documented that MOS competitively adsorbs to the mannosespecific type 1 fimbriae of *Escherichia coli* and other pathogens, thereby limiting their colonization of the intestinal epithelium. This phenomenon results in the pathogens ultimately being excreted from the intestine [39,40]. Muthusamy et al. [41] reported that dietary MOS lowered *Salmonella* spp. and *Escherichia coli* number in the small intestine (duodenum, jejunum and ileum) of broilers with poor health or *Salmonella* challenged. In this study, MOS had a linear decreasing effect on *Salmonella* colony in the cecal content at 21 days, indicating that the prepared MOS can decrease colonization by enteric pathobionts. Different results were found by Li et al. [36] whereby MOS supplementation did not alter *Escherichia coli* and *Salmonella* colonies in the cecal content (only a decreased tendency was noted). Thus, oligosaccharides from different sources and different chain lengths may have different results on different intestinal microorganisms. This hypothesis requires further research to prove it.

4.4. Intestinal Immunoglobulins

The immune system guards the body against foreign substances and protects it from invasion by pathogenic organisms. In chickens, three classes of immunoglobulins participate in immune system maintenance. These immunoglobulins have been identified as IgM, IgG and IgA [42]. sIgA plays an important role in the protection and homeostatic regulation of intestinal mucosal epithelia separating the outside environment from the inside of the body. The primary function of sIgA is referred to as immune exclusion, a process that limits the access of numerous microorganisms and mucosal antigens to the thin and vulnerable mucosal barriers [43]. Savage et al. [44] reported that when feeding MOS to broilers, the concentration of IgA in the bile increased 14.2%, and that the MOS may have a mechanism that directly protects the mucosa. The present study showed that MOS linearly increased jejunal IgM and IgG contents at 21 days, while it quadratically increased sIgA and IgG contents at 42 days. Simultaneously, ileal sIgA, IgM and IgG contents were quadratically increased at 42 days. Similar results were also observed by Li et al. [36] and Gao et al. [33]. We assumed that the main target of the prepared MOS is located in the intestine, and it may simulate the development of intestinal cells in the jejunum and ileum to secrete more immunoglobulins. This result indicates that the prepared MOS can improve intestinal immune status.

4.5. Intestinal Oxidative Status

Reactive oxygen species (ROS) are produced during normal metabolism in cells, but concentration of ROS exceeding the antioxidant protection levels of cells can cause widespread damage to DNA, proteins and endogenous lipids [45]. SOD is generally regarded as one of the main antioxidant enzymes in scavenging the oxygen free radical [46]. The MDA is the main end product of lipid peroxidation by ROS, and increased MDA accumulation is an important indication of lipid peroxidation [47]. MOS from konjac has been reported to display relatively good antioxidative properties [48]. In poultry, enhanced SOD activity in the serum of broilers fed dietary MOS has recently been found by Attia et al. [49]. Bozkurt et al. [7] reported that dietary MOS supplementation could decrease MDA concentration in both eggs and liver, and increase SOD activity in the liver in laying hens. In this study, MOS

linearly and quadratically decreased jejunal MDA accumulation in 42 days and it had quadratic effect on ileal MDA accumulation at 42 days. This was in agreement with the results of Liu et al. [10], who demonstrated that dietary MOS inclusion decreased MDA accumulation in fish under adverse conditions. According to the literature, dietary MOS supplementation can accelerate gastrointestinal maturation and increase nutrient absorption for better growth performance in organisms [50–52], which may simultaneously and indirectly contribute to improving the adsorption and utilization of small molecules related to the synthesis of antioxidants. Thus, in the current study, elevated oxidative status in the intestinal mucosa by MOS supplementation might also be related to the promotion of MOS addition on the gut ecology and digestive function in animals [51,52].

5. Conclusions

In this study, MOS did not affect growth performance whereas it improved immune function (enhanced relative weight of bursa of Fabricius, enhanced jejunal sIgA and IgG contents and ileal sIgA and IgG levels), intestinal oxidative status (decreased jejunal MDA content), and regulated the cecal microflora population (reduced cecal *Salmonella* population) in Partridge Shank chickens.

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animals

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Article

Effects of Chromium-Loaded Chitosan Nanoparticles on the Intestinal Electrophysiological Indices and Glucose Transporters in Broilers

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Simple Summary: Chromium is an important trace element responsible for the metabolism of glucose by enhancing insulin activity. This study was planned to evaluate the effects of chromium-loaded chitosan nanoparticles on the transport of glucose or amino acid across jejunum, gene expression of glucose transporters, and glycogen contents of liver and muscle. The results revealed that an increase in the supplemented dose of chromium-loaded chitosan nanoparticles decreased liver glycogen content and glucose transport across jejunum, while the muscle glycogen, gene expression of glucose transporters, and amino acid transport remained unaffected.

Abstract: The present study aimed to evaluate the effect of chromium-loaded chitosan nanoparticles (Cr-CNPs) on the electrophysiological indices, gene expression of glucose transporters, and tissue glycogen in broilers. A total of 200 one-day-old broilers were randomly divided into five groups, with each having five replicates (n = 8). Group A was fed a corn–soybean meal diet, while the diets of groups B, C, D, and E were supplemented with 200, 400, 800, and 1200 μg/kg of Cr as Cr-CNPs, respectively. On day 35, the jejunum was collected for electrophysiological study, gene expression of glucose transporters, and tissues glycogen determination. The basal short-circuit current and tissue conductance before the addition of glucose was the same in all groups. Following the addition of glucose, the change in short-circuit current decreased ($p < 0.05$) in the jejunal tissues of birds supplemented with 400 and 1200 μg Cr-CNPs compared with the control group. Gene expression of SGLT-1 and GLUT-2 remained unaffected with supplementation. The serum glucose and liver glycogen concentration decreased ($p < 0.05$) linearly with supplementation, while no effect was observed on muscle glycogen. In conclusion, Cr-CNPs supplementation decreases the glucose absorption and liver glycogen content, without affecting the gene expression of glucose transporters.

Keywords: nanoparticles; chromium; supplementation; electrophysiology; Ussing chamber; poultry

1. Introduction

Glucose metabolism of avian species differs from that of mammals; i.e., the birds have higher blood glucose concentration and lower insulin levels [1]. Furthermore, avian species are considered

less sensitive to insulin than mammals [2]. Chromium (Cr), a trace element, is known to increase insulin sensitivity in mammals [3] whereas, in broilers, Cr supplementation has reduced the serum glucose concentration [4]. The Cr level in poultry feed is yet to be appropriately recommended [5]. It is believed that poultry diets containing Cr can meet the requirements of the birds reared under the standard management conditions specific to broilers. However, several studies reported a positive effect of Cr supplementation on the production performance and carcass traits of broilers [6–8].

Intestinal absorption of carbohydrates occurs via glucose transporters [9]. In birds, most of the glucose or amino acid transport occurs in the jejunum [10]. The sodium-dependent glucose co-transporter transports glucose, along with sodium, from the intestinal lumen, while the sodium-independent glucose transporter is responsible for the transport of glucose along the basolateral side [11]. The transport of Glucose and amino acid through the intestine can be evaluated by studying the electrical variables with the help of an Ussing chamber. The effect of chromium-loaded chitosan nanoparticles (Cr-CNPs) on the electrophysiological indices and gene expression of glucose transporters is yet to be reported in poultry. Limited studies are available regarding the effect of organic chromium picolinate and chromium histidinate on the glucose transporters in layers exposed to heat stress [9]. The current research is aimed to explore the effect of Cr-CNPs supplementation on the electrophysiological parameters, gene expression of glucose transporters, and tissue glycogen content in broilers reared under standard management conditions.

2. Materials and Methods

All the experimental procedures used were approved by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan, vide letter No. DR/498.

2.1. Preparation of Chromium-Loaded Chitosan Nanoparticles

The Cr-CNPs were prepared and characterized at the Interdisciplinary Research Center in Biomedical Research, COMSATS University Islamabad (Lahore Campus), Pakistan, according to the method described by Wang et al. [12]. Briefly, 1% (*w/v*) solution of chitosan was prepared by dissolving chitosan into a 0.5% acetic acid solution with the pH adjusted to 3.5. The chitosan solution was then stirred for one hour. During stirring, 200 mg/L of chromium chloride solution was added to the chitosan solution to get a suspension of chitosan and chromium chloride. The pH of the suspension was adjusted to 6.5, and stirring was continued for five hours. Subsequently, the precipitate was centrifuged at 12,000 g for 15 min at room temperature and washed with distilled water to get Cr-CNPs.

2.2. Experimental Animals, Grouping, Diet, and Management

Two hundred male broiler chicks (Hubbard), were randomly divided into five groups, with each having five replicates (n = 8). Birds in group A (control) were given the non-supplemented basal diet, as shown in Table 1 [13], while the birds in the groups B, C, D, and E were fed the same diet but supplemented with graded levels of Cr-CNPs i.e., 200, 400, 800, and 1200 μg/kg of Cr as Cr-CNPs, respectively, for 35 days. The feed and water were provided ad libitum. Temperature and relative humidity on day 1 was kept at 35 ± 1.1 °C and 65 ± 5%, respectively. The temperature was decreased by 3 °C per week until it reached 26 °C on day 21.

Table 1. Composition of the diet (g/kg).

Ingredients	Percentage
Corn	58.50
Soybean meal 44%	25.00
Sunflower meal	3.50
Canola meal	8.00
Vegetable oil	1.50
Dicalcium phosphate	0.90
Limestone	1.51
Common salt	0.50
DL-Methionine	0.21
L-Lysine HCl	0.12
Vitamin premix [1]	0.13
Micro mineral premix [2]	0.13
Total	100.00
Nutrient contents	
Crude protein	20.72
Metabolizable energy (MJ)	12.2
Calcium	0.91
Phosphorus	0.61
* Available P	0.33

[1] Provided vitamins per kg of the feed: vitamin A (retinol), 11000 IU; vitamin B-12 (cyanocobalamin), 0.0132 mg; vitamin D_3 (cholecalciferol), 2200 IU; vitamin E (alpha-tocopherol), 22 IU; choline chloride, 440 mg; riboflavin, 8.8 mg; pantothenic acid, 22 mg; ethoxyquin, 250 mg; menadione, 2.2 mg; pyridoxine, 4.4 mg; folic acid, 1.1 mg; biotin, 0.22; thiamin, 4.4 mg. [2] Supplied minerals per kg of the feed: Cu ($CuSO_4$), 20 mg; Zn (ZnO), 200 mg; Mn ($MnSO_4$), 240 mg; Fe ($FeSO_4$), 120 mg; I (KI), 0.92 mg; Ca, 150–180 mg. Measured Chromium 4.05 mg/kg. * Calculated according to NRC 1994.

2.3. Sample Collection and Processing of Tissue

On day 35, eight birds per group were randomly selected and their jejunal segments were prepared as described earlier by Rehman et al. [14]. Briefly, after exsanguination, a segment of jejunum was removed, washed thoroughly with an ice-cold Ringer's buffer solution, and transferred to the laboratory in buffer. The serosal layer was stripped off the jejunum. The jejunum was subsequently opened longitudinally along the mesenteric border, rinsed with Ringer's solution to remove the luminal contents, and then gassed with carbogen (95% O_2 and 5% CO_2) till mounting on the Ussing chamber.

Blood samples were taken to determine the glucose level. One hundred milligrams of tissue from each liver and pectoral muscle was collected for quantification of glycogen contents. For mRNA quantification of glucose transporters, the collected jejunal segments were washed with ice-cold normal saline. All the samples were stored at −80 °C for further analyses.

2.4. Measurement of Electrophysiological Indices

The jejunal mucosa (with stripped-off serosal layer) was cut into four pieces of 1 cm^2 and mounted in between two compartments of the Ussing chamber. The exposed area of the chamber was 0.95 cm^2 [15]. Damage to the edge of the tissue was minimized by silicon rubber rings on both sides of the tissue. Buffer solution (16 mL) was added to the chambers on each side. The buffer solution contained (in mM) 1.2 $CaCl_2$, 115 NaCl, 25 $NaHCO_3$, 20 Mannitol, 5 KCl, 2.4 Na_2HPO_4, 1.2 $MgCl_2$, and 0.4 NaH_2PO_4, with the pH adjusted to 7.4. The buffer solution was continuously aerated with carbogen (95% O_2 and 5% CO_2) and maintained at 37 °C. Buffer osmolarity was measured (Osmomat 030, Gonotec GmbH, Berlin, Germany) and adjusted to 300 mOsmol/L, using mannitol. Tissues were allowed to equilibrate for 20 min under open circuit and then short-circuited by clamping the voltage at 0 mV for 5 min. Following equilibrium, 10.0 mM glucose or L-glutamine was added to the mucosal side, and the peak electrical response was measured. Electrical measurements like short-circuit current (Isc) and transmural tissue conductance (Gt) were observed with an automatic

computer-controlled voltage-clamp device (Mussler, Aachen, Germany) to assess the electrogenic transport of glucose or L-glutamine linked to sodium across the jejunal mucosa.

2.5. Extraction of RNA and Quantification of Glucose Transporters

The mRNA expression of glucose transporters (sodium-dependent glucose transporter-SGLT-1 and sodium-independent glucose transporter-GLUT-2) were determined by real-time PCR (Router gene 5 plex real-time PCR, Qiagen, Hilden, Germany). The oligonucleotide primers sequence for SGLT-1 [16], GLUT-2 [17], and ß-actin [17] (housekeeping gene) used for PCR amplification are shown in Table 2. The RNA extraction of the jejunal mucosa was done by using Trizol (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. The total RNA was quantified by using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). A total of 5 µg RNA was reverse transcribed to complementary DNA (cDNA), using First-Strand cDNA synthesis kit (Thermo Scientific™, Waltham, MA, USA). Real-time PCR was performed using the SYBER green maxima PCR kit (Thermo Scientific™, Waltham, MA, USA), as per manufacturer's instructions. The following PCR program was set on the machine to amplify the target mRNA in tissue extracts: 95 °C for 3 min, followed by 40 cycles of 95 °C for the 30 s, 60 °C for 30 s, and 72 °C for 30 s. To determine the melting points of the amplified cDNA and to confirm the production of a single product, a dissociation curve was generated after 40 cycles. Relative mRNA expression levels of SGLT-1 and GLUT-2 were determined using the $2^{-\Delta\Delta CT}$ method [18].

Table 2. Primer sequences used during real-time PCR.

Name of Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Annealing Temperature (°C)
SGLT-1	GTCCTGGCAGTGGGAGTATG	AAGAGTGAAGCACCGATCGG	61
GLUT-2	CACACTATGGGCGCATGCT	ATTGTCCCTGGAGGTGTTGGTG	60
β-Actin	ATGAAGCCCAGAGCAAAAGA	GGGGTGTTGAAGGTCTCAAA	60

SGLT-1 (Na^+-dependent glucose and galactose transporter); GLUT-2 (Na^+-independent glucose, galactose and fructose transporter).

2.6. Quantification of Tissues Glycogen and Serm Glucose

Liver and muscle glycogen contents were quantified by using iodine assay described by Bennet et al. [19] and Dreiling et al. [20], with some modifications. Briefly, 100 mg liver and muscle samples were homogenized using chilled perchloric acid to solubilize the glycogen. The homogenate was then centrifuged at 2500 rpm for 10 min at 4 °C. The supernatant was collected, and the pellet was re-homogenized with chilled perchloric acid. After another round of centrifugation, the supernatant thus collected was added to the previously collected supernatant and subjected to iodine assay. The absorbance of samples or glycogen standards was measured at 460 nm, using an EPOCH^{TM} microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA). The serum glucose concentration was estimated by the commercially available kit (DiaSys, Germany Germany), according to manufacturer's instruction using the same EPOCH^{TM} microplate spectrophotometer.

2.7. Statistical Analysis

Data were statistically analyzed using Statistical Package for Social Sciences (SPSS for windows version 20.0, IBM). Data were presented as means ±SEM and were analyzed using one-way analysis of variance (ANOVA). For group differences, Tukey's post hoc test was used. Polynomial contrasts were used to determine the linear, quadratic, and cubic effects of Cr-CNPs supplementation at $p < 0.05$.

3. Results

The basal short-circuit current (Isc) and transmural tissue conductance (Gt) before the addition of glucose did not vary between the supplemented groups and the control group (Table 3). After the addition of glucose to the mucosal side, the change in short-circuit current decreased linearly ($p < 0.05$) in the groups C and E compared with the control group (Figure 1). However, no effect on the change in transmural tissue conductance was observed with Cr-CNPs supplementation after addition of glucose (Figure 1).

Table 3. Effect of Cr-CNPs supplementation on initial short-circuit current and tissue conductance before the addition of glucose.

Variables	Group A	Group B	Group C	Group D	Group E	SEM	*p*-Value	Linear	Quadratic	Cubic
ISci ($\mu A/cm^2$)	7.71	5.57	6.81	4.93	4.08	0.50	0.158	0.095	0.746	0.077
Gti (mS/cm^2)	4.24	2.90	2.72	4.53	3.51	0.28	0.135	0.935	0.260	0.039

Data are presented as Mean ± SEM. Group A = control—without Cr-CNPs supplementation. Group B = offered 200 µg Cr-CNPs/kg of feed. Group C = offered 400 µg Cr-CNPs/kg of feed. Group D = offered 800 µg Cr-CNPs/kg of feed. Group E = offered 1200 µg Cr-CNPs/kg of feed. ISci = initial short circuit current. Gti = initial tissue conductance.

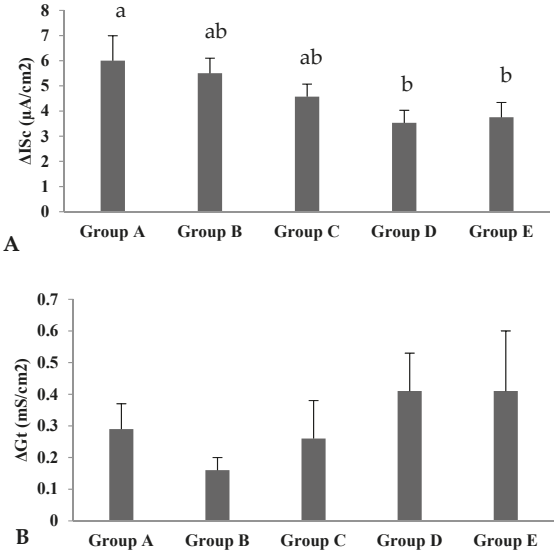

Figure 1. Effect of Cr-CNPs supplementation on (**A**) change in short-circuit current (ΔISc) and (**B**) change in tissue conductance (ΔGt) after addition of glucose to the jejunum in broilers. Labeled bars without a common letter differ significantly, $p < 0.05$. Data are presented as Mean ± SEM. Group A = control—without Cr-CNPs supplementation. Group B = offered 200 µg Cr-CNPs/kg of feed. Group C = offered 400 µg Cr-CNPs/kg of feed. Group D = offered 800 µg Cr-CNPs/kg of feed. Group E = offered 1200 µg Cr-CNPs/kg of feed.

Prior to the addition of glutamine addition, the basal Isc and Gt were similar in all supplemented groups compared to the control group (Table 4). After the addition of glutamine to the mucosal side, no effect of Cr-CNPs supplementation was observed on the change in short-circuit current and change in transmural tissue conductance, as shown in Figure 2.

Table 4. Effect of Cr-CNPs supplementation on initial short-circuit current and tissue conductance before the addition of L-glutamine.

Variables	Group A	Group B	Group C	Group D	Group E	SEM	*p*-Value	Linear	Quadratic	Cubic
ISci (μA/cm^2)	9.55	6.67	6.67	9.56	8.67	0.62	0.353	0.812	0.221	0.129
Gti (mS/cm^2)	4.50	3.09	2.78	4.45	4.45	0.29	0.057	0.415	0.080	0.059

Data are presented as Mean ± SEM. Group A = control—without Cr-CNPs supplementation. Group B = offered 200 μg Cr-CNPs/kg of feed. Group C = offered 400 μg Cr-CNPs/kg of feed. Group D = offered 800 μg Cr-CNPs/kg of feed. Group E = offered 1200 μg Cr-CNPs/kg of feed. ISci = initial short circuit current. Gti = initial tissue conductance.

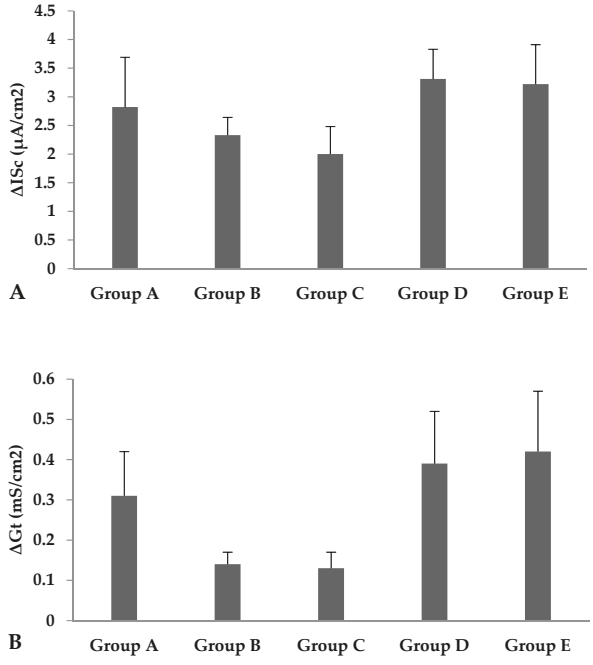

Figure 2. Effect of Cr-CNPs supplementation on (**A**) change in short circuit current (ΔISc) and (**B**) change in tissue conductance (ΔGt) after addition of L-glutamine to the jejunum in broilers. Mean values with different small letters on the same bar differ significantly at $p < 0.05$. Data are presented as Mean ± SEM. Group A = control—without Cr-CNPs supplementation. Group B = offered 200 μg Cr-CNPs/kg of feed. Group C = offered 400 μg Cr-CNPs/kg of feed. Group D = offered 800 μg Cr-CNPs/kg of feed. Group E = offered 1200 μg Cr-CNPs/kg of feed.

The mRNA expression of glucose transporters, i.e., SGLT-1 and GLUT-2, remained unaffected by the Cr-CNPs supplementation compared to the control group, as shown in Figure 3.

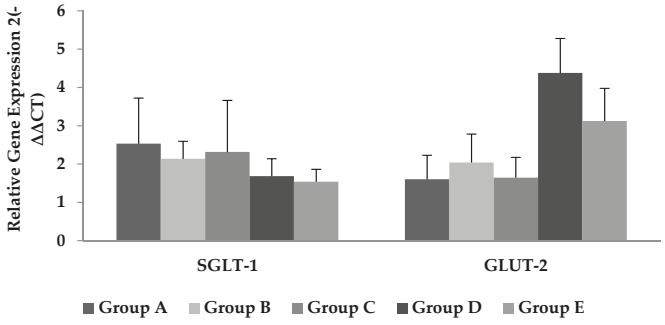

Figure 3. Effect of Cr-CNPs supplementation on gene expression of glucose transporters, i.e., (left side) SGLT-1, and (right side) GLUT-2 in broilers. Data are presented as mean ± SEM. Group A = control—without Cr-CNPs supplementation. Group B = offered 200 µg Cr-CNPs/kg of feed. Group C = offered 400 µg Cr-CNPs/kg of feed. Group D = offered 800 µg Cr-CNPs/kg of feed. Group E = offered 1200 µg Cr-CNPs/kg of feed.

The liver glycogen concentration in groups D and E decreased linearly ($p < 0.05$) with the Cr-CNPs supplementation compared with the control group. No significant effect of Cr-CNPs supplementation was found on muscle glycogen concentration (Table 5). The serum glucose level decreased linearly ($p < 0.05$) with the Cr-CNPs supplementation (Table 5).

Table 5. Effect of Cr-CNPs supplementation on liver and muscle glycogen concentration (mg/100g) and blood glucose concentration (mg/dL) in broilers.

Variables	Group A	Group B	Group C	Group D	Group E	SEM	*p*-Value	Linear	Quadratic	Cubic
Liver	13.38 ^{a}	13.89 ^{a}	8.71 ^{ab}	7.36 ^{b}	6.71 ^{b}	0.93	0.014	0.002	0.826	0.230
Muscle	3.37	3.75	3.11	3.24	3.05	0.17	0.756	0.382	0.807	0.592
Blood Glucose	252 ^{a}	267 ^{a}	229 ^{a}	218 ^{ab}	164 ^{b}	14.5	<0.001	<0.001	0.046	0.796

Data are presented as mean ± SEM. $^{a–b}$ Within the same row, different superscript indicates significantly different means at $p < 0.05$. Group A = control—without Cr-CNPs supplementation. Group B = offered 200 µg Cr-CNPs/kg of feed. Group C = offered 400 µg Cr-CNPs/kg of feed. Group D = offered 800 µg Cr-CNPs/kg of feed. Group E = offered 1200 µg Cr-CNPs/kg of feed.

4. Discussion

Chromium (Cr) is a biologically active trace element that plays a key role in metabolic activities in the body. Upon absorption, Cr is found in the blood either in free form or in bound-to-globulin proteins, transferrin, or complexes like glucose tolerance factor [21]. The bioavailability of organic chromium is higher than the inorganic form due to its increased absorption rate [22]. The inorganic form irreversibly binds with the undigested content in the intestine, and, hence, its absorption and bioavailability are limited [23]. The absorption of Cr can be enhanced by chelation, as it prevents the precipitation of chromium at alkaline pH in the poultry intestine [24].

The intestine of birds is highly absorptive for water and electrolytes. The electrical current across the epithelium is due to the net movement of ions. The transport of glucose and amino acids across the membrane occurs by either the paracellular or transcellular pathway [14]. Paracellular transport occurs without the expense of energy, while transcellular transport utilizes energy to transport glucose via sodium-dependent glucose transport (SGLT-1) and amino acids by carrier proteins in the luminal and basolateral membranes [25]. Transcellular transport of glucose and amino acids in poultry occurs in the small intestine and colon [26,27]. Most of the sodium-dependent uptake of glucose and amino acids via carrier proteins takes place in the jejunum [10]. The addition of glucose and amino acids to the luminal side of the intestine potentiates carrier-mediated transport, along with the enhanced uptake

of luminal sodium. In response, the intestinal membrane depolarizes, and increased cytoplasmic sodium stimulates the sodium–potassium ATPase pump in the basolateral membrane, which, in turn, increases the net movement of sodium from the mucosal to the serosal side. These events bring changes in the electrical variable of the intestine and increase the short-circuit current [14,28,29]. In the present study, the basal short-circuit current and tissue conductance remained unaffected with Cr-CNPs supplementation before the addition of glucose or glutamine, which indicates good preservation and preparation of the tissues [30]. After the addition of glucose to the mucosal side, the change in short-circuit current (ΔISc) decreased linearly in jejunal tissues of Cr-CNPs-supplemented birds, but no effects were observed in ΔISc upon the addition of glutamine to the mucosal side. The decline in ΔISc after glucose addition reflects a decrease in sodium transport across the intestinal membrane. The change in tissue conductance did not vary in tissues of Cr-CNPs-supplemented birds after the addition of glucose or glutamine. To the best of our knowledge, no data are available regarding the effects of the Cr-CNPs on the electrophysiological indices in poultry. Gammelgaard et al. [31] conducted in vitro permeation studies with an Ussing chamber by using pig intestine to compare the absorption of organic and inorganic chromium. They found no response due to adsorption of chromium to the chambers.

The uptake of carbohydrates at the level of the intestine is facilitated by glucose transporters. The GLUT-2 is responsible for the exit of monosaccharides from the enterocytes by facilitated diffusion, while the SGLT-1 mediates the uptake of monosaccharides [32]. The SGLT-1 is expressed in the intestine and kidney [33]. It was reported that glucose absorption was decreased in SGLT-1-deficient mice, which depicts the role of SGLT-1 in maintaining the sodium–glucose homeostasis [15]. In our study, no effect was observed on the expression of GLUT-2 and SGLT-1 with the Cr-CNPs supplementation. Contrary to our study, Orhan et al. [9] reported an increase in the expression of SGLT-1 and GLUT-2 with chromium picolinate and chromium histidinate in layers subjected to heat-stress conditions. The possible reasons could be difference in chromium sources or environmental conditions. In our study, the ΔISc on mucosal addition of glucose decreased linearly with Cr-CNPs supplementation, but gene expression of SGLT-1 remained unaffected. The expression of GLUT-2 is, however, upregulated ($p > 0.05$) with the increase in Cr-CNPs concentration, which might have facilitated transportation of other monosaccharides, including fructose or galactose. However, translation of mRNA to GLUT-2 protein is still debatable and calls for further investigations into the role of Cr-CNPs. It may be due to the lack of consistency between mRNA and protein concentration data [34].

Chromium is a cofactor of glucose tolerance factor and enhances insulin function to increase the cellular uptake of glucose [35]. In our study, the liver glycogen concentration decreased linearly with Cr-CNPs supplementation, but no effect of Cr-CNPs supplementation was found on muscle glycogen concentration. Brooks et al. [1] reported no effects on muscle glycogen or liver glycogen in broilers supplemented with 200, 400, or 800 μg/kg of Cr as chromium propionate. Also, Cr supplementation at 1 mg/kg diet to a low-Cr diet increased liver glycogen synthetase activity but did not affect liver glycogen concentrations in rats [36]. Chromium supplementation also did not affect muscle glycogen in broilers [37], rats [36], humans [38], and sheep [39]. In birds, the liver is the major site of glycogen storage, and liver glycogen is a readily available source of glucose for homeostasis [19]. In our study, the linear decrease in liver glycogen may be the result of glycogenolysis in order to maintain homeostasis that was affected by the linear decrease in glucose absorption from the intestine following Cr-CNPs supplementation.

5. Conclusions

In conclusion, Cr-CNPs supplementation decreases the absorption of glucose across the jejunal mucosa, with a concomitant decrease in liver glycogen concentration. However, it does not affect the expression of glucose transporters. Further insights are required to explore the effect of Cr-CNPs on the pathways involved in glucose metabolism and transportation.

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animals

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Article

Effects of Probiotics as Antibiotics Substitutes on Growth Performance, Serum Biochemical Parameters, Intestinal Morphology, and Barrier Function of Broilers

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Simple Summary: The abuse of antibiotics in animals feed may cause antibiotic-resistant microbes and antibiotic residue in animal products. Probiotics (PB) have been used in the feed industry for several decades due to their beneficial effects on immunity and the growth of livestock and poultry. However, the efficiency of PB on animals varies due to the types and dose of PB. Therefore, investigating the effects of PB (*Bacillus subtilis*, *Bacillus licheniformis*, and *Saccharomyces cerevisiae*) as an antibiotic substitute on growth performance and intestinal health status in broilers is valuable and meaningful.

Abstract: The aim of this study was to investigate the effects of the combination of probiotics replacing antibiotics on growth performance, serum biochemical parameters, intestinal morphology, and expression of tight junction proteins in intestinal mucosa of broilers. A total of 168 Arbor Acres broilers (45.04 ± 0.92 g) were randomly divided into three treatments, with seven replicates per treatment, and eight broilers per replicate. The experiment included phases 1 (d 0 to 21) and 2 (d 21 to 42). The dietary treatments contained a corn soybean meal-based diet (control group; CON); an antibiotic group (basal diet + 75 mg/kg chlortetracycline; CTC), and a probiotics group (basal diet + probiotics (500 mg/kg in phase 1 and 300 mg/kg in phase 2; *Bacillus subtilis* 5×10^9 CFU/g, *Bacillus licheniformis* 2.5×10^{10} CFU/g and *Saccharomyces cerevisiae* 1×10^9 CFU/g; PB). The results showed broilers fed PB had improved ($p < 0.05$) feed conversion ratio (FCR) in phase 1 and increased ($p < 0.05$) average daily gain (ADG) in phase 2, as well as improved ($p < 0.05$) ADG and FCR overall (d 0 to 42). The apparent total tract digestibility (ATTD) of dry matter, organic matter, gross energy, and crude protein was increased ($p < 0.05$) in broilers fed PB, while the ATTD of dry matter and organic matter was enhanced in broilers fed CTC compared with CON. Broilers fed PB showed increased ($p < 0.05$) serum total antioxidant capacity concentrations and tended to have higher ($p = 0.06$) level of serum immunoglobulin M in phase 1 compared with CON. These broilers also had increased ($p < 0.05$) level of serum immunoglobulin A in phase 2 in comparison with CON and CTC. Moreover, broilers fed CTC and PB showed increased ($p = 0.05$) villus height to crypt depth ratio in duodenum, as well as higher ($p < 0.05$) mRNA expression of zonula occludens-1 in jejunum compared with CON. In conclusion, dietary supplementation with PB as chlortetracycline substitute could improve the growth performance, nutrient digestibility, serum antioxidant capacity, jejunal mucosal barrier function, and intestinal morphology of broilers.

Keywords: antibiotics; broiler; growth performance; intestinal health; probiotics

1. Introduction

The wide application of antibiotics has greatly improved the growth performance of livestock and poultry, whereas the abuse of antibiotics in animal feeds may cause antibiotic residue in animal products and the direct selection of antibiotic-resistant microbes, which may cause harm in humans [1]. Broilers, which are one of the fastest growing applications of animal husbandry, face significant problems that impact their growth performance and intestinal health [2]. Therefore, seeking alternatives for in-feed antibiotics for broilers has gained enormous interest currently.

Studies show that herb extracts [3], essential oils [4], and probiotics (PB) [5] could be used as antibiotic substitutes in animals. Among these, PB have been used in feed processing for decades due to their beneficial effects on immune function and growth rate, as well as their low production cost [2]. *Bacillus licheniformis*, which is generally recognized as safe, has been extensively used for a long time in the poultry industry and has demonstrated a positive effect in aiding nutrient digestion and absorption in the host's body [6,7]. In addition, research has proved that *Bacillus subtilis* improves broiler growth and performance equally as well as antibiotics such as bacitracin methylene disalicylate and avilamycin, and supplementation of *Bacillus subtilis* not only improves broiler performance but also positively impacts villi histomorphometry [8]. These bacteria can also produce digestive enzymes, such as protease, amylase, and lipase, and promote the digestion and absorption of nutrients. Bacterial components, such as cell wall sugar and peptidoglycan, can also promote the growth and development of immune organs in poultry [2]. *Saccharomyces cerevisiae* is a type of anaerobic bacteria, which is rich in protein, nucleic acid, vitamins, polysaccharides, and other nutrients, and its cell wall has a special spatial structure, which can reduce the toxicity of mycotoxins in animals [2]. However, less is known about the effect of the mixture of these three probiotics (*Bacillus subtilis*, *Bacillus licheniformis*, and *Saccharomyces cerevisiae*) on ameliorating impairment of growth performance and intestinal health in broilers.

Therefore, the aim of this study was to explore the effect of dietary inclusion of *Bacillus subtilis, Bacillus licheniformis*, and *Saccharomyces cerevisiae* in broiler diets, on growth performance, nutrient digestibility, serum immunoglobulin, antioxidant function, intestinal barrier function, and intestinal morphology.

2. Materials and Methods

The experimental protocols used in this experiment were approved by the Institutional Animal Care and Use Committee of China Agricultural University (Beijing, China) (No. AW09089102-1). The experiment was carried out at the National Feed Engineering Technology Research Center of the Ministry of Agriculture Feed Industry Center Animal Farm (Hebei, China).

2.1. Experimental Products

The main components of the PB were *Bacillus subtilis* 5×10^9 CFU/g, *Bacillus licheniformis* 2.5×10^{10} CFU/g, and *Saccharomyces cerevisiae* 1×10^9 CFU/g, which were provided by Beijing Smistyle Sci. and Tech. Development Co., Ltd.

2.2. Experimental Animals and Design

A total of 168 one-day-old as-hatched Arbor Acres chicks (weighing 45.04 ± 0.92 g) were purchased from Arbor Acres Poultry Breeding Company (Beijing, China). All the broilers were randomly divided into 3 treatments, 7 replicates per treatment, and 8 chickens per replicate. The trial was divided into two phases: phase 1 (day 0 to 21) and 2 (day 21 to 42). The test period was 42 days. The dietary treatments contained a corn soybean meal-based diet (control group, CON); an antibiotic group (basal diet + 75 mg/kg chlortetracycline, CTC), and a probiotics group (basal diet + probiotics (500 mg/kg in phase 1 and 300 mg/kg in phase 2; PB). The feed formulation was based on National Research Council (NRC, 1994) [9] and the formulation is shown in Table 1.

Table 1. Composition and nutrient levels of basal diets (%, as-fed basis).

Ingredients	Day 0 to 21	Day 21 to 42
Corn	58.17	64.26
Soybean meal, 43%	30.44	24.05
Corn gluten meal	2.00	2.50
Fish meal, 64.6%	2.00	2.00
Soybean oil	3.38	3.60
Dicalcium phosphate	1.50	1.04
Limestone	1.30	1.35
Salt	0.30	0.30
L-lysine HCl, 78%	0.01	0.08
DL-Methionine, 98%	0.14	0.04
L-Threonine, 98%	0.01	0.03
Chromic oxide	0.25	0.25
Vitamin-mineral premix [1]	0.50	0.50
Total	100.00	100.00
Calculated nutrient levels [2]		
Metabolizable energy, MJ/kg	12.76	13.17
Crude protein	21.00	19.00
Calcium	1.00	0.90
Available phosphorus	0.45	0.35
Standardized ileal digestible lysine	0.86	0.73
Standardized ileal digestible methionine	0.30	0.28
Standardized ileal digestible threonine	0.63	0.56
Standardized ileal digestible tryptophan	0.28	0.24

[1.] Vitamin A, 11,000 IU; vitamin D, 3025 IU; vitamin E, 22 mg; vitamin K_3, 2.2 mg; thiamine, 1.65 mg; riboflavin, 6.6 mg; pyridoxine, 3.3 mg; cobalamin, 17.6 μg; nicotinic acid, 22 mg; pantothenic acid, 13.2 mg; folic acid, 0.33 mg; biotin, 88 μg; choline chloride, 500 mg; iron, 48 mg; zinc, 96.6 mg; manganese, 101.76 mg; copper, 10 mg; selenium, 0.05 mg; iodine, 0.96 mg; cobalt, 0.3 mg. [2.] Crude protein was the analyzed value. Other values were calculated.

2.3. Detection Index and Measuring Method

2.3.1. Growth Performance

The body weight and feed intake of the broilers were registered on day 0, 21, and 42, and the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated.

2.3.2. Nutrient Retention

At day 39–42, 200 g of the excreta sample was collected for each replicate, feathers and the like in the excreta were removed, and the sample was then oven dried at 65 °C for 72 h. All samples were ground to pass through a 1 mm screen (40 mesh) before analysis. Feed or fecal samples were analyzed for dry matter (DM), crude protein (CP), crude fat (EE), and ash according to Association of Official Agricultural Chemists (AOAC, 2012) [10]. The gross energy (GE) in feed and fecal samples were determined by an automatic isoperibol oxygen bomb calorimeter (Parr 1281, Automatic Energy Analyzer; Moline, IL, USA). Organic matter (OM) was calculated as 1 − ash content (DM-base). Nutrient retention was determined by the equation as follows: Apparent total tract digestibility$_{nutrient}$ (ATTD) = 1 − (Cr_{diet} × $Nutrient_{feces}$)/(Cr_{feces} × $Nutrient_{diet}$).

2.3.3. Serum Antioxidant and Immune Function

At day 21 and 42, one broiler chicken with a body weight close to the average was selected for each replicate. A quantity of 4 mL of blood was collected from the wing vein and centrifuged at 3000 r/min for 10 min, and the supernatant was dispensed into a 0.5 mL Eppendorf tube and stored at −80 °C. The contents of serum total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and glutathione

peroxidase (GSH-Px) were determined by spectrophotometric methods using a spectrophotometer (Leng Guang SFZ1606017568, Shanghai, China) following the instructions provided by manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The contents of serum malondialdehyde (MDA) were determined using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The contents of serum immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) were measured by an ELISA kit (IgG, IgM, and IgA quantitation kit; Bethyl Laboratories, Inc., Montgomery, TX, USA).

2.3.4. Intestinal Morphology

On day 42 of this experiment, two broilers were slaughtered from each replicate. The abdominal cavity was dissected and the intestine was separated. Segments of the mid-duodenum, mid-jejunum, and mid-ileum were taken and rinsed with cold physiological saline (0.9% saline), then immediately stored in 10% buffered formalin. Conventional paraffin embedding, sectioning, HE staining, and six straight and complete fluffs were selected for each section, and the height of the villi and the depth of the crypt corresponding to the villi were determined. The height of random orientated villi and their adjoined crypts were determined with a light microscope using a calibrated eyepiece graticule [11].

2.3.5. The Level of Claudin-1, Occludin and ZO-1 Gene Expression in Jejunal Mucosa

On day 42 of this experiment, the jejunal mucosa was taken from the broilers and then stored in liquid nitrogen. Total RNA extraction was done using Trizol Reagent (TaKaRa, Dalian, China), and the purity and concentration of total RNA were measured by ultraviolet spectrophotometer. Total RNA (1μg) was reverse-transcribed into cDNA using Prime Script RT Reagent Kit (TaKaRa, Dalian, China) according to the direction of the manufacturer's protocol. The primers were synthesized by TaKaRa Biotechnology (TaKaRa, Dalian, China), which were obtained from the published works of Shao et al and Li et al [12,13], and are shown in Table 2. Real-time PCR was conducted according to Li et al [14].

Table 2. Sequences of the primers used for the determination of gene expression levels.

Genes	Primer Sequence (5′-3′)	Gene Accession No.	References
Claudin-1	F: TGGAGGATGACCAGGTGAAGA R: CGAGCCACTCTGTTGCCATA	NM_001013611.2	[11]
Occludin	F: TCATCGCCTCCATCGTCTAC R: TCTTACTGCGCGTCTTCTGG	NM_205128.1	[11]
ZO-1	F: TGTAGCCACAGCAAGAGGTG R: CTGGAATGGCTCCTTGTGGT	XM 413773.4	[12]

ZO-1: zona occludens-1.

2.4. Statistical Methods

Data was subjected to Analysis of variance (ANOVA) using the GLM procedure of SAS (SAS Institute, 2008) [15]. The replicate was the experimental unit. Significantly different means were separated by Duncan's multiple range test. Results were expressed as least squares means and SEM. Significance was designated at $p \leq 0.05$, while a tendency for significance was designated at $0.05 < p \leq 0.10$.

3. Results

3.1. Growth Performance

As can be seen from Table 3, dietary supplementation with CTC and PB had no significant effect on the ADFI of broilers compared with CON. In phase 1, broilers fed PB showed improved FCR compared with CON and CTC ($p < 0.05$). In phase 2, broilers fed PB showed improved ADG in comparison with

CON ($p < 0.05$) and had no significant difference with CTC. Overall (day 0 to 42), broilers fed PB had improved ADG and FCR compared with CON ($p < 0.05$) and enhanced ADG compared with CTC ($p < 0.05$).

Table 3. Effects of probiotics on growth performance of broilers [1].

Item	CON	CTC	PB	SEM	*p*-Value			
					Treatment [2]	CON vs. CTC	CON vs. PB	CTC vs. PB
day 0 to 21								
Average daily gain, g	27.32	26.96	28.41	0.47	0.15	0.45	0.56	0.11
Average daily feed intake, g	36.99	36.42	35.93	0.62	0.76	0.73	0.49	0.80
Feed conversion ratio	1.35 [a]	1.35 [a]	1.26 [b]	0.02	0.02	0.96	0.01	0.02
day 21 to 42								
Average daily gain, g	68.85 [b]	71.44 [a,b]	76.62 [a]	1.12	<0.01	0.12	<0.01	0.34
Average daily feed intake, g	105.87	105.05	112.35	2.55	0.12	0.89	0.16	0.11
Feed conversion ratio	1.54	1.47	1.46	0.03	0.24	0.43	0.36	0.85
day 0 to 42								
Average daily gain, g	48.08 [b]	49.20 [b]	52.52 [a]	0.52	<0.01	0.79	<0.01	<0.01
Average daily feed intake, g	71.43	70.73	74.14	1.10	0.09	0.82	0.10	0.08
Feed conversion ratio	1.49 [a]	1.44 [a,b]	1.41 [b]	0.02	0.04	0.23	0.02	0.15

[1] CON, control; CTC, chlorotetracycline (75 mg/kg); PB, probiotic (500 mg/kg in phase 1, 300 mg/kg in phase 2); SEM, standard error of the mean. [2] Treatment, the specific *p* value of the diet effect in the ANOVAs analysis. [a,b] values in the same row with different letters are significantly different at $p < 0.05$.

3.2. The ATTD of Nutrients

The effects of PB on the ATTD of nutrients in broilers are shown in Table 4. Compared with CON, the DM and OM were increased ($p < 0.05$) in broilers fed PB and CTC. In addition, broilers fed PB also showed enhanced ($p < 0.05$) GE and CP compared with CON.

Table 4. Effects of probiotics on the nutrient retention of broilers (%, day 42) [1].

Items	CON	CTC	PB	SEM	*p*-Value			
					Treatment [2]	CON vs. CTC	CON vs. PB	CTC vs. PB
Dry matter	73.80 [b]	75.42 [a]	75.92 [a]	0.30	<0.01	<0.01	<0.01	0.72
Gross energy	76.58 [b]	78.07 [a,b]	78.38 [a]	0.39	0.03	0.16	0.01	0.84
Crude protein	64.04 [b]	66.83 [a,b]	67.95 [a]	0.95	0.06	0.09	0.03	0.42
Ether extract	91.57	93.05	91.71	0.68	0.31	0.52	0.66	0.13
Organic matter	76.49 [b]	77.93 [a]	78.33 [a]	0.30	0.01	0.02	<0.01	0.63

[1] CON, control; CTC, chlorotetracycline (75 mg/kg); PB, probiotic (500 mg/kg in phase 1, 300 mg/kg in phase 2); SEM, standard error of the mean. [2] Treatment, the specific *p* value of the diet effect in the ANOVAs analysis. [a,b] values in the same row with different letters are significantly different at $p < 0.05$.

3.3. Serum Antioxidant Status

The effects of PB on the antioxidant status of broilers are shown in Table 5. Compared with CON, broilers fed CTC and PB showed increased serum T-AOC concentration in phase 1 ($p < 0.05$). There was a tendency of enhancing concentration of SOD ($p = 0.06$), GSH-Px ($p = 0.06$), and reducing ($p = 0.07$) level of MDA in serum of broilers fed PB compared with CON in phase 1. In phase 2, broilers fed PB had higher ($p < 0.05$) concentration of GSH-Px and lower ($p < 0.05$) level of MDA in serum in comparison with CON.

Table 5. Effects of probiotics on the serum antioxidant function of broilers [1].

Items	CON	CTC	PB	SEM	*p*-Value			
					Treatment [2]	CON vs. CTC	CON vs. PB	CTC vs. PB
d 0 to 21								
T-AOC (U/ml)	1.00 [b]	2.50 [a]	2.37 [a]	0.40	0.05	0.02	0.04	0.53
SOD (U/ml)	55.21	56.61	57.13	0.55	0.08	0.24	0.06	0.36
GSH-Px (μmol/L)	27.39	30.16	36.02	2.56	0.09	0.28	0.06	0.14
MDA (nmol/ml)	7.64	7.10	6.90	0.22	0.10	0.25	0.07	0.44
d 21 to 42								
T-AOC (U/ml)	3.33	2.99	3.35	0.52	0.77	0.41	0.89	0.37
SOD (U/ml)	87.24	75.05	87.99	4.35	0.12	0.11	0.74	0.14
GSH-Px (μmol/L)	19.42 [b]	23.92 [a,b]	26.75 [a]	1.90	0.06	0.23	0.04	0.46
MDA (nmol/ml)	7.58 [a]	6.46 [a,b]	6.09 [b]	0.41	0.07	0.06	0.03	0.75

[1] CON, control; CTC, chlorotetracycline (75 mg/kg); PB, probiotic (500 mg/kg in phase 1, 300 mg/kg in phase 2); SEM, standard error of the mean. [2] Treatment, the specific *p* value of the diet effect in the ANOVAs analysis. [a,b] values in the same row with different letters are significantly different at $p < 0.05$.

3.4. Serum Immunoglobulins

The effects of PB on serum immunoglobulins of broilers are shown in Table 6. In phase 1, broilers fed PB ($p = 0.07$) and CON ($p = 0.06$) tended to show enhanced level of IgM compared with CTC, while broilers fed PB increased ($p < 0.05$) level of IgA in phase 2 in comparison with CTC and CON.

Table 6. Effects of probiotics on the serum immunoglobulins function of broilers (ug/mL) [1].

Items	CON	CTC	PB	SEM	*p*-Value			
					Treatment [2]	CON vs. CTC	CON vs. PB	CTC vs. PB
day 0 to 21								
Immunoglobulin A	4.24	4.10	4.40	0.12	0.24	0.68	0.57	0.32
Immunoglobulin G	2.27	2.23	2.28	0.03	0.45	0.43	0.51	0.39
Immunoglobulin M	1.71	1.65	1.70	0.02	0.07	0.06	0.88	0.07
day 21 to 42								
Immunoglobulin A	4.27 [b]	4.25 [b]	4.98 [a]	0.22	0.07	0.76	0.04	0.04
Immunoglobulin G	2.28	2.25	2.51	0.14	0.39	0.65	0.22	0.18
Immunoglobulin M	1.68	1.68	1.91	0.14	0.44	0.83	0.26	0.24

[1] CON, control; CTC, chlorotetracycline (75 mg/kg); PB, probiotic (500 mg/kg in phase 1, 300 mg/kg in phase 2); SEM, standard error of the mean. [2] Treatment, the specific *p* value of the diet effect in the ANOVAs analysis. [a,b] values in the same row with different letters are significantly different at $p < 0.05$.

3.5. Intestinal Morphology

The effects of PB on the intestinal morphology of broilers are shown in Table 7. Compared with CON, the duodenal villus height to crypt depth ratio was significantly increased ($p < 0.05$) in broilers fed CTC and PB. In addition, these broilers tended to showed lower crypt depth in duodenum, as well as higher villus height to crypt depth ratio in ileum compared with CON.

Table 7. Effects of probiotics on intestinal morphology of broilers (day 42) [1].

Items	CON	CTC	PB	SEM	*p*-Value			
					Treatment [2]	CON vs. CTC	CON vs. PB	CTC vs. PB
Duodenum								
Villus height (μm)	1905	2234	2134	128	0.26	0.14	0.21	0.37
Crypt depth (μm)	269	172	181	26	0.07	0.06	0.09	0.26
Villus height/ Crypt depth	7.95 [b]	13.10 [a]	12.04 [a]	1.19	0.05	0.03	0.04	0.64
Jejunum								
Villus height (μm)	1372	1561	1360	95	0.32	0.14	0.72	0.11
Crypt depth (μm)	253	195	177	35	0.34	0.16	0.11	0.61
Villus height/ Crypt depth	6.00	8.19	8.25	1.10	0.33	0.45	0.28	0.76
Ileum								
Villus height (μm)	1204	1103	1097	84	0.62	0.32	0.25	0.84
Crypt depth (μm)	159	138	105	20	0.25	0.36	0.14	0.62
Villus height/ Crypt depth	8.14	8.27	10.58	0.72	0.09	0.62	0.06	0.13

[1] CON, control; CTC, chlorotetracycline (75 mg/kg); PB, probiotic (500 mg/kg in phase 1, 300 mg/kg in phase 2); SEM, standard error of the mean. [2] Treatment, the specific *p* value of the diet effect in the ANOVAs analysis. [a,b] values in the same row with different letters are significantly different at $p < 0.05$.

3.6. Jejunal Mucosal Barrier Functions

The effects of PB on jejunal mucosal barrier function of broilers are shown in Table 8. Compared with CON, broilers fed CTC and PB showed higher gene expression of zonula occludens-1 (ZO-1) in jejunum ($p < 0.01$), and had no significant effect on the gene expression of claudin-1 and occludin in jejunum.

Table 8. Effects of probiotics on gene expression levels of claudin-1, occludin and ZO-1 genes in the jejunum barrier function of broilers (day 42) [1].

Items	CON	CTC	PB	SEM	*p*-Value			
					Treatment [2]	CON vs. CTC	CON vs. PB	CTC vs. PB
Claudin-1	1.00	0.93	0.53	0.18	0.18	0.72	0.12	0.14
Occludin	0.99	1.41	0.98	0.31	0.27	0.13	0.87	0.11
ZO-1	1.02 a,b	5.98 a	5.66 a	0.57	<0.01	<0.01	<0.01	0.92

[1] CON, control; CTC, chlorotetracycline (75 mg/kg); PB, probiotic (500 mg/kg in phase 1, 300 mg/kg in phase 2); SEM, standard error of the mean. [2] Treatment, the specific *p* value of the diet effect in the ANOVAs analysis. [a,b] values in the same row with different letters are significantly different at $p < 0.05$.

4. Discussion

The current study showed that broilers fed PB improved ADG in phase 2, and ADG and FCR overall. Our results are consistent with the study of Kalia et al. [16], who reported that a diet supplemented with mixed PB could improve the body weight gain and feed efficiency, and decrease mortality in broilers. However, studies conducted by Ahmad et al. [17] and Fathi et al. [18] showed PB had no significant effects on improving FCR. This difference might be due to the variation of survivability of PB in the intestine of broilers and the dose rate of PB used for broilers. The possible reason for the current positive effect on performance could be explained by the *Bacillus subtilis* in PB improving the immune response [19] and the positive effect of PB on modulating the microbiota structure (such as reducing the content of *Salmonella Enteritidis*) [20]. The improvement of performance might also be due to PB increasing nutrient retention (GE, CP, DM, and OM). Research has shown that PB is able to improve the activity of digestive enzymes of animals [21]. Moreover, dietary PB supplementation could produce some metabolites, including organic acids, to enhance the nutrient retention in broilers [22]. The current study showed dietary inclusion of PB, namely, *Bacillus subtilis*, *Bacillus licheniformis*, and *Saccharomyces cerevisiae*, has the same effects as CTC in improving growth performance, which indicates that PB could be a potential antibiotics substitute.

Current research indicates that addition of PB had a positive role on antioxidant functions in broilers. In agreement with our results, Capcarova et al. and Wen et al. [23,24] found that some probiotics could be beneficial in oxidation resistance, scavenging reactive oxygen species, and promoting antioxidant capability. With regard to antioxidant capacity, the endogenous antioxidant defense system in animals also relies on other external sources, such as probiotics, which are the natural source for prevention of the oxidative stress induced by reactive oxygen species [25]. Collectively, this study suggested that PB can possess antioxidant capacity in broilers.

The current study also showed dietary PB supplementation had a positive effect on serum immunoglobulin, which is in agreement with Fathi et al. [18], who reported improving effects of PB on IgM and cell-mediated immunity. The reason may be that *Bacillus subtilis* had a positive effect on enhancing antibodies against the Newcastle disease of broiler chicks [19]. PB *Bacillus subtilis* could also enhance humoral immune responses and stimulate the host's mucosal immune system by interacting with intestinal epithelial cells in broilers [26]. The mechanism of PB on the immunity of broilers may also result because PB can protect animals from pathogen colonization by competing for epithelial binding sites and nutrients, strengthening the intestinal immune response, and producing antimicrobial bacteriocins. [22]

The current study showed that dietary PB supplementation can increase the ratio of villus height to crypt depth, which indicates that PB can promote the development of the absorptive surface of duodenum and ileum in broilers. This might be due to the beneficial bacteria in PB, which may improve crypt cell proliferation in the small intestine, and thus help increase the growth rate in broilers [17]. In addition, the *Bacillus licheniformis* in PB can colonize and form niches in the small intestine, which positively protects the villi from pathogens and improves the growth of villi [27]. However, Sohail et al. [28] found that PB had no effect on stress-induced injury in the intestinal morphology of 42-day-old chickens, which might be due to the variation of types and amounts of PB used in different studies. Moreover, the improvement of intestinal morphology and integrated intestinal barrier are important for epithelial cell function, which might be the reason for the improved ATTD of nutrients [29].

The function of the intestinal barrier and the absorption of nutrients can be directly affected by the damage of the mucosal epithelium, and PB can regulate intestinal immunity and tight junction protein mRNA expression of broilers [30]. Current research indicates that the addition of PB to diets can promote the gene expression of ZO-1 in jejunal mucosa of broilers and improve the jejunal mucosal barrier function of broilers. PB in diets can decrease the feed weight gain ratio and intestinal coliform, and can also increase the duodenal villus height to crypt depth ratio. These results suggest that the supplementation of a PB mixture in the diet can effectively improve part of the intestinal barrier function. PB has been shown to be adherent to the intestinal epithelium, resistant to acidic conditions, and capable of antagonizing and competitively eliminating certain pathogens in vivo [31]. In contrast, the PB mixture used in this study consisted of *Bacillus licheniformis*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*. *Bacillus licheniformis* and *Bacillus subtilis* are aerobic bacteria that use oxygen in the intestine to provide an anaerobic environment for the colonization of anaerobic bacteria, such as *Lactobacilli* and *Bifidobacteria*. Therefore, these lactic acid-producing bacteria produce a more acidic environment, which impairs the growth of opportunistic pathogens [32].

5. Conclusions

The results of this experiment showed that the addition of probiotics (500 mg/kg in phase 1, 300 mg/kg in phase 2) could improve broilers' growth performance, nutrient retention, and serum antioxidant capacity, and improve their intestinal health via improving jejunal mucosal barrier function and intestinal morphology. The results indicated that the current probiotics could be used as a chlortetracycline substitute in the diet of broiler chickens.

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Review

Use of Medicinal Mushrooms in Layer Ration

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Simple Summary: The extensive use of antibiotics in the poultry industry to increase production performance has led to human health hazards. The use of natural herbs as antibiotic substitutes has been reported in the poultry feed industry. Therefore, the objective of this review was to determine the effect of different levels of mushrooms and their extract in diet on laying performance and health status. On the basis of previous findings, dietary supplementation using mushrooms as a natural feed supplement sustained laying performance and improved immunity in laying hens.

Abstract: Application of different medicinal mushrooms intended to enhance production performance and health status has created an importance demand in poultry production. One goal of using medicinal mushrooms is to get rid of antibiotics in poultry feed without affecting the optimum performance. Increasing concerns about this issue have led to more attention on antibiotic substitutes and a significant demand for them for organic egg production. Thus, supplementation with medicinal mushrooms is a new concept for research in layer production, however, there is still a great deal of confusion about inclusion levels and the mode of action of medicinal mushrooms on production performance and health status in laying hens. Taking this into account, this review outlines the experimental uses of medicinal fungi on the growth performance, laying performance, egg quality, and health status of layer birds based on previous findings to date. Finally, we highlight that supplementation with medicinal fungi can play a role on the immunity, health, and production performance in laying hens.

Keywords: medicinal mushrooms; laying hens; health status; performance

1. Introduction

Traditionally, mushrooms have been used for highly valued food and pharmaceutical purposes because of their role as a tonic and their benefit to health [1]. Cultivated edible mushrooms are good sources of protein, have low-fat content, and are cholesterol free [2]. Mushrooms are also very popular as a quality protein containing essential amino acids, adequate vitamins, minerals, and are rich source of different unsaturated fatty acids [3]. Different bioactive components have been extracted from the fruiting body and mycelium part of mushroom and tested in invitro studies. Polysaccharides are considered to be the most activate component in mushrooms which have immune stimulating activities [3]. In addition, the polysaccharides in mushroom have been found to produce different cytokines and increase the weight of immune stimulating organs in laboratory animals [4,5]. Presently, researchers have become interested in the role of medicinal mushrooms in poultry production systems.

Antibiotics as feed additives have been used as growth and health promoters in poultry production [6], however, because of the appearance of microorganisms that are resistant to specific antibiotics, the application of antibiotics in poultry ration has been forbidden or restricted in the developed countries [7,8]. As a result, exploring new growth-promoting alternatives to antibiotics has become a hot topic of research for several years [9]. Chickens are very sensitive to immunosuppressive

stressors and infectious diseases [10]. Different infections are responsible for reduction in growth rates, poor egg production, and mortality, which have resulted in huge economic losses in the poultry industry. There is a direct relationship between feeding and the immune system of the host [11]. Various attempts, through genetic manipulation, dietary alterations, various medicinal supplements, etc. have been tried to reduce the cholesterol content in meat and eggs, and therefore improve their health status [12].

At present, there are several scientific works about the health promoting benefits of involving mushrooms in farm animals. Currently, poultry researchers are committed to using unconventional natural feed supplement as a substitute for antibiotics that have been proven as possible ways to enhance the health and to improve the production in poultry. Although it is known that mushrooms are medicinally importance for chickens health, unfortunately, the inclusion level of mushrooms in poultry diets is still under consideration. Findings from past reports have highlighted that their inclusion may enhance production performance and health in laying hens [13]. Taking this into consideration, this review is focused on the importance of the medicinal mushrooms as an alternative for antibiotics that can improve the performance and the immunity in laying hens.

2. Common Medicinal Mushrooms Used in Layer Study

A group of mushrooms have been identified as medicinal mushrooms, in recent years, due to their biological properties both invivo and invitro studies. The phylum Basidiomycota is the most predominant among the mushrooms species that has been proven to be a medicinal mushroom [14]. On the basis of some previous studies, we have identified some common medicinal mushrooms that can be used as a source of active substances for optimum performance and health status in layer chickens. A list of major medicinal mushrooms that were used in poultry ration during the previous years is presented in Table 1 and Figure 1.

Figure 1. Photographs of different medicinal mushrooms: (**a**) Flammulina velutipes, (**b**) *Hericium erinaceus*, (**c**) Agaricus brasiliensis, (**d**) Pleurotus ostreatus, (**e**) *Lentinula edodes*, (**f**) Ganoderma lucidum, (**g**) *Cordyceps sinensis*, and (**h**) Tremella fuciformis.

Table 1. Botanical classification and distribution of medicinal mushrooms used in poultry ration [1].

Common Name/Local Name	Scientific Classification	Distribution
Golden needle mushroom/Winter mushroom/Lily mushroom/Velvent shank/Enoki mushroom/Jingen Gu	K: Fungi P: Basidiomycota C: Agaricomycetes O: Agaricales F: Physalacriaceae G: Flammulina Sp: *Flammulina velutipes*	Europe, USA, and Asia, especially China, Japan, Korea, and Vietnam
Monkey's head/Lion's mane/Bear's head/Yamabushitake (Japan)/Houtou or Shishigashira (China)	K: Fungi P: Basidiomycota C: Agaricomycetes O: Russulales F: Hericiaceae G: *Hericium* Sp: *Hericium erinaceus/Hericium caput-medusae*	Europe, Asia, and North America
White button mushroom/Almond mushroom/ Mushroom of sun/God's mushroom	K: Fungi P: Basidiomycota C: Agaricomycetes O: Agaricales F: Agaricaceae G: Agaricus Sp: *Agaricus brasiliensis/Agaricus bisporus*	California, Hawaii, Great Britain, The Netherlands, Taiwan, Philippines, Australia, Brazil, China, Japan, Korea and Vietnam
Oyster mushroom	K: Fungi P: Basidiomycota C: Agaricomycetes O: Agaricales F: Pleurotaceae G: Pleurotus Sp: *Pleurotus ostreatus/Pleurotus eryngii*	All over the world, especially Germany, India, China, Japan, and Korea
Shiitake mushroom	K: Fungi P: Basidiomycota C: Agaricomycetes O: Agaricales F: Marasmiaceae G: Lentinula Sp: *Lentinulaedodes*	Southeast Asia, especially China and Japan
Reishi/Lingzhi mushroom	K: Fungi P: Basidiomycota C: Agaricomycetes O: Polyporales F: Ganodermataceae G: Ganoderma Sp: *Ganoderma lucidum/Ganoderma applanatum*,	Southeast Asia especially China, Japan, and Korea
Caterpillar Mushroom/ Cordyceps mushroom	K: Fungi P: Ascomycota C: Sordariomycetes O: Hypocreales F: Cordycipitaceae G: *Cordyceps* Sp: *Cordyceps sinensis/Cordyceps militaris*	Asian countries, e.g., Nepal, China, Japan, Bhutan, Korea, Vietnam, and Thailand
Snow fungus/ Snow ear/Silver ear fungus/White jelly mushroom.	K: Fungi P: Basidiomycota C: Tremellomycetes O: Tremellales F: Tremellaceae G: Tremella Sp: *Tremella fuciformis*	North America, Africa, Australia, New Zealand, Asia including Korea, Japan, and China

[1] K, kingdom; P, phylum; C, class; O, order; F, family; G, genus; and Sp, species.

3. Biological Role of Medicinal Mushrooms

Mushrooms have been reported to have many useful functions including antitumor, anticancer, antihypertensive, cholesterol lowering effect, antioxidant properties, anti-inflammatory, immune-modulatory function, as well as anti-bacterial, antiviral, and antifungal activities on human and animal health [15,16].

3.1. Antitumor Activities

The shiitake mushroom (*Lentinus edodes*) is rich in antitumor agents which play a role inhibiting cancer cell growth [17]. Aqueous extracts from the vegetative submerged mycelia of cultivated *Ganoderma lucidu*, and *Lentinus edodes* have been reported to have antitumor activities [18]. *F. velutipes* mushrooms have been reported to hold bioactive compound having antitumor functions [19]. The extract of *F. velutipes* mushroom has been used to oppose breast cancer cells [20]. Recently, significant novel components with anticancer function were discovered in *F. velutipes* by Chinese researchers. These researchers discovered a sesquiterpene, which is known as flammulinol A, along with other flammulinolides A–G derived from *F. velutipes* mushroom that were effective against several cancer cell lines [21]. A recent study by Dong et al. [22] reported that polysaccharide, purified from *Ganoderma applanatum* mushroom, was effective against human breast cancer in an invitro study.

3.2. Antioxidant Activities

Today, the antioxidant properties of different medicinal mushrooms are well-known. Some previous studies have reported that the polysaccharides and oligosaccharide present in medicinal mushrooms show antioxidant functions [23]. Conventional uses of butylated hydroxyanisole and butylated hydroxytoluene as synthetic antioxidants can be hazardous to humans, and therefore there is a need to discover natural antioxidant products [24]. Tang et al. [3] stated that the phenolic ingredients present in mushrooms may have the capacity to withdraw the oxidation of the LDL for their anti-inflammatory activities. A fibrinolytic enzyme that was successfully purified and derived from the culture supernatant of needle mushroom was reported by Park et al. [25]. The antioxidant activities depend on different parts and varieties of mushrooms. Zeng et al. [26] stated that *F. velutipes* mushroom hold a higher phenolic amount with the highest antioxidant activities. Different mushrooms were found to exhibit vitaminC and selenium that can play a role in antioxidant functions [14]. A recent study by Lin et al. [27] found that the *Cordyceps sobolifera* (Ascomycetes) mushroom exhibits antioxidant properties as a functional food and dietary supplement. In addition, *Agaricus brasiliensis* are considered potential auxiliaries for the treatment of patients with rheumatoid arthritis due to their capacity to reduce oxidative stress [28]. The anti-inflammatory and antioxidant properties of *A. bisporus* biomass extracts from an in vitro culture were reported by Muszynska et al. [29]. In their studies, incubation of Caco-2 cells with *A. bisporus* extracts resulted in decreased expression of cyclooxygenase-2 and prostaglandin F2α receptor as compared with the lipopolysaccharide (LPS) or TNF-α-activated cells. The antioxidant activity of *Pleurotus ostreatoroseus* (Agaricomycetes) mushroom was also noted by Brugnari et al. [16].

3.3. Lipid Metabolism Activities

The positive role of golden needle mushroom on lipid metabolism in male hamsters was reported by Yeh et al. [30]. Their study showed that both the extract and the powder originating from needle mushroom were capable of reducing serum and liver tissue cholesterol level in hamsters. Another study by Yang et al. [31] found a lower level of plasma triglyceride, total cholesterol (TC), and low-density lipoprotein cholesterol in diet-induced hyperlipidemic rats fed *Hericiumerinaceus* mushroom exo-polymer. Lovastatin, as well as γ-aminobutyric acid (GABA), were identified from *F. velutipes* fruiting bodies [32]. Lovastatin is used to reduce cholesterol production that can diminish risks of heart diseases [32,33]. Another study by Harada et al. [34] reported very effective results

by decreasing the systolic pressure in rats using GABA-mediated *F. velutipes* mushroom powder. *ß*-D-glucan and its derivatives present in medicinal mushrooms ensured their cholesterol lowering effects by reducing the absorption or increasing the faecal excretion [35]. The oyster mushroom is also famous for its cholesterol reducing functions [36].

3.4. Antimicrobial Activities

The antimicrobial properties of medicinal mushrooms are well established. The extracts derived from medicinal *Pleurotus* species mushroom have been reported to have potential antibacterial and antifungal functions [37,38]. An invitro experiment was conducted by Sknepnek et al. [39] with reishi mushroom (*Ganoderma lucidum*) on antimicrobial functions. Their studies concluded that the liquid *Ganoderma lucidum* mushroom beverage at a 0.04 mg/mL concentration was very useful against *Staphylococcus epidermidis* and *Rhodococcus equi*. In addition, it was very useful against *Bacillus spizizenii*, *B. cereus*, and *R. equi* at a 0.16 mg/mL concentration. Nedelkoska et al. [40] reported that the mushroom fruiting body was very effective against different bacteria. Kashina et al. [41] stated that the mushroom, *F. velutipes*, exhibited inhibitory activities in opposition to two different harmful fungi (*Sporothrix schenckii* and *Candida albicans*). Enokipodins have been found in the needle mushroom that has antimicrobial functions [42].

3.5. Immune Functions

The immune functions of mushrooms are well known. Different protein and various peptides present in mushrooms are able to modify the immune response positively [14]. Invitro immune-modulatory studies with *F velutipes* showed that raw 264.7 cells were stimulated to secret nitric oxide upon administration of 200 to 500 μg/mL *F.velutipes* polysaccharide (FVPA2). The FVPA2 also encouraged the proliferation of the spleen lymphocytes and B lymphocytes in experimental mice [43]. Manayi et al. [44] used the extract of *Ganoderma applanatum* mushroom at a concentration of 1000 mg/kg diet on the defense mechanisms in rainbow trout. This study found the potential ability of *G. applanatum* mushroom extract to activate immunologic parameters in rainbow trout. Lee et al. [45] found that the mushroom could increase the concentration of IFNγ that has a toxic function against lymphoma cell. The polysaccharides of needle mushroom were found to produce different cytokines and increase the weight of immune stimulating organs in laboratory animals [4,5]. The mushroom polysaccharides increased the body weight of experimental mice and the weight ratio of the thymus and spleen, as well as it could modulate the T cell subpopulation of thymocytes and splenocytes [30]. Moreover, the polysaccharides of mushroom increased NO (nitric oxide), TNF-a, IL-1b, and IL-6 production, and lymphocyte proliferation in mice model [46].

3.6. Nutritional Roles

Mushrooms are very popular for their nutritional values. Mushrooms have been reported as a good source of six major nutrients which include carbohydrates, especially dietary fiber, proteins, vitamins, minerals, lipids, and water. Rich in proteins, carbohydrates, and fiber with low fat are the unique features of the medicinal mushroom. In addition, different types of essential amino acids (AA) have been found in mushroom [47–49]. The nutritional component of different mushroom showed dry matter (DM) 74% to 89.6%; crude protein (CP) 8.9% to 14.8%; carbohydrate 43.33% to 69.40%; total detergent fiber (TDF) 1.9% to 7.40%; crude fat (EE) 1.75% to 3.91%; ash (total mineral) 4.91% to 8.40%; calcium (Ca) 2.21% to 3.05%, and phosphorus (P) 1.68% to 1.88% [3,50,51].

4. Medicinal Mushrooms in Layer Chicken Ration

The data regarding the role of medicinal mushrooms in layer chicken performance are summarized in Table 2. Hence, there has been considerable debate regarding current findings on laying hens' performance, as well as many variables that have been associated with the current findings such as mushroom species, use dosage, method of application (either non-fermented or fermented with

beneficial organisms), part of the mushroom (either fruiting bodies or stem base), and the treatment period. However, collectively, many scientists agreed that mushrooms could have a positive role by improving the laying percent, table egg quality, egg yolk cholesterol level, as well as immunity in laying hens. Further studies are needed to detect the actual dose for optimum performance in layer chickens.

Table 2. Role of medicinal mushrooms on performance in layer chickens [1].

Mushroom Species	Study Design	Main Finding	References
Flammulina velutipes	ISA Brown layer pullet from 10 weeks to 16 weeks (42 days) form: dried mushroom, dose: mushroom 2%, 4%, 6% (inclusion type)	• increased final live weight • increased nutrient retention • higher dry matter content in excreta • lower pH in excreta • higher bursa weight • higher antibody titers against ND, IB, and AI • higher serum immunoglobulin IgA, IgG, and IgM	Mahfuz et al. [52]
Flammulina velutipes	ISA Brown layer from 19 weeks to 29 weeks (70 days) form: dried mushroom, dose: mushroom 2%, 4%, 6% (inclusion type)	• increased marketable egg number • increased calcium retention • higher antibody titers against ND and IB • higher serum immunoglobulin sIgA, IgG, and IgM • higher serum cytokines IL-2, IL-4, IL-6, and TNF-α	Mahfuz et al. [53]
Flammulina velutipes	Hy-line Brown layer from 60 weeks to 65 weeks (35 days) form: mushroom fermented by *Bacillus subtilis* and *Klebsiella spp*, dose: mushroom 1%, 2%, 3%, 4%, 5% (supplementation type)	• higher egg weight • higher albumen height, haugh unit, eggshell weight, and shell thickness • lower cecal *Salmonella spp* and *E. coli* number • lower excreta ammonia (NH_3) concentration	Lee et al. [13]
Lentinula edodes	Tetran Brown layer from 22 weeks to 30 weeks (56 days) form: dried mushroom powder, dose: mushroom 0.25%, 0.5% (supplementation type)	• higher egg production • higher haugh unit • higher linoleic acid, total n-6 and polyunsaturated fatty acid in egg yolk • lower egg yolk cholesterol	Hwang et al. [54]
Ganoderma lucidum	Lorman Brown pullet from 0 to 20 weeks form: dried mushroom powder dose: mushroom 2 g/kg, 1 g/kg, 0.5 g/kg (supplementation type)	• improve FCR • higher antibody titers	Ogbe et al. [55]
Pleurotus eryngii	Hendrix layer from 22weeks to 30 weeks (56 days) form: dried mushroom powder dose: mushroom 0.5%, 1%, 2% (supplementation type)	• lower egg yolk and serum cholesterol • higher haugh unit • higher antioxidant enzyme activities	Lee et al. [56]
Cordyceps militaris	Hendrix layer from 22 weeks to 34 weeks (84 days), Form: dried mushroom waste dose: mushroom 5 g/kg, 10 g/kg, 20 g/kg, (supplementation type)	• lower egg yolk cholesterol • higher egg mass • improved FCR	Wang et al. [57]

[1] ND, Newcastle disease; IB, infectious bronchitis (IB); AI, Avian influenza; Ig, immunoglobulin; IL, interleukin; n-6, omega-6 fatty acid; and FCR, feed conversion ratio.

4.1. Application of Medicinal Mushrooms on Performance and Egg Quality

There have been limited studies conducted, in previous years, to evaluate the effects of medicinal mushrooms in laying hens. Mahfuz et al. [52] conducted a study to examine the role of *Fammulina velutipes* mushroom stem wastes (FVW) on growth performance, and immunity in pullet birds on basic of different levels (2%, 4%, and 6%). They found that the final live weight was greater ($p < 0.05$) in mushroom fed groups at all levels (2%, 4%, and 6%) than that of the control and antibiotics diets. No differences ($p > 0.05$) were found for the average daily feed intake, average daily weight gain, and feed conversion ratio (FCR) among treatments. Dry matter (DM), crude protein (CP), and ether extract (EE) retention were higher ($p < 0.05$) in FVW diets than the control and antibiotic diets. Excreta DM was higher ($p < 0.05$) and pH was lower ($p < 0.05$) in FVW diets than the control and antibiotic

diets. The higher body weight, in this study, must be related to higher nutrient retention in mushroom supplemented groups. In addition, the Excreta DM was higher in the mushroom supplemented groups which suggests that incorporated FVW reduced excreta moisture, which can prevent the wet litter in poultry house, as well as increase the absorption of nutrients and reduce the ammonia gas production from excreta in chicken house. Teye et al. [58] stated that high moisture content, high temperatures, and high pH can facilitate the production of ammonia from excreta. Mahfuz et al. [53], consequently, assessed the role of *Flammulina velutipes* in laying hens ration with different levels of mushrooms (2%, 4%, and 6%) in experimental diets and did not find any differences ($p > 0.05$) in laying performance parameters such as average daily egg production percentage, egg mass, FCR, etc., in laying hens. The number of unmarketable table eggs was fewer ($p < 0.05$) in mushroom fed diets as compared with the control diets. This study also found suitability for calcium retention in eggshells with FVW diets as compared with the control and the antibiotic diets. It was hypothesized that the higher calcium retention could be related to a higher number of marketable eggs in mushroom fed groups. No effects on egg production percentage, egg mass, and FCR ensured the fact that feeding mushroom did not have any adverse effects on laying performance. Lee et al. [13] found that feeding *F. velutipes* mycelium had no adverse effects on egg production percentage, feed intake, and FCR, in laying hens, but the egg weight was found greater ($p < 0.05$) in the 1% and 3% mushroom feeding groups than the control diets. Furthermore, feeding mushroom at the 4% level resulted in significantly higher ($p < 0.05$) egg albumen height, haugh unit, eggshell weight, and shell thickness. It was thought that mushrooms contain higher level of CP that leads to increased egg albumin and might have an effect on shell gland for continuous eggshell formation. On the contrary, Na et al. [59] found that dietary inclusion of mushroom had no effect on eggshell weight, shell thickness, and haugh unit. Finally, the authors suggested that mushroom, as a natural resource of feed for laying hens, can be used at the 5% level without affecting normal performance.

Lentinula edodes are commonly known as Shiitake mushroom which has long been considered to be a medicinal mushroom. An experiment was conducted with the shiitake mushroom on laying performance and egg quality by Hwang et al. [54]. Higher ($p < 0.05$) egg production and higher ($p < 0.05$) haugh unit in eggs were reported by feeding shiitake mushroom than that of the control group. However, the other laying parameters including egg weight, shape index, shell thickness, albumen height, yolk color, and the egg sensory evaluation (e.g., appearance, color, flavor, oily nature) were not affected ($p > 0.05$). Egg yolk fatty acids, especially linoleic acid, total omega-6 fatty acid (n-6), and the polyunsaturated fatty acid were found to be higher ($p < 0.05$) in 0.5% mushroom feeding groups than the control group, however, palmitoleic acid and α-linolenic acid were lower ($p < 0.05$) in the 0.5% mushroom feeding group than the control fed group. In addition, the cholesterol concentration of egg yolk was lower ($p < 0.05$) in the 0.5% mushroom fed diet than the control group [54]. Foods rich in total omega-3 fatty acid (n-3), total omega-6 fatty acid (n-6), as well as polyunsaturated fatty acid (PUFA) are very helpful for health. A diet enriched withn-3 PUFA is considered to have preventative functions for people with vascular diseases [60]. Willis et al. [61] reported that adding *Lentinula edodes* mushroom mycelium extract to layer diets had no significant effects on laying performance. In a subsequent study, Willis et al. [62] further investigated that birds fed with fungus myceliated grain could successfully induce molting and allowed egg production earlier than the control group. This study concluded that fungus myceliated meal can be an effective alternative to conventional feed withdrawal methods, for the successful initiation of molt and maintenance of post-molt performance.

The mushroom, *Cordyceps militaris*, was used in layer diets to evaluate the performance and egg yolk cholesterol level by Wang et al. [57]. The results showed significantly lower ($p < 0.05$) egg cholesterol in mushroom fed groups with 1% and 2% levels than the control fed groups. In addition, improved ($p < 0.05$) FCR with greater ($p < 0.05$) egg weight were found at the 2% level mushroom fed group than in the control group. However, no significant differences were observed on the eggshell weight, egg yolk weight, shell thickness, and egg yolk color, among the treatment groups.

Lee et al. [13] used the spent mushroom (*Hypsizygus marmoreus*) substrates in layer ration to evaluate the feeding effects on egg production and table egg quality. None of the production performance parameters were affected by feeding mushroom *Hypsizygus marmoreus* during the entire study period. However, the egg yolk color scores were higher ($p < 0.05$) in mushroom fed groups than the control group. They concluded that fermented spent mushroom can be used, up to15% in layer ration, without affecting normal laying percent and table egg quality.

A wild medicinal mushroom, *Ganoderma lucidum*, was used for pullet performance in a study by Ogbe et al. [55]. No significant effects were observed for the feed intake by feeding *Ganoderma lucidum* mushroom in pullets. However, FCR was improved ($p < 0.05$) in mushroom groups than the non supplemented control group. Lee et al. [56] used *Pleurotus eryngii* mushroom to evaluate the performance in laying hens. Egg cholesterol level was lower ($p < 0.05$) in the mushroom groups than the control diets, however they did not observe any significant differences on egg production performance by feeding mushroom base diets in laying hens. The haugh unit was greater ($p < 0.05$) in the 1% and 2% experimental diet groups. The authors finally concluded that laying hens fed with the residue of *Pleurotus eryngii* mushroom could produce lower cholesterol in eggs.

4.2. Application of Medicinal Mushrooms on Health Status in Layer Chickens

Body immunity and inner organ weight are good indicator of health status in chickens. No significant difference was found for inner relative organ (liver, gizzard, spleen, and abdominal fat) weights between control and antibiotic fed diets [52], however, the bursa weight was higher ($p < 0.05$) in the mushroom fed diets than the control and antibiotic fed diets [52]. No effects on inner organ weights ensured that feeding mushroom did not have any toxic effects on pullet chickens, whereas the higher bursa weight ensured the improved immune status in experimental chickens. Higher bursa weight is an indicator of better health status and a sound physiological response to body immune system [63]. The appropriate level of immune sub-parameters such as immunoglobulin, cytokines, protein, and some biochemical index are important to maintaining the immune response in host. Antibody titers against Newcastle disease (ND), infectious bronchitis (IB), and Avian influenza (AI) virus vaccines were found to be higher ($p < 0.05$) in mushroom stem waste fed diets in pullet [52]. In addition, serum immunoglobulin parameters (IgA, IgG, and IgM) were found to be higher ($p < 0.05$) in mushroom fed diets than the control and antibiotic fed diets in the experimental pullet. Bai et al. [64] stated that serum immunoglobulin concentrations can generate humoral immune response in animals due to their important roles on immune function fighting against various infections. In addition, supplementation of β-glucan from edible mushroom had a significant immune stimulatory effect in chickens [65]. Similarly, the antibody titers against infectious bursal diseases virus were greater in mushroom *Ganoderma lucidum* fed groups than the control diets, in pullet [55]. In another study by Mahfuz et al. [66] antibody response on ND was greater ($p < 0.05$) in the 6% mushroom stem fed group and IB were greater ($p < 0.05$) in all levels of mushroom fed groups than both the positive and negative control diets, in laying hens. This study further demonstrated that the serum cytokines concentrations (IL-2, IL-6, IL-4, and TNF-α) were higher ($p < 0.05$) in mushroom feed groups than the control and antibiotic fed groups, in laying hens. The polysaccharides in medicinal mushrooms have strong immune modulatory activity and possess antioxidant activity that could enhance nonspecific and specific immune responses invitro [46]. In addition, cytokines are known to be regulators of the immune status. The function of IL-2 relies on the commencement of B and T lymphocytes cells. However, the activation of Th1 depends on secretion of IL-2, TNF-α, along with other cytokines that create the cellular immunity [67]. Similarly, Jarosz et al. [68] reported that the function of Th2 depends on IL-4, with other cytokines secretion that stimulates humoral immunity.

Lee et al. [13] found that the number of pathogenic bacteria, especially *Salmonella* spp., *E coli*, and *Clostridium* spp. were lower ($p < 0.05$) in mushroom (*Flammulina velutipes*) fed groups than the control diets, in laying hens. Similarly, Willis et al. [61] reported that adding *Lentinula edodes* mushroom mycelium extract in layer diets, could decrease the number of pathogenic bacteria *Salmonella* spp. in the

caecum and crop of birds fed with mushroom extracts. Lee et al. [56] used mushroom *Pleurotus eryngii* in layer ration. This study found that both the serum triglyceride and the serum cholesterol were lowered ($p < 0.05$) in mushroom fed groups than the control. Moreover, the dietary inclusion of dried mushroom at the 1% and 2% level showed greater ($p < 0.05$) serum antioxidant enzyme activities, in laying hens. This is due to a higher content of phenolic substance and different minerals, especially selenium, in mushrooms. Dietary supplementing selenium could enhance the body weight gain and antioxidant enzyme activities in chickens [69]. The author finally concluded that the residue of *Pleurotus eryngii* mushroom could improve the antioxidant status in layer chickens. Sun et al. [70] reported that edible mushrooms have a hypo-cholesterolemic effect on health and suggested its use as an oral medicine. The improved antioxidant status of chickens fed with different medicinal mushrooms was due to the presence of phenolic compounds, especially phenolic acid, which is the major naturally occurring antioxidant components found in mushrooms.

5. Conclusions and Future Perspectives

This review highlights that medicinal mushrooms could be fruitfully used as an effective natural growth promoter, as well as an immune boosting agent, in layer birds. In spite of the brood uses of medicinal mushrooms in layer diets, further studies by various researchers are recommended regarding the dosages of medicinal mushrooms on optimum performance and immune response, in laying hens. Therefore, future study should examine the use of medicinal mushroom in reaction to a pathogen challenge, as well as dosages. We suggest future research on medicinal mushrooms as alternates for antibiotics in laying hens so that it can be an effective strategy for organic egg production and encourage future researchers to discover the aspects of medicinal mushrooms that are important to immunity and health status that previous studies were not able to explore.

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animals

MDPI

Review

The Genus *Allium* as Poultry Feed Additive: A Review

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Simple Summary: The routine and unregulated use of in-feed antibiotics as growth promoters in poultry have been linked to the development of antimicrobial resistance, a serious global threat to the human, animal, and environment health. Growing public health concerns about food and environmental safety intensified the search for effective antibiotic alternatives in poultry production. The aim of this review is to present the current state of knowledge on the use of alliums as effective poultry feed additives in relation to their effects on growth performance, disease infections, gut and immune modulation, and product quality.

Abstract: The genus *Allium*, belonging to the family Amaryllidaceae has been known since ancient times for their therapeutic potentials. As the number of multi-drug resistant infections has increased due to in-feed antibiotic usage in poultry, the relevance of alliums as feed additives has been critically assessed. Garlic and the other *Allium* species, such as onions, leek, shallot, scallion, and chives, have been characterized to contain a plethora of bioactive compounds such as organosulfur compounds, polyphenols, saponins, fructans, and fructo-oligosaccharides. Consequently, alliums have been validated to confer antioxidant, antibacterial, antiviral, immunostimulatory, gut homeostasis, and lipid- as well as cholesterol-lowering properties in poultry. This review intends to summarize recent progress on the use of edible alliums as poultry feed additives, their beneficial effects, and the underlying mechanisms of their involvement in poultry nutrition. Perspectives for future research and limitations are also briefly discussed.

Keywords: *Allium*; feed additive; beneficial effects; organosulfur compounds; polyphenols; poultry

1. Introduction

The prolonged and unregulated use of antibiotics driven by a growing demand for animal products lead to the emergence of antibiotic resistance, a global threat to the animal and human health [1–3]. Poultry is the world's primary source of animal protein and it represents one of the highest consumers of antibiotics as growth promoters [3]. The European ban on sub-therapeutic use of antibiotics (1831/2003/EC, 2006) and the growing awareness among the consumers of the fatalistic effects of antibiotic resistance as well as residues in animal products intensified the hunt for effective in-feed antibiotic surrogates without affecting animal productivity or product quality [4–6]. However, the major challenges associated with antibiotic-free poultry production are poor growth performance, lower productivity, and increased morbidity as well as mortality in birds [7,8]. Many reviews shedding light on efficient and cost-effective antibiotic alternatives in poultry have been published in recent times [2,6,9–11]. Recently, plant-derived feed additives have gained considerable interest as sustainable substitutes in poultry diets [12,13]. An effective plant-derived additive in poultry (broilers, layers, and quails) is expected to stimulate feed intake, improve digestive enzyme secretions, activate

immune system, modulate gut microbiota, as well as have antibacterial, coccidiostatical, antiviral, antioxidant and/or anti-inflammatory activities [12–14]. In this context, *Allium* holds immense promise due to a variety of bioactive compounds including organosulfur compounds (OSCs), polyphenols, saponins, fructans, fructo-oligosaccharides (FOS), among many others. The genus *Allium* of the Amaryllidaceae family consists of ca. 850 species and represents one of the most studied plants of medicinal importance [15]. Extensive literature is available on the therapeutic properties of *Allium* spp. in humans, however, there is poor evidence in the poultry counterpart.

In the last three decades, alliums, in particular onion (*A. cepa*) and garlic (*A. sativum*), as well as garlic chives (*A. hookeri*) more recently have been reported to be incorporated into poultry diets to investigate their effects. However, the published literature on the effects of allium feeding in poultry have generated great inconsistency, making it impossible to draw a generalized conclusion on the efficacy of such feed additives. The discrepancies may be due to the heterogeneity of the composition of allium preparations, subject recruitment (broiler, layers, quails, etc.), dosage, duration of study, and so forth. This review combs the existing literature and gleans information to present an updated relevance of *Allium* spp. as effective poultry feed additives. We discuss the vast array of allium compounds in relation to their bio-functionalities. Emphasis was given to the dietary effect of *Allium* spp. on growth performance, infectious diseases, immunomodulatory properties, gut microbiota as well as gut morphology, and product quality in poultry. Moreover, this review discusses the lacunae to be surmounted for optimal application of alliums in poultry.

2. Overview of Major Bioactive Compounds in *Allium*

2.1. Organosulfur Compounds

The genus *Allium* is a rich source of organosulfur compounds (OSCs), which are one the main bioactive compounds of the plants [16,17]. The major OSCs in *Allium* spp. include allyl cysteines, S-alk(en)yl-L-cysteine sulfoxides (ACSOs), thiosulfinates, and sulfides in varying amounts [18]. The characteristic aroma in different *Allium* spp. are mainly associated with the different levels of ACSO precursor, namely alliin (S-allyl-L-cysteine sulfoxide; garlic and elephant garlic), methiin (S-methyl-L-cysteine sulfoxide; garlic, onions, leeks, and shallots), propiin (S-propyl-L-cysteine sulfoxide; shallots), and isoalliin (S-1-propenyl-L-cysteine sulfoxide; onions and shallots) [19,20]. The synthesis of the OSCs is depicted in Figure 1 and starts with the transformation of γ-glutamyl peptides into ACSOs by the action of γ-glutamyl transpeptidase and oxidase in the cytoplasm of plant cells. When the bulbs are cut or crushed alliin is transformed into the allicin (alkenyl alkene thiosulfinate) by the action of a vacuolar lyase, alliinase. Allicin immediately decomposes into diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), diallyl tetrasulfide (DATTS), dipropyl disulfide (DPDS), ajoenes, and vinyldithiins depending on their manufacturing process [21,22]. The direct catabolism of γ-glutamyl cysteine leads to the formation of water-soluble S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC) [23]. The OSCs and their transformation products are well-studied antimicrobial agents [24]. Several antimicrobial compounds have been extracted and identified from many spp. of *Allium* including garlic (*A. sativum* L.), onion (*A. cepa* L.), shallot (*A. ascalonicum* L.), elephant garlic (*A. ampeloprasum* L. var. *ampeloprasum* auct.), rosy garlic (*A. roseum*) [24], garlic chives (*A. hookeri*) [25], and wild garlic (*A. ursinum*) [26]. Although the antimicrobial mechanism of these compounds has not been well defined, it seems that it is associated with the inhibition of important thiol-dependent enzymatic systems (alcohol dehydrogenase, thioredoxin reductase, trypsin, other proteases, RNA and DNA polymerases) and antioxidant activity, which have a multiple inhibitory effect on the microbial cell [27–29]. The potent antimicrobial activities of OSCs is also related to the number of disulfide bounds, i.e., DATTS > DATS > DADS > DAS [30].

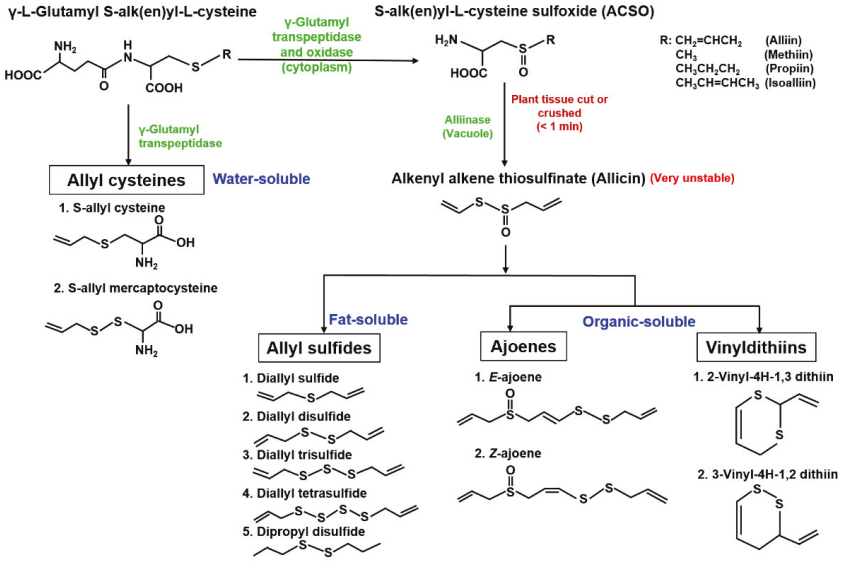

Figure 1. Major organosulfur compounds (OSCs) of *Allium* spp.

2.2. Polyphenolic Compounds

Another important class of bioactive compounds in alliums includes polyphenols [31,32]. The health-promoting activity of dietary polyphenols seems to be related to their antioxidant and anti-inflammatory activities [33]. Allium vegetables contain high levels of polyphenolic compounds, particularly phenolic acids, flavonoids, and their derivatives. *Allium* spp. are amongst the richest sources of dietary flavonoids [34]. Leighton et al. [35] found that flavonoid levels in the edible portion of allium vegetables (leeks, shallots, green onions, garlic, and onions) range from > 0.03 to 1 g/kg of vegetables. Flavonoids identified in onions were quercetin di-glucosides, quercetin 4′-glucoside, quercetin aglycone, and in some cases, isorhamnetin monoglucosides or kaempferol monoglucosides [36]. Quercetin glucosides of onion are more bioavailable than other quercetin-rich foods such as tea and apples [37]. The main phenolic acids found in alliums include *p*-Coumaric acid, ferulic acid, sinapic acid, gallic acid, and protocatechuic acid [38]. However, very few studies used allium flavonoids as feed additives to promote growth, immune, and antioxidant response for animals [39,40].

2.3. Saponins

Saponins are surface-active glycosides with triterpenoid or steroidal aglycone. Allium plants contain steroidal saponins, which are mainly divided into three groups based on their structure: spirostanols, furostanols, and open-chain (cholestane-type) saponins [41]. Saponin accumulation in the root organs is reported to be higher than in the aerial parts (stem and leaves) of alliums [42]. Until now, as many as 290 steroidal saponins (130 spirostanols, 140 furostanol, and 18 cholestane-type) have been identified in more than 40 different *Allium* species [41]. Allium saponins are not pungent and have many biological properties including antispasmodic, antifungal, haemolytic, anti-inflammatory, cholesterol-lowering, and cytotoxic activities. Moreover, saponins have the advantage of being more stable to food processing and cooking than the relatively unstable OSCs [43].

2.4. Fructans and Fructo-Oligosaccharides

Water-soluble fructans and fructo-oligosaccharides (FOS) together with glucose, fructose, and sucrose constitute the main non-structural carbohydrates in *Allium* species [44]. Fructans from various spp. of *Allium* including *A. cepa* (onion), *A. cepa* L. var. *ascalonicum* (shallot), *A. ampeloprasum* L. var. *porrum* (leek, 3 cvs.), *A. schoenoprasum* L. (chives), *A. sativum* L. (garlic), *A. fistulosum* L. (Japanese bunching onion/Welsh onion), *A. tuberosum* Rottl. ex. spr. (Chinese chives) have been characterized [44]. Several in vitro and in vivo studies witnessed the immunomodulatory [45–47], prebiotic [48], antiviral [49], and gastroprotective [50] effects of allium poly- and oligosaccharides. Lee et al. [46] reported the influenza A virus inhibitory activity of the fructan from *A. fistulosum* in an animal model and it was suggested to be mediated by host immune functions since the polysaccharide did not show any direct inhibitory effect on the virus replication in vitro. The immunomodulatory effect was attributed to promotion of phagocytosis, release of NO, and expressions of several immune-related cytokines [interleukin (IL), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ)] [47,48,51].

3. *Allium* spp. as Poultry Feed Additives

Literature search was conducted to accumulate the latest findings in implication of alliums as poultry feed additives and their role in growth performance, lipid metabolism, poultry infectious diseases, immunomodulation, gut modulation, and product quality (Table 1). The sections below outline the key components and mechanisms responsible for these functions.

3.1. Effects on Growth Performance

Several studies have documented the benefits of *Allium* spp. (in particular onion and garlic) on growth performance in poultry by improving weight gain, feed intake, and/or feed efficiency [52–54]. Farhani et al. [52] found that onion extract (1%) in drinking water improved growth performance and blood biochemical characteristics. They attributed the effect to the onion FOS, which might help in maintaining beneficial gut microorganisms and improve nutrient absorption. Goodarzi et al. [54] speculated that the OSCs of onion have increased nutrient absorption and thereby improved growth performance in broilers. In addition, onion in diet can reduce blood glucose stimulating the nervous system for higher feed intake, which can lead to increased weight gain [52,54].

The precise mechanisms behind the improved growth performance in poultry fed alliums remain unclear. However, some researchers have linked this improvement to the increased feed intake of allium supplemented diets [55]. Generally, garlic is used as seasonings to improve the flavor and hence it might improve the palatability of feed, thus increasing voluntary feed intake. Brzóska et al. [55] reported that garlic extract (2.25 mL/kg of feed) stimulated the appetite of chickens, which resulted in significantly greater feed intake and thereby higher body weight gains. Sheoran et al. [56] and Kirubakaran et al. [57] hypothesized that the improvement in weight gain of the birds using garlic in their rations may probably be due to allicin. Kirubakaran et al. [57] postulated that garlic in broiler diet may increase salivary flow rate and gastric juice secretion, resulting in improved digestibility and higher body weight. Negative effects on growth performance in broilers were also observed with the supplementation of 1 g of garlic powder/kg feed and 15 g of garlic bulb/kg feed [58,59]. The inclusion of alliums may reduce diet palatability due to their pungency and as a result the feed intake and body weight of animals decrease [58,60,61]. While Aji et al. [62] reported ineffectiveness of low doses (0.025 and 0.05 g of onion and garlic/kg feed) to produce any observable effects and suggested that the dosage of alliums as an important factor. However, several studies reported no significant effects on growth performance parameters such as feed intake, body weight gain, or feed efficiency in broilers by the dietary supplementation of alliums [14,60,62–64]. In the case of layers, most of the studies found no significant changes in performance when layer diets were supplemented with alliums [65–68]. Some researchers suggested that well-nourished healthy poultry reared under clean and ideal conditions,

often do not respond to growth-promoting supplements, while the stressed or challenged birds may give better results with the same supplements [49,66,67,69–72]. Intriguingly, Ao et al. [66] indicated garlic supplementation could increase growth performance in broilers by reducing the concentration of cortisol, the stress hormone. The variability in the efficacy of alliums on animal performance could also be attributed to the variation in the product fed, dosage, duration, and subjects used among the studies.

Table 1. Effect of dietary supplementation of *Allium* spp. in poultry.

Animals	*Allium* spp. & treatments	Formulation	Main active components	Effects	Ref.
Male Shaver Starbo broilers (7-d-old)	0.5 or 5 g *A. sativum*/kg feed; 8 weeks	Raw and boiled Powder	ND	↑ BWG; ↑ Oxidative stability of meat during storage	[64]
104 Mixed sex broilers (4-week-old)	*A. cepa* (0, 0.025, 0.05, or 0.1 g) and *A. sativum* (0, 0.025, 0.05, or 0.1 g)/kg feed; 3 weeks	Powder	ND	↑ BWG; ↑ Feed and water intake; ↑ FCR	[62]
320 Ross-308 broilers (1-d-old)	1.5, 2.0, or 2.5 g *A. cepa*/kg feed; 6 weeks	Powder	ND	↑ Body weight; ↑ Feed intake; ↑ *Lactobacillus* and *Streptococcus*; ↓ *E. coli*; ↑ Intestinal histomorphology	[53]
60 Ross broilers (1-d-old) (Challenged by live *Eimeria acervulina*)	0.01 g Garlicon40® (*A. sativum*)/kg feed; 20 days	Powder	67% Propyl thiosulfinate and 33% propyl thiosulfinate oxide	↑ BWG; ↓ Fecal oocyst shedding; ↑ *E. acervulina* profilin antibody responses	[73]
144 Male Cobb broilers (1-d-old)	0.045 or 0.09 g Proallium-SO-DMC® (*A. sativum*)/kg feed; 3 weeks	Powder	11.3% Propyl propane thiosulfonate	↑ Body weight; ↓ Feed intake; ↓ Feed/gain ratio; ↓ Enteropathogens; ↑ Ileal histological structure	[74]
144 Male Cobb broilers (1-d-old)	0.045 or 0.09 g Proallium-SO-DMC® (*A. sativum*)/kg feed; 3 weeks	Powder	11.3% Propyl propane thiosulfonate	↑ Nutrients digestibility; Modulate intestinal microbiota	[75]
300 Male Arbor Acres broilers (1-d-old)	10–50 g *A. sativum*/kg feed; 5 weeks	Powder	ND	↓ Total serum cholesterol levels; ↓ LDL-C; ↑ HDL-C; ↑ Meat quality (↑ Color stability; ↓ TBARS; ↑ Total unsaturated fatty acid:total saturated fatty acid ratios)	[63]
150 Hy-Line Brown layers (50-week-old)	10–50 g *A. sativum*/kg feed; 5 weeks	Powder	ND	↑ Haugh Unit; ↓ Total serum cholesterol; ↓ Total egg yolk cholesterol	[76]
24 Mixed sex broilers (1-d-old)	15 or 30 g *A. sativum*/kg feed; 8 weeks	Powder	ND	Regulate lipid metabolism (↓ Plasma cholesterol; ↓ triglycerides; ↓ VLDL-C; ↓ LDL-C; ↑ HDL-C)	[77]
150 Male Ross-708 broilers (1-d-old) (Challenged by LPS	10 g or 50 g *A. hookeri*/kg feed; 8 days	Powdered fermented roots	ND	↑ Body weight; ↓ Inflammatory response (↑ Expression of intestinal tight junction proteins and mucin; ↓ Serum α-1-AGP; ↓ Pro-inflammatory cytokines)	[49]
125 Male Ross-708 broilers (1-d-old)	10 g *A. hookeri*/kg feed; 3 weeks	Powdered roots	ND	↑ Body weight; ↑ antioxidant activity (↑Gene expression of heme oxygenase, aflatoxin B1 aldehyde reductase, SOD 1, and CAT; ↑Serum levels of SOD, CAT, and MDA)	[70]
300 male Ross-708 broiler chicks (1-d-old) (Challenged by NE)	10 g or 30 g *A. hookeri*/kg feed; 20 days	Powdered roots	ND	↓ Loss of BWG; ↓ Lesion; ↓ Fecal oocyst shedding; ↑ Innate immunity (↓ Pro-inflammatory cytokines; ↑ Expression of tight gut junction proteins and mucin)	[71]
500 Arbor Acres broiler chicks (1-d-old)	1–4 g *A. sativum*/kg feed; 5 weeks	Fermented powder	ND	↑ WBC, lymphocyte and IgG; ↓ Total cholesterol; ↓ Triglyceride; ↓ LDL-C; ↓ Cortisol; ↑ Meat quality (↓ TBARS and pH)	[66]
600 White mini broiler chicks (1-d-old)	3 or 5 mL *A. cepa*/kg feed; 5 weeks	Fermented liquid	ND	↑ Final body weight; ↑BWG; ↓ Serum cholesterol; ↓ Triacylglycerol	[78]
400 Male Ross-308 broiler chicks (3-d-old)	5, 7.5, or 10 g *A. cepa*/kg feed; 4 weeks	Liquid extract	ND	↑ BWG; ↑ Feed intake; ↑ ATTR; ↑ Serum IgG; ↓ TBARS	[79]

Table 1. *Cont.*

Animals	*Allium* spp. & treatments	Formulation	Main active components	Effects	Ref.
240 Cobb-400 broiler chicks (1 day old)	10 g *A. sativum*/kg feed; 6 weeks	Powder	ND	↑ BWG; ↑ Final weight; ↑ Villi length and width; ↑ Cryptal depth	[80]
288 commercial broiler chicks (1-d-old)	5 or 10 g *A. sativum*+1 or 2 g *Piper nigram*/kg feed; 6 weeks	Powder	ND	↑ BWG; ↑ Final weight; ↑ FCR	[57]
36 layers of six different strains (Hisex Brown, Isa Brown, Lohmann, Starcross, Babcock, and Starcross-579 strains) (28-week-old)	20, 40, 60, 80, or 100 g *A. sativum*/kg feed; 6 weeks.	Paste (sun-dried)	ND	↑ Egg production; ↑ Egg yolk weight; ↓ Egg yolk cholesterol; ↓ Serum cholesterol	[81]
162 SHSY-type brown layers (21-week-old)	5 or 10 g *A. sativum*/kg feed; 22 weeks	Powder	ND	↑ Egg weight; ↓ Egg yolk cholesterol; ↓ Serum triglyceride and cholesterol	[82]
300 Quails (9-week-old)	5 or 10 g *A. sativum*/kg feed; 21 weeks	Powder	ND	↑ Egg weight; ↓ Egg yolk cholesterol; ↓ Serum cholesterol	[68]
72 Dekalb white layers (30-week-old)	30 or 50 mg *A. sativum*/kg feed; 7 weeks	Powder	ND	↑ Albumen height; ↑ Haugh units; ↑ Egg and albumen weight	[67]
120 laying Japanese quails (10-week-old)	10, 20, or 40 g *A. sativum*/kg feed; 12 weeks	Powder	ND	↓ Egg yolk cholesterol; ↓ Serum cholesterol	[83]
240 Isa brown laying hens (41-week-old)	10, 20, or 40 g *A. sativum*/kg feed; 5 weeks	Fermented powder	ND	↑ Yolk height and color; ↑ Haugh Unit; ↓ Serum total cholesterol; ↑ Yolk PUFA:SFA ratio	[69]
108 laying hens (30-week-old)	5 or 10 g *A. sativum* and 10 g *A. cepa*/kg feed; 4 weeks	Powder	ND	↑ FCR; ↑ Egg production; ↑ Egg weight; ↓ Total cholesterol; ↓ Creatinine	[84]
180 Isa Brown hens (18-week-old)	10, 20, 30, 40, or 50 g *A. sativum*/kg feed; 20 weeks	Paste (Raw)	ND	↑ HDL-C; ↓ Total cholesterol; ↓ Yolk cholesterol; ↓ LDL-C	[65]
640 Mixed sex Ross 308 broiler chickens (body weight 45 ± 7 g)	1, 1.5, or 2.25 mL/kg feed; 6 weeks	Liquid	ND	↓ Mortality; ↓ FCR; ↑ EPEF; ↑ Dressing percentage; ↑ Weight of breast muscles; ↑ Liver weight; ↑ Protein and crude ash content of breast meat; ↑ Total protein content of serum	[55]

ND: Not determined; BWG: Body weight gain; FCR: Feed-conversion ratio; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol; VLDL-C: Very low-density lipoprotein-cholesterol; LPS: Lipopolysaccharide; α-1-AGP: Alpha-1-acid glycoprotein; SOD: Superoxide dismutase; CAT: Catalase; MDA: Malondialdehyde; NE: Necrotic enteritis; WBC: White blood cells; IgG: Immunoglobulin G; TBARS: Thiobarbituric acid reactive substances; ATTR: Apparent total tract retention of nutrients; PUFA: Polyunsaturated fatty acid; SFA: Saturated fatty acid; EPEF: European production efficacy factor.

3.2. Hypolipidemic and Hypocholesterolemic Effects

Alliums (especially garlic) have a traditional place in folk medicine as hypolipidemic and hypocholesterolemic agents in many cultures. Elevated blood cholesterol and triacylglycerides in animal proteins are associated with the increased risk of cardiovascular disease in humans. Several researchers have opined that garlic exhibited hypocholesterolemic effects in poultry including broilers, layers, and quails through the inhibition of key enzymes such as malic enzyme, fatty acid synthase, glucose-6-phosphate dehydrogenase, and 3-hyydroxy-methyl-glutaryl-CoA (HMG-CoA) reductase involved in cholesterol and lipid synthesis [81,83,85–87]. Allicin is thought to be the potentially active component [81,87]. However, Lanzotti et al. [43] reported that allicin is very unstable and not present in intact garlic or in any garlic products. Moreover, the acidity of the stomach prevents the formation of allicin [88]. Some researchers attributed the cholesterol-lowering effect of garlic to the steroidal saponins possibly by inhibiting cholesterol absorption in the intestine or a direct effect on cholesterol metabolism [41]. The exact mechanism by which garlic reduces plasma cholesterol concentration is remaining elusive.

3.3. Effects on Infectious Diseases

Recently, allium derived feed additives have been given useful results against several infectious diseases in broilers such as the infection of *Escherichia coli*, *Salmonella*, *Clostridium perfringens*, and *Eimeria* [27,71,72,89–91]. Elmowalid et al. [89] reported that garlic dietary supplementation for three weeks provided in vivo protection against multi-drug resistant *E. coli* O78 challenge in broilers by reducing the mortality rates to >10% from 60% (control, non-supplemented birds). The authors suggested that the bioactive phenolic and non-phenolic compounds in garlic are responsible for this effect. Lee et al. [71] reported less loss of body weight gain, decreased lesion score, and oocyst shedding by *A. hookeri* dietary supplementation in necrotic enteritis (NE) challenged (*Clostridium*/*Eimeria* co-infection) commercial broilers. Another in vivo study with the dietary supplementation of two garlic metabolites (10 ppm) namely propyl thiosulfinate (PTS) and propyl thiosulfinate oxide (PTSO) revealed increased body weight gain, decreased fecal oocyst excretion, a higher profilin antibody response, and greater spleen cell proliferation in *E. acervulina*-infected chickens as compared with the infected birds fed a non-supplemented control diet [73]. Ali et al. [91] also reported that the dietary supplementation of garlic powder (15g/kg of feed) reduced oocysts shedding and lesion score as well as lowering mortality, and improved histopathology of the small intestines in the supplemented group. They ascribed these effects to the presence of allicin and phenolic compounds in garlic. The allicin has antioxidant and antiparasitic activity which directly kill the sporozoites [73]. The phenolic compounds in garlic act on the cytoplasmic membrane of *Eimeria* and make changes in their cation permeability, leading to the death of pathogens [91].

Salem et al. [27] assessed the efficacy of garlic extract (40 mg/mL) in experimentally *S. typhimurium* induced salmonellosis in Cobb broiler chicks. The garlic extract used in the study contained allicin, alliin, allylsulfide, E-ajoene, and vinyldithiin. The mortality rate was decreased from 53.3% to 13.3% after treatment with garlic extract. The body weight of the infected chickens was significantly improved with the treatment of garlic extract when compared with infected non-treated groups. The post-mortem lesions were less severe in the garlic-treated infected chicks as compared with control infected chicks. The authors suggested the efficacy of garlic against multidrug resistant *Salmonella* by reducing its invasion, resistance to antimicrobial agents, and biofilm formation ability. Jimoh et al. [90] reported that garlic at the various supplementation levels reduced the caecal load of *C. perfringens* as compared with the control group and attributed to the OSCs.

Kavindra and Shalini [92] reported in vitro anthelminthic potential of garlic oil (2%, 4%, and 6%) against *Ascaridia galli* diseases in poultry birds. Mechanistically, the garlic oil reduced significantly the glucose uptake, glycogen content, oxygen consumption, and relative activity of acid and alkaline phosphomonoesterases in the parasite. However, an in vivo study by Velkers et al. [28] failed to observe efficacy of allicin from garlic against experimentally induced *A. galli* infection in chickens with no significant effect on worm load. Shojai et al. [93] observed an inhibitory effect of garlic extract against infectious bronchitis virus in specific pathogen-free (SPF) embryonic eggs and they suggested that the garlic extract could have an effect on the virus in replication phase. From the above discussion, it can be inferred that allium compounds at a certain inclusion rate can alleviate the negative effects of infections in chickens and mediate multiple disease-related signaling pathways.

3.4. Effects on Intestinal Microbiota and Morphology

The gastrointestinal tract of poultry harbors complex assemblages of microorganisms (microbiome) mainly dominated by the phyla Firmicutes (*Lactobacillus*, *Streptococcus*, *Bacillus*, *Enterococcus*), Bacteroidetes (*Bacteroides*, *Bifidobacterium*), Proteobacteria (*Escherichia*, *Salmonella*, *Campylobacter*, *Shigella)* and Actinobacteria [94]. This gut microbiome is recognized as a key player in governing host growth performance and health by providing nutrients from indigestible dietary substrates, competitive exclusion of pathogens, detoxification, strengthening the gut barrier, and modulation of immune system [95,96]. Pan and Yu [97] suggested an intertwined relationship of the gut microbiome with poultry host and diet. Therefore, any perturbation in the taxonomic composition of gut

microbiota (called dysbiosis) may underlie its contribution to symptoms of a disease condition like that in humans. Recently, few studies strengthened the applicability of alliums (mainly garlic and onion) as poultry feed additive in the gut microbiota modulation in regard to diversity and composition [53,54,74,75,80,98]. Supplementation of onion showed a significant reduction in the population of *E. coli* and increased significantly *Lactobacillus* and *Streptococcus* species. Similarly, Goodarzi et al. [99] and Shargh et al. [100] also reported higher *Lactobacilli* spp. and reduced *E. coli* load in ileum of onion fed broilers. Shin et al. [101] hypothesized that the phylum Proteobacteria may potentially serve as biomarker for gut dysbiosis in humans. Intriguingly, Kim et al. [102] demonstrated that lower numbers of certain gut pathogens such as *E. coli* may improve broiler performance. Sheoran et al. [56] and Kirubakaran et al. [57] also indicated that the lower *Staphylococcus aureus* and *E. coli* as well as aflatoxins producing fungi in the intestine fostered nutrient digestibility which in turn improve weight gain of the birds.

Allicin has also been reported to improve and regenerate the physiological structure of the intestinal epithelium layer, and enhance crypt depth and villus height, which ultimately support its digestive capacity through increased absorption of nutrients and assimilation [103]. However, the instability and poor bioavailability of allicin question its effects in vivo [23]. Ur Rahman et al. [53] observed that onion supplementation significantly increased dimensions (villus height, width, crypt depth, and surface area) of duodenum, jejunum, and ileum. The authors hypothesized that larger intestinal villi are associated with higher absorption of the nutrients and reduction of *E. coli* in the intestine. Mehmood et al. [104] also reported that supplementation of onion in the feed significantly increased villus height, crypt depth, and surface area of the jejunum in broilers.

Karangiya et al. [80] indicated that garlic supplementation (10g/kg feed) increased the absorptive surface area of the intestine (villus height, width, and crypt depth) and correlated with the higher body weight gain in broilers. Diets containing garlic-derived propyl propane thiosulfonate (PTS-O) (0.045 and 0.090 g/kg feed) has also been shown to improve absorption surface at the ileal level in broilers [74]. In an extended study, Peinado et al. [75] observed a decrease in the numbers of enterobacteria, in particular lactobacilli and an increase in bacteroides in the broiler intestine with the dietary supplementation of PTS-O (0.045 and 0.090 g/kg feed). Although generally regarded as a beneficial group, the higher number of lactobacilli is linked to the impairment in fat digestion or absorption in poultry due to their bile-deconjugation activity [105,106]. Thomas et al. [107] suggested that the higher bacteroidetes was responsible for the improved performance in chickens since bacteroidetes are involved in fermentation of high molecular weight carbohydrates, activation of T-cell mediated immune responses, prevention of potential pathogens, bile acid metabolism, and transformation of toxic and/or mutagenic compounds. Likewise, Ruiz et al. [98] observed lower diversity indices of ileal mucosa-associated microbiota in chickens fed the PTS-O–supplemented diet, which was ascribed to the bactericidal effect of PTS-O against enterobacteria, coliforms, *E. coli*, *C. jejuni*, and *Salmonella* spp., as also observed by Peinado et al. [75]. In addition, PTS-O was able to significantly increase and modulate the composition of bifidobacteria in growing broilers; which are considered as excellent candidates of probiotics in broilers [108]. Another study involving PTS-O supplementation indicated negative correlations between relative abundances of *Escherichia–Shigella* or enterobacteria (crop, ileum and caeca) and growth performance as well as fat digestibility in PTS-O fed broilers [109]. When garlic extract (0.04 or 0.06 g/kg feed) was gavaged to broilers reduced number of *E. coli* and *Staphylococcus aureus* in the ileo-caecal digesta and improved nutrient digestibility were observed [110]. Kırkpınar et al. [111] reported that garlic oil alone or in combination with oregano, reduced *Clostridium* counts in the ileum of broilers. However, total organism, *Streptococcus*, *Lactobacillus* spp., and coliform counts were not affected by the dietary treatments. The lower *Clostridium* counts were ascribed to the antibacterial effects of essential oils.

Notwithstanding the fact that specific mechanistic studies how dietary alliums affect chicken gut health and physiology are limited; it is clear from the above-cited findings that alliums participate in gut homeostasis to foster an intestinal environment conducive to commensals by reducing the expansion

of pathogenic microorganisms. However, a better understanding of the gut/microbe interactions and gut microbial diversity using next generation sequencing will provide new opportunities for the improvement of poultry health and production.

3.5. Effects on Immune Response

Poultry diet and nutrition are critical determinants of birds' immune response. Several studies have advocated disease prevention or immune enhancing effects of alliums in poultry, however, very few studies investigated the underpinning mechanisms for their specific immunomodulatory effects. For instance, Kim et al. [73] investigated the effects of two garlic secondary metabolites (10 ppm) namely PTS and PTSO on the in vitro and in vivo parameters of chicken gut immunity during experimental *E. acervulina* infection. In vitro, PTSO/PTS treatment dose-dependently killed invasive *E. acervulina* sporozoites and stimulated splenocyte proliferation. In vivo feeding of PTSO/PTS provided increased protective immunity following live *E. acervulina* challenge infection, as indicated by improved bodyweight gains, reduced fecal oocyst shedding, and higher anti-profilin serum antibody titers, compared with the non-supplemented controls. In PTSO/PTS-fed birds, microarray hybridization identified 1227 transcripts, whose levels were significantly altered (552 up-regulated and 675 down-regulated) in the intestinal intraepithelial lymphocytes (IEL) involving immune- and cardiovascular-related gene pathways and networks. The authors observed a simultaneous and interactive effects of PTSO/PTS dietary supplementation on adaptive (increased splenocyte proliferation and anti-prolifin titers) and innate immunity [downregulation of toll-like receptors (TLR) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)] in chickens against coccidiosis.

Hanieh et al. [112] reported that the dietary alliums (garlic and onion) have a potential to enhance the humoral immune functions in White Leghorn chickens following immunization with Newcastle disease virus (NDV), sheep red blood cells (SRBC) and *Brucella abortus* (BA). The authors observed that alliums (10 g/kg feed) enhanced anti-NDV, anti-SRBC, and anti-BA antibody production, which might be due to increased CD4/CD8 cells, following immunization. Moreover, the relative weight of spleen and thymus were increased in case of garlic supplementation, which was ascribed to the enhanced lymphocyte proliferation and the increase in WBC counts. The mechanism of improved humoral immune functions by the dietary alliums against three antigens was further delineated by a subsequent in vitro study on the lymphocytes and peritoneal macrophages from white Leghorn chickens (male) [113]. The authors observed that garlic and onion extract augmented concanavalin A (ConA)-induced splenocyte and thymocyte proliferations, and gene expression of IL-2 and IFN-γ as well as higher microbicidal activity and reactive oxygen species production in macrophages. They speculated different mechanisms of immune modulation by garlic and onion. Garlic had a direct stimulatory effect on the immune cells, whereas onion had an indirect stimulatory effect, the antioxidant activity of high flavonoids of onion may be a plausible explanation [112,113]. In contrast, Jafari et al. [114] and Goodarzi et al. [54] failed to report any significant effect on antibody titers against NDV in garlic and onion fed broilers, respectively.

Another study investigated the effect of dietary supplementation of *A. hookeri* on the inflammatory immune activities in the jejunum during the immunological stress induced by *Clostridium/Eimeria* co-infected commercial broilers [71]. The authors observed downregulated expression of pro-inflammatory cytokines such as IL-1β, IL-8, IL-17A, inducible nitric oxide synthase (iNOS), and lipopolysaccharide induced TNF-α (LITAF) in jejunum of NE challenged group by the dietary supplementation of *A. hookeri* as compared with control. In addition, the dietary supplementation of *A. hookeri* significantly increased expressions of tight junction (TJ) proteins [junctional adhesion molecule (JAM), occludin, and zonula occludens 1 (ZO1)] and intestinal mucin 2 (MUC2). These proteins play crucial roles in the regulation of intestinal permeability and barrier function [49].

Garlic dietary supplementation modulated chicks' innate immune response via various mechanisms including phagocytosis augmentation, bactericidal activity enhancement and nitric

oxide (NO) production reduction, together with triggering the IL-1β, IL-6 and IFN-γ cytokines expression levels in comparison with the non-supplemented chicks against multi-drug resistant *E. coli* O78 challenge [89].

Lee et al. [49] reported that the dietary supplementation of *A. hookeri* promoted gut integrity and enhanced innate immunity during an immunological stress induced by lipopolysaccharide (LPS) in young broiler chicken. They observed decreased levels of alpha-1-acid glycoprotein (α-1-AGP), a marker for systemic non-specific inflammation or gut barrier health. In LPS-challenged groups, chickens fed diets supplemented with *A. hookeri* (1 or 3g/kg feed) exhibited lower transcript levels of pro-inflammatory cytokines (IL-1b, IL-8, TNF superfamily member 15, and LITAF) as compared with the non-supplemented fed chickens. Furthermore, the dietary supplementation of *A. hookeri* significantly upregulated the expression levels of TJ proteins (JAM, occludin, and ZO1) and MUC2.

Garlic powder and holy basil leaf powder either alone or in combination in the broiler's diet have potent immune modulating activity by showing a stimulatory effect on relative mRNA expression of TLR 2, TLR 4, and TLR 7 in the commercial broilers [56]. However, Toghyani et al. [14] reported no influence of dietary garlic (4g/kg feed) on immune-related parameters such as antibody titers, lymphoid organs' weight, albumin to globulin ratio, and heterophil to lymphocyte ratio in broilers and speculated that a higher dose is required to elicit any immune response. Indeed, the aforementioned data related to the immune effects of dietary alliums in poultry form the basis of further studies involving the mechanisms of molecular signaling and immune response initiation. Therefore, in a more long-term perspective, the assessment of variation among immune system components of poultry in response to allium supplementation will offer a better understanding of nutritional immunomodulation to reduce risk and manage field infections.

3.6. Effects on Product Quality

Dietary strategies are valuable options to improve nutritional value as well as oxidative stability and sensory properties of poultry meats and eggs. The antibacterial, anticoccidial, antifungal, antiviral, and antioxidant activity, as well as the immune-enhancing activity of allium-derived compounds have garnered attention in improving the poultry product quality. The plasticity and extraordinary responsiveness of poultry eggs to dietary factors make them the most attractive targets for nutrition modulation [115]. Several studies reported the use of alliums toward the improvement of egg quality [67,69,81–83,85]. Ao et al. [69] found a better fatty acid profile in egg yolk with higher poly-unsaturated fatty acid and lower saturated fatty acid by the dietary garlic (30 g/kg feed). Damaziak et al. [116] indicated that administration of the dietary onion extract to hens resulted in heavier eggs, with a higher content of egg yolk and better quality of albumen. The genus *Allium* has an exceptional ability to absorb, metabolize, and store selenium as organoselenium compounds such as selenomethionine and selenocysteine [117]. Olobatoke and Mulugeta [67] had given the possible explanation to the increased egg weight in laying hens is the absorption of garlic compounds (selenomethionine and selenocysteine) and their subsequent deposition in the egg yolk. Additionally, alliums are rich source of polyphenols (gallic acid, ferulic acid, quecetin, kaempferol, and flavonoid glycosides), potent antioxidants. The Haugh unit, albumen height, and pH are the indicators of the freshness of eggs, which tends to decrease during storage. Lim et al. [76] reported that with the dietary garlic in layers, the Haugh unit was improved during storage possibly due to the antioxidant effect from allicin and organoseleniums. Allicin inhibits the formation of superoxide by the xanthine/xanthine oxidase system, probably via a thiol exchange mechanism [118]. Wakebe [119] reported that the inclusion of selenomethionine in the layer diet (0.3 ppm/kg feed) resulted in higher Haugh units, which was ascribed to increased glutathione peroxidase activity in the egg yolk and white. Mahmoud et al. [85] proposed another explanation in that the garlic enhances the egg's antioxidant status by upgrading the glutathione peroxidase activity in yolk and albumen; this thereby increased egg quality during storage with better albumen height, Haugh unit, and pH probably because of less lipid and protein oxidation. In an organoleptic assessment, Motozono et al. [120] reported an off flavor in eggs with garlic dietary

supplementation (20 g crushed garlic/kg feed) in layers, while Birrenkott et al. [121] and Olobatoke and Mulugeta [67] reported no differences in color and flavor in eggs from hens consuming up to 30 g dietary garlic powder per kg feed. Damaziak et al. [116] indicated that the effect of dietary allium on the taste of eggs may be determined by both supplementation level and duration.

The inclusion of alliums in poultry diets has also been reported to improve color stability, fatty acid composition [63], sensory properties [14,111], and the anti-oxidative ability of meat [64,66,79]. Choi et al. [63] reported the color stability of meat (highest redness and yellowness) by the incremental levels of garlic powder (1–5%) dietary supplementation and the effect was ascribed to the reduced metmyoglobin formation and oxidation in thigh muscle of chicks. The same study observed better fatty acid profile in garlic supplemented groups by protecting the oxidation of unsaturated fatty acid. However, Abdullah et al. [122] reported no effects of garlic supplementation on the meat quality such as cooking loss percentage, shear force, color coordinates). The improved anti-oxidative capability of chicken meat by the dietary allium supplementation was attributed to the accumulation of antioxidant compounds such as flavonoids and OSCs [66,79]. Indeed, the utilization of dietary alliums to improve the quality of poultry products should be done carefully because high doses of the allium may reduce overall acceptability with altered taste and odor.

4. Factors Determining the Effectiveness of Alliums in Poultry Feed

Several variables need to be considered while recognizing the efficacy and safety of alliums in poultry (Table 2). From the above-cited studies, it is evident that six different kinds of allium formulations were mainly used in poultry, i.e., powder (sun- or air-dried), juice, purified extract, oil, aged extract, and paste. These processed alliums contain a variety of OSCs (major bioactive constituents) which differ greatly from their intact forms, depending on their manufacturing process. Most of these preparations were not chemically characterized and thus cannot be generalized under a single umbrella to have a biological response in poultry. Thiosulfinates, the most bioactive OSCs, are volatile and can evaporate rapidly, leading to largely varied final concentrations in the feed [123]. The pungent smell of thiosulfinates [21] might also affect the feed palatability, depending on the applied dosage. In the published literature, the inclusion rates of alliums in poultry have been reported to be very wide ranging from 0.001% to 10% (Table 1). For instance, Aji et al. [62] reported ineffectiveness of a low dose (25 and 50 mg of allium/kg feed) of onion and garlic supplementation while Varmaghany et al. [58] indicated negative effects of a high dose of garlic supplementation. Therefore, identification of an optimal dosage of alliums will also determine its effectiveness in poultry. Fujisawa et al. [124] reported that thiosulfinates might lose antimicrobial activity by reacting with sulfhydryl (SH) compounds of other feed components (proteins). The thermally unstable nature of allium bioactive constituents [16,20] also affects their application in feed production, since thermal processing is an important step to decontaminate the harmful microorganisms of feed. The OSCs have poor water solubility [125], which further limits their application in feed. While considering these factors, the higher cost of allium-based feed cannot be overlooked. Apart from these, the poultry responses might also be affected by various factors such as the feed type (pellet or mash) and quality, duration of study, hygiene, subject recruitment (broiler, layers, quails, etc.), age, health status (healthy or challenged) and environmental factors among many others [126]. Indeed, without proper standardized formulation, in practice the choice of an economically feasible allium-based feed additive is compromised in poultry diets.

Table 2. Factors determining the effectiveness of alliums in poultry.

Items	Characteristics	Effects
Thiosulfinates	Volatile	Varied final concentration in feed
	Pungent smell	Feed palatability
	Reacts with SH groups of other feed constituents	Loss of antimicrobial activity
	Thermal instability	Difficult in feed processing
	Poor water solubility	
Formulation	Variation in chemical constituents	Variation in biological response
Dosage	Pungent smell	Feed palatability
Other factors	Subjects (broilers, layers, quails, etc.), age, health status, feed type and quality, environmental conditions, and duration of study	Inconsistent results

5. Fortification/Preservation of *Allium* Bioactivity

The instability and volatility of allium bioactive compounds prompted animal nutritionists to devote intensive efforts in the search for new stabilization techniques that could ensure feed safety and quality as well as enhance modern preservation methods in the feed industry.

5.1. Fermentation

Fermentation has significantly improved bioactivities and organoleptic properties of alliums (Table 3). Allium fermentation resulted in higher polyphenolic content via the deglycosylation of complex phenolic glycosides to their simpler derivatives by the action of glucosidases, thereby increasing their antioxidant activity as well as bioavailability [127–129]. Fermentation could also reduce the pungent smell of alliums and hence expected to improve the palatability of feed [128–130]. Furthermore, fermented alliums can act as a viable source of probiotics and provide host health benefits [131]. Bernaert et al. [132] hypothesized that fermentation can be used as a stabilization technique for the preservation of antioxidant activity in *A. ampeloprasum* var. *porrum*. Hossain et al. [133] reported an increase in feed intake with the fermented garlic supplementation as compared with the control diet in broilers. Thus, the allium 'probiotication' may offer a cost-effective approach in the manufacture and storage processes of a feed additive by extending shelf-life and maintaining desired sensory properties in addition to the host health benefits.

Table 3. Fermentation of *Allium* spp. with respect to compositional changes and bioactivities.

Plants	Microorganisms	Fermentation Conditions	Compositional Changes	Study	Biological Activities	Ref.
A. sativum	*Saccharomyces cerevisiae*; *Lactobacillus plantarum*; *Mimulus pilosus*	*S. cerevisiae* and *L. plantarum* at 25 and 37 °C, respectively, for 48 h and *M. pilosus* at 25 °C for 7 days	↑ S-allyl-l-cysteine and cycloalliin; ↓ γ-Glutamyl peptide	-	ND	[130]
A. sativum	*L. plantarum*	37 °C for 24 h	Alliin content ↓; ↑ Cycloalliin content; ↑ S-allyl cysteine content	In vivo (mice model)	Lipid metabolism and antioxidant	[134]
A. sativum	Spontaneous	40 days at 60–70 °C and 85–95% relative humidity	↑ Polyphenol content	In vitro	Antioxidant	[135]
A. cepa	*Aspergillus kawachii* crude enzyme extract	30 °C for 24 h	↑ Quercetin and quercetin-3-glucoside; ↓ quercetin-3,4′-diglucoside and -4′-glucoside	In vitro	Antioxidant and neuroprotection	[136]

Table 3. *Cont.*

Plants	Microorganisms	Fermentation Conditions	Compositional Changes	Study	Biological Activities	Ref.
A. cepa	Spontaneous	37 °C for 3.5 days	Flavonoid profile changed	In vitro	Antibacterial, antigenotoxic, and antiproliferative	[137]
A. cepa	*Aspergillus oryzae*, *Bacillus subtilis*, *L. plantarum*, and *S. cerevisiae*	30 °C for 3 days	↑ Isoquercitrin	In vitro		[138]
A. cepa	Spontaneous	96–108 h with or without 1% (*w*/*v*) salt	↑ Lactic acid and acetic acid; ↓ amino acids; ↑ esters, alcohols, and aldehydes	In vitro	Flavor	[139]
A. cepa	*L. plantarum*	19 °C for 48 h	↑ Quercetin diglucoside	In vitro	Antioxidant	[140]
A. ampeloprasum var. *porrum*	*L. plantarum*, *Leuconostoc mesenteroides*, and *Lactobacillus sakei*	18 °C for 3 weeks	ND	In vitro	Sensory properties	[141]
A. ampeloprasum var. *porrum*	Spontaneous	18 °C for 21 days	↑ Polyphenol; ↓ methiin and isoalliin	In vitro	Antioxidant	[142]
A. tuberosum	*L. mesenteroides*	30 °C for 3 days	↑ Polyphenol	In vitro	Antibacterial and antioxidant	[127]
A. chinense	*L. plantarum*	37 °C for 7 days	↑ Polyphenol content; ↑ Free amino acid content; ↓ Sulfur containing compounds	In vitro	Antioxidant and flavor	[128]

5.2. Microencapsulation/Nanotechnology

Microencapsulation is one of the most effective approaches for protecting bioactive compounds against oxidation, heat and evaporation, controlled delivery, uniform distribution, storage stability, masking off-flavours, and extending the shelf life without affecting their physical, chemical or functional properties [143]. Milea et al. [143] and Akdeniz et al. [144] successfully encapsulated phenolic compounds extracted from onion skin. Piletti et al. [145] reported that β-cyclodextrin encapsulation of garlic oil increased thermal stability and water solubility, as well as preserved antimicrobial activity. However, the use of microencapsulation is based on several factors, including feasibility, practicability, and cost [146].

Nanoparticles can be used as possible feed supplements for poultry to improve overall health and feed conversion ratio [147]. The formulation of plant-derived bioactive compounds using nanotechnology may result in their improved activity at low dosage [148]. Sundari et al. [149] reported turmeric extract nanoparticle as a feed additive which improved meat quality at low dosage without affecting performance in broilers. Xu et al. [125] converted natural organosulfur compounds into nanometer-sized iron sulfides (nFeS) with improved antibacterial activity and antibiofilm efficacy in vitro. Jini and Sharmila [150] synthesized silver nanoparticles from *A. cepa*, which have higher in vitro antidiabetic and antioxidant activities. However, the toxicity of nanoparticles due to nano size and the high cost hinder their practical application in poultry.

6. Future Perspectives

The aforementioned findings are testimony to the fact that the appreciation of alliums as poultry feed additives exhibits tremendous opportunities as well as hurdles. Therefore, the scientists, veterinarians, and commercial partners must work together to thwart the limitations for optimal efficacy of alliums, from poultry health and economic perspectives. Future research in this field will help us to better understand their mechanism of action and optimal dosage as well as efficient delivery methods (fermentation, microencapsulation and/or nanotechnology). Since alliums are a hub of bioactive compounds which might affect poultry production synergistically, the dietary supplementation of dried alliums or their extracts poses an advantage over the single extracted compound. Moreover, the synergistic effect of alliums with other antibiotic alternatives such as prebiotic, probiotic, organic

acid, etc. together with good management and farming practices will be the key to achieve sustainable poultry production. Moreover, researchers in this field should be encouraged to publish even the negative or no effects of alliums in poultry.

7. Conclusions

It is evident from our discussion that alliums harbor a variety of bioactive compounds such as organosulfur compounds, flavonoids, fructans, fructo-oligosaccharides, saponins, etc., and thereby justifying their usefulness as feed additives for poultry production. Recently, several studies have established that alliums in poultry diets have a significant modulatory effect on their growth performance indices, lipid metabolism, gut ecosystem as well as immune responses, especially when poultry are experiencing stress and disease challenge conditions. In addition, the alliums also improve the nutritional quality of poultry products via their enrichment in antioxidant (flavonoids) and organoselenium compounds. However, their application in poultry production has been largely circumvented due to inconsistent efficacy among studies, lack of a clear understanding of the mechanisms of action, non-availability of a standard as well as chemically characterized formulation, and higher cost. The processing methods, such as extraction, encapsulation, fermentation, and heating strongly influence the chemical composition, ergo, the biological activity of alliums. Therefore, poultry nutritionists must understand the inherent differences among the allium products used in various studies, along with their potential role in providing desired potential effects when added to poultry feed. A standardized procedure should be developed for an allium-based feed additive retaining its bioactive components. The OSCs and polyphenol contents of allium products may serve as proxy for their strategic application in poultry nutrition feeding programs. In addition, to preserve the effectiveness of alliums as a poultry feed additive, the optimization of dosage regimens that encompasses bioavailability could also be a suitable strategy. This review is expected to inspire investigations on alliums as feed additives for poultry health and disease management.

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Growth Performance of Broilers as Influenced by Different Levels and Sources of Methionine Plus Cysteine

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Simple Summary: The current work evaluated the utilization of different sources of methionine either from DL-methionine (DL-Met) or L-methionine (L-Met) using different concentrations of dietary methionine plus cystine (Met + Cyst) in broiler chickens. Results showed that a better edible meat yield could be obtained by supplementing Met + Cyst at the rate of 80% of the digestible lysine.

Abstract: The objective of this work was to evaluate the utilization of methionine from DL-methionine (DL-Met) and L-methionine (L-Met) with different levels of dietary methionine plus cystine (Met + Cyst) in broilers. The experimental diets were formulated by using three levels of Met + Cyst, i.e., 74%, 77% and 80% of digestible lysine. Met + Cyst was provided either from DL-Met or L-Met. A total of 450 day-old broilers were divided into six groups (five replicates of 15 birds each) in a 3 × 2 factorial arrangement under completely randomized design. Weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) was determined. At the end of the experiment (35 days), two birds from each replicate were slaughtered to determine carcass characteristics and serum homocysteine. Results indicate that the combined effect of L-Met and DL-Met significantly affected ($p < 0.05$) the WG in the starter period and FI in the finisher period. Neither source nor level of methionine influenced ($p > 0.05$) the FI, WG and FCR of broilers during the starter, finisher or overall phase of growth. The interaction between sources and levels of methionine did not influence ($p > 0.05$) the feed intake, weight gain and FCR during the overall phase of growth. Source of methionine had no ($p > 0.05$) effect on carcass characteristics. Methionine levels had a significant effect ($p < 0.05$) on carcass weight, chest weight and thigh weight. The interaction between sources and levels of methionine had a significant ($p < 0.05$) effect on the liver weight. The sources of methionine had significant ($p < 0.05$) effects on the liver and heart weight, while methionine levels significantly influenced ($p < 0.05$) the liver and gizzard weight. Finally, it was concluded that if DL-Met and L-Met are included in feed at a standard level, they are equally effective as a source of methionine for broilers.

Keywords: methionine; levels; sources; growth; carcass; broiler

1. Introduction

Methionine has a vital role in the metabolic functioning of animals and humans, which is why it is also known as functional amino acid. Methionine is considered as the first limiting amino acid in broilers and its deficiency may cause reduced growth performance, metabolic disorder and impaired immune system [1,2]. It plays a vital role in the production of energy through the synthesis of protein; it also enhances the broilers' livability, efficiency of feed and growth performance [3–5]. Also, a methyl group that is provided by sulfur-adenosyl methionine is required for many metabolic reactions such as epinephrine, carnitine, choline and creatine synthesis [5,6]. Synthetic sources of methionine (L-methionine (L-Met), DL-methionine (DL-Met) and DL-2 hydroxy–4-(methyl) butanoic acid (LMA)) are included in commercial broiler feed to optimize the dietary level of methionine. However, synthetic methionine is very expensive and the availability of methionine from different synthetic sources is controversial [7]. The availability of methionine from L-Met, DL-Met and LMA is 100%, 99% and 88%, respectively. L-Met is directly used by the animal as a precursor for protein synthesis and metabolized through the trans-sulfuration pathway to produce cysteine and glutathione [8,9]. Methionine hydroxy analog free acid (MHA-FA) is chemically different from DL-Met because it has a hydroxyl group at the asymmetric carbon atom, whereas DL-Met has an amino group. This chemical difference lowers the bio-availability of MHA compared to DL-Met [10,11].

Different levels of methionine in the diet of poultry have been reported by researchers, ranging from 0.3–1.2% during the initial period and 0.3–0.9% during the growth period of poultry. It has been suggested that commercial poultry production does not need more than 0.38% and 0.50% methionine in grower and starter diets, respectively, for the optimum feed efficiency and growth of broilers, although high rates of methionine are necessary to boost the immune system [12]. Reports regarding the dietary level of methionine are controversial. Kalinowski [13] studied the effect of DL-Met levels (0.32%, 0.38%, 0.44%, and 0.50%) with a constant level of L-cystine (0.40%) on slow and fast growing broilers from 3 to 6 weeks of age, and observed that weight gain was not affected and the feed conversion ratio (FCR) was improved with the highest level of methionine. However, Xie et al. [14] reported that increasing levels of DL-methionine (0.285%, 0.385%, 0.485%, 0.585% or 0.685%) resulted in decreased feed intake and weight gain because of higher plasma homocysteine concentration. This might be related to differences in the source of methionine used. Because of deamination of other amino acids during conversion of D-Met to L-Met, different sources of methionine may perform differently. Ribeiro et al. [8] observed that L-Met addition in broiler diet provided better FCR as compared to DL-Met, and MHA. Lui et al. [15] observed that the bioavailability of MHA-FA was greater than DL-Met. Data regarding the use of different methionine sources with varying dietary Met + Cyst levels are scarce, thus the main objective of this study was to evaluate the utilization of methionine from DL-Met and L-Met with different levels of dietary Met + Cyst in broilers.

2. Materials and Methods

The animal experiment was conducted in accordance with the recommendations and guidelines of the Committee on the Ethics of Animal Experiments of Sargodha University, Sargodha, Pakistan.

2.1. Experimental Design, Birds and Diets

The experiment was conducted at the poultry research center at the College of Agriculture, University of Sargodha (Sargodha, Pakistan). A total of 450 day-old broiler chickens (Ross 308-mixed sex) with similar body weight were randomly divided into 6 groups in a 3 × 2 factorial arrangement under completely randomized design (CRD). Each group had five replicates (pens) of 15 birds. Six experimental diets were formulated (Tables 1 and 2) by using 3 levels of Met + Cyst (74%, 77% and 80% of digestible lysine) and two sources (DL-Met and L-Met) of methionine. Chickens were reared in suitable pens, under the same managerial, hygienic and environmental conditions. Each diet was randomly allotted to each group for five consecutive successive weeks.

Table 1. Ingredients and nutrients of starter diets (1–21 days).

Ingredients (%)	[1] Diets					
	DLM74	**DLM77**	**DLM80**	**LM74**	**LM77**	**LM80**
Maize	18.85	18.85	18.85	18.85	18.85	18.85
Rice Tips	28	28	28	28	28	28
Soybean Meal	26.23	26.23	26.23	26.23	26.23	26.23
Canola	16	16	16	16	16	16
Rice Polish	4.9	4.9	4.9	4.9	4.9	4.9
Vegetable Oil	2.85	2.85	2.85	2.85	2.83	2.8
Limestone	1.01	1.01	1.01	1.01	1.01	1.01
Di-calcium phosphate	1	1	1	1	1	1
Salt	0.3	0.3	0.3	0.3	0.3	0.3
Lysine	0.408	0.408	0.408	0.408	0.408	0.408
DL-Methionine	0.245	0.282	0.319	0	0	0
L-Methionine	0	0	0	0.245	0.282	0.319
Premix *	0.1	0.1	0.1	0.1	0.1	0.1
L-Threonine	0.085	0.085	0.085	0.085	0.085	0.085
Extra XAP	0.01	0.01	0.01	0.01	0.01	0.01
Extra Phytase	0.01	0.01	0.01	0.01	0.01	0.01
	Nutrients (%)					
ME (Kcal/Kg)	2870	2870	2870	2870	2870	2870
Crude Protein	22.1	22.1	22.1	22.1	22.1	22.1
Calcium	1	1	1	1	1	1
Available P	0.45	0.45	0.45	0.45	0.45	0.45
D-Lysine	1.22	1.22	1.22	1.22	1.22	1.22
D-Methionine	0.57	0.606	0.643	0.57	0.606	0.643
Methionine + Cysteine	0.903	0.940	0.976	0.903	0.940	0.976

[1] LM 74, 77 and 80 and DLM 74, 77 and 80 indicate inclusion of L-methionine and DL-methionine at the rate of 74%, 77% and 80% of digestible lysine, respectively. ME = Metabolizable energy. * Provides per kg of diet: 20 MIU Vitamin A; 5 MIU Vitamin D3; 60 g Vitamin E 50; 2 g Vitamin K3; 6 g Vitamin B2; 45 g Vitamin B3; 12 g Vitamin B5; 5 g Vitamin B6; 12.5 g Vitamin B9; 12.5 g Vitamin B12; 275 g Manganese ($MnSO_4$); 150 g Ferrous ($FeSO_4$); 200 g Zn ($ZnSO_4$); 75 g Cu ($CuSO_4$); 75 g Selenium; 4 g Potassium iodide.

2.2. Housing and Management

The housing area was cleaned and fumigated before the arrival of the chicks. Fumigation was done by using $KMnO_4$ and formalin. Similar management conditions (floor space, temperature, relative humidity, light and ventilation) were provided to all replicates. Feed and water were provided ad libtium.

Table 2. Ingredients and nutrients of finisher diets (22–35 days).

Ingredients (%)	[1] Diets					
	DLM74	**DLM77**	**DLM80**	**LM74**	**LM77**	**LM80**
Maize	22.2	22.2	22.2	22.2	22.1	22
Rice Tips	32	32	32	32	32	32
Soybean Meal	24.33	24.33	24.33	24.33	24.33	24.33
Canola	7.462	7.462	7.462	7.462	7.462	7.462
Rice Polish	4.9	4.9	4.9	4.9	4.9	4.9
Vegetable Oil	3.75	3.75	3.75	3.75	3.75	3.75
Limestone	1.09	1.09	1.09	1.09	1.09	1.09
Di-calcium phosphate	0.8	0.8	0.8	0.8	0.8	0.8
Salt	0.3	0.3	0.3	0.3	0.3	0.3
Lysine	0.458	0.458	0.458	0.458	0.458	0.458
DL-Methionine	0.247	0.282	0.317	0	0	0
L-Methionine	0	0	0	0.247	0.282	0.317
L-Threonine	0.15	0.15	0.15	0.15	0.15	0.15
Premix *	0.1	0.1	0.1	0.1	0.1	0.1
Extra XAP	0.01	0.01	0.01	0.01	0.01	0.01
Extra Phytase	0.01	0.01	0.01	0.01	0.01	0.01
	Nutrients (%)					
ME (Kcal/Kg)	3040	3040	3040	3040	3040	3040
Crude Protein	20	20	20	20	20	20
Calcium	0.95	0.95	0.95	0.95	0.95	0.95
Available P	0.44	0.44	0.44	0.44	0.44	0.44
D-Lysine	1.1	1.1	1.1	1.1	1.1	1.1
D-Methionine	0.529	0.563	0.597	0.529	0.563	0.597
Methionine + Cystine	0.814	0.847	0.880	0.814	0.847	0.880

[1] LM 74, 77 and 80 and DLM 74, 77 and 80 indicate inclusion of L-methionine and DL-methionine at the rate of 74%, 77% and 80% of digestible lysine, respectively. * Provides per kg of diet: 20 MIU Vitamin A; 5 MIU Vitamin D3; 60 g Vitamin E 50; 2 g Vitamin K3; 6 g Vitamin B2; 45 g Vitamin B3; 12 g Vitamin B5; 5 g Vitamin B6; 12.5 g Vitamin B9; 12.5 g Vitamin B12; 275 g Manganese ($MnSO_4$); 150 g Ferrous ($FeSO_4$); 200 g Zn ($ZnSO_4$); 75 g Cu ($CuSO_4$); 75 g Selenium; 4 g Potassium Iodide.

2.3. Growth Performance

Feed intake and weight gain were recorded through the test periods (the starter period corresponds to 1–21 days of age, the finisher period to 22–35 days of age, and the overall period to 1–35 days of age). The feed intake was calculated by the difference between feed supplied and refusal at each period. The feed conversion ratio (FCR) was calculated by dividing feed intake by weight gain [16].

2.4. Carcass Evaluation and Serum Homocysteine

At the end of the experiment, two birds of average body weight from each replicate were randomly selected and slaughtered to determine the carcass characteristics (live weight, carcass weight, eviscerated weight, chest weight and thigh weight) and weight of visceral organs (liver, heart and gizzard).

2.5. Blood Sampling

Blood samples (n = 5) were collected from the wing vein at 35 days of age without anticoagulant for serum separation. Samples were centrifuged at 1435× g for 5 min at 4 °C to obtain clear sera, which was collected for homocysteine analysis using chromatographic assay [17].

2.6. Statistical Analysis

Data collected were analyzed by using the analysis of variance technique in a 3 × 2 factorial arrangement under CRD. Means of all parameters were separated by using Tukey's test with the assistance of software (SAS® 9.3 Software).

3. Results

3.1. Growth Performance

The combined effect of L-Met and DL-Met significantly affected ($p < 0.05$) the weight gain of broilers in the starter period. Neither the source nor levels of methionine influenced ($p > 0.05$) the feed intake, weight gain and FCR of broilers during the starter, finisher or the whole period (Table 3).

Table 3. Effect of different sources and levels of methionine plus cystine on growth performance of broilers during the starter and finisher phases.

Treatments	Feed Intake (g)		Weight Gain (g)		FCR (g Feed/g Gain)	
Source × Level	**1–21 days**	**22–35 days**	**1–21 days**	**22–35 days**	**1–21 days**	**22–35 days**
LM 74	1321.5	2105.0 ^{b}	934.8 ^{b}	1282.4	1.4191	1.9386
LM 77	1342.8	2149.1 ^{a,b}	1011.8 ^{a}	1209.1	1.3291	1.7873
LM 80	1338.2	2154.9 ^{a}	1023.4 ^{a}	1282.5	1.3076	1.8108
DLM 74	1335.1	2077.3 ^{b}	914.1 ^{b}	1129.0	1.3293	1.6395
DLM 77	1320.8	2097.3 ^{b}	1005.7 ^{a}	1188.9	1.4054	1.8183
DLM 80	1331.5	2150.7 ^{a}	1008.1 ^{a}	1214.9	1.3225	1.6195
SEM	5.857	15.630	18.002	68.759	0.0262	0.0142
			Source			
LM	1334.2	2134.9	990.00	1258.0	1.3524	1.8455
DLM	1329.1	2109.8	984.97	1177.6	1.3519	1.6925
SEM	3.382	9.0243	10.393	39.698	0.0151	0.0601
			Level			
74	1328.3	2123.2	970.3	1205.7	1.3742	1.7891
77	1331.8	2129.9	976.4	1199.0	1.3672	1.8028
80	1334.8	2114.0	1015.8	1248.7	1.3150	1.7151
SEM	4.142	11.052	12.72	48.620	0.0185	0.0737
			p-Values			
Source × Level	NS	*	*	NS	NS	NS
Source	NS	NS	NS	NS	NS	NS
Level	NS	NS	NS	NS	NS	NS

LM 74, 77 and 80 and DLM 74, 77 and 80 indicate inclusion of L-methionine and DL-methionine at the rate of 74%, 77% and 80% of digestible lysine, respectively. ^{a,b} Means sharing different superscripts differ significantly ($p < 0.05$). NS = Non-significant ($p > 0.05$). * = Significant ($p < 0.05$).

Data presented in Table 3 indicate that the interaction between source and levels of methionine had a significant effect ($p < 0.05$) on the feed intake and weight gain during the finisher and starter period, respectively. The results regarding feed intake in the finisher period revealed that the best values ($p < 0.05$) were achieved at an 80% ratio of L-Met. However, no significant differences in feed intake were observed between LM80 and DLM80. During the starter period, the highest values of weight gain ($p < 0.05$) were achieved with a 77% or 80% ratio of L-Met and DLM in comparison with a ratio of 74%.

As shown in Table 4, the interaction between sources and levels of methionine did not influence ($p > 0.05$) the feed intake, weight gain and FCR during the overall phase of growth.

Table 4. Effect of different sources and levels of methionine plus cystine on growth performance of broilers during overall experimental period (1–35 days).

Treatments	Feed Intake (g)	Weight Gain (g)	FCR (g Feed/g Gain)
		Source × Level	
LM 74	3470.5	2288.1	1.6931
LM 77	3447.8	2150.2	1.5739
LM 80	3488.9	2290.6	1.5692
DLM 74	3432.5	2063.8	1.5015
DLM 77	3475.6	2200.7	1.6284
DLM 80	3408.8	2238.3	1.4891
SEM	18.641	78.525	0.0570
		Source	
LM	3469.1	2243.0	1.6120
DLM	3439.0	2167.6	1.5397
SEM	10.762	45.337	0.0329
		Level	
74	3451.5	2176.0	1.5973
77	3461.7	2175.5	1.6011
80	3448.8	2264.5	1.5292
SEM	13.181	55.526	0.0403
		p-Values	
Source × Level	NS	NS	NS
Source	NS	NS	NS
Level	NS	NS	NS

LM 74, 77 and 80 and DLM 74, 77 and 80 indicate inclusion of L-methionine and DL-methionine at the rate of 74%, 77% and 80% of digestible lysine, respectively. NS = Non-significant ($p > 0.05$).

3.2. Carcass Characteristics

The source × level of methionine had a significant ($p < 0.05$) effect on thigh weight and non-significant ($p > 0.05$) effect on live weight, carcass weight, after skin removal, eviscerated weight and chest weight (Table 5). The source of methionine had a non-significant effect ($p > 0.05$) on the carcass characteristics of broilers. Level of methionine had a significant ($p < 0.05$) effect on carcass weight, chest weight and thigh weight and a non-significant ($p > 0.05$) effect on live weight, after skin removal and eviscerated weight.

3.3. Weight of the Visceral Organs

As presented in Table 6, the interaction between sources and levels of methionine had a significant ($p < 0.05$) effect on liver weight, while the effect on heart and gizzard weight was non-significant ($p > 0.05$). On the other hand, with regard to liver weight, there was no significant difference between LM80 and DLM80. The sources of methionine had a significant ($p < 0.05$) effect on the liver and heart weight while the effect on the gizzard weight was non-significant ($p > 0.05$), since LM increases liver and heart weight when compared to DLM. Liver and gizzard weights were gradually increased as the levels of methionine increased from 74% to 77% to 80% ($p < 0.05$).

Table 5. Effect of different sources and levels of methionine plus cystine on carcass characteristics of broilers.

Treatments	Live Weight (g)	Carcass Weight (g)	After Skin Removal (g)	Eviscerated Weight (g)	Chest Weight (g)	Thigh Weight (g)
			Source × Level			
LM 74	2051.2	1979.4	1721.0	1329.2	581.60	528.20 ^{b}
LM 77	2096.0	2027.2	1801.0	1560.2	642.00	548.00 ^{a,b}
LM 80	2368.4	2288.8	2002.8	1747.4	685.00	620.60 ^{a}
DLM 74	2067.2	1992.8	1729.0	1804.0	584.60	489.90 ^{b}
DLM 77	2157.4	2087.6	1830.4	1491.0	614.60	531.00 ^{a,b}
DLM 80	2180.0	2118.6	1792.0	1538.0	643.00	573.80 ^{a}
SEM	93.01	85.12	82.45	166.58	27.27	26.68
			Source			
LM	2171.9	2098.5	1841.5	1545.6	636.20	552.83
DLM	2134.9	2066.3	1783.8	1611.0	614.0	544.33
SEM	53.70	49.14	47.60	96.17	15.74	15.40
			Level			
74	2059.2	1981.1 ^{b}	1725.0	1566.6	583.10 ^{b}	509.05 ^{b}
77	2126.7	2057.4 ^{a,b}	1815.7	1525.6	628.30 ^{a,b}	539.50 ^{a,b}
80	2274.2	2203.7 ^{a}	1897.3	1642.7	664.00 ^{a}	597.20 ^{a}
SEM	65.77	60.19	58.30	117.8	19.28	18.87
			p-Value			
Source × Level	NS	NS	NS	NS	NS	*
Source	NS	NS	NS	NS	NS	NS
Level	NS	*	NS	NS	*	*

LM 74, 77 and 80 and DLM 74, 77 and 80 indicate inclusion of L-methionine and DL-methionine at the rate of 74%, 77% and 80% of digestible lysine, respectively. ^{a,b} Means sharing different superscripts differ significantly ($p < 0.05$). NS = Non-significant ($p > 0.05$). * = Significant ($p < 0.05$).

Table 6. Effect of different sources and levels of methionine plus cystine on visceral organs and serum homocysteine of broilers.

Treatments	Liver (g)	Heart (g)	Gizzard (g)	Serum Homocysteine μmol/L
		Source × Level		
LM 74	51.0 ^{b}	21.4	65.4	47.40
LM 77	54.1 ^{a,b}	21.0	73.0	54.62
LM 80	60.6 ^{a}	17.2	78.8	58.74
DLM 74	43.4 ^{b}	13.8	69.1	52.46
DLM 77	52.4 ^{a,b}	14.0	76.0	44.76
DLM 80	54.0 ^{a}	12.6	74.4	52.48
SEM	2.78	1.98	3.67	4.686
		Source		
LM	55.523 ^{a}	19.867 ^{a}	72.400	59.900
DLM	49.933 ^{b}	13.467 ^{b}	73.167	53.687
SEM	1.6059	1.1470	2.1236	2.706
		Level		
74	48.75 ^{b}	17.6	67.25 ^{b}	50.080
77	52.50 ^{a,b}	17.5	74.50 ^{a,b}	49.690
80	56.50 ^{a}	14.9	76.60 ^{a}	55.610
SEM	1.966	1.404	2.600	3.3141
p-Value				
Source × Level	*	NS	NS	NS
Source	*	*	NS	NS
Level	*	NS	*	NS

LM 74, 77 and 80 and DLM 74, 77 and 80 indicate inclusion of L-methionine and DL-methionine at the rate of 74%, 77% and 80% of digestible lysine, respectively. ^{a,b} Means sharing different superscripts differ significantly ($p < 0.05$). NS = Non-significant ($p > 0.05$). * = Significant ($p < 0.05$).

3.4. Serum Homocysteine

The combined effect of L-Met and DL-Met had no ($p > 0.05$) effect on serum homocysteine level (Table 6). Neither source nor level of methionine had a significant ($p > 0.05$) influence on serum homocysteine level.

4. Discussion

The body weight gain of broilers was significantly increased ($p < 0.05$) in the starter period due to the combined effect of L-Met and DL-Met. These findings of growth performance confirmed the reports of earlier researchers, Ahmed and Abbas [18] who studied the effect of dietary methionine levels above the nutrient requirements of poultry (NRC) [19] recommendation on performance and carcass traits in broiler birds. Four dietary levels of methionine, 0%, 100%, 120% and 130% of the NRC recommendation were used. Weight gain was significantly higher by 110% and 130% of NRC methionine than that of the control diet. Better weight gain with L-Met than DL-Met is also supported by the findings of Katz and Baker [20] who observed that L-Met provided better and more efficient weight gain than D-Met or DL-Met. At a level of supplementation near the requirement, equal efficiency was attained because L-Met is 100% absorbed in the body as compared to DL-Met.

The feed intake and FCR in the starter period remained unchanged by the combined effect of L-Met and DL-Met; this is supported by other researchers [21,22] who have also observed no significant difference in feed intake and FCR due to supplementation of L-Met and DL-Met because when the diet was supplemented with methionine alone, some methionine was converted to cysteine. The presence of small excess amounts of cysteine depressed the feed intake without a proportional reduction in weight gain because the presence of cysteine reduces the metabolic damage. The combined expression of methionine and cystine as sulfur amino acids restricts the efficient use of feedstuff, and also results in inconsistency in requirements. The conversion of methionine to cysteine was nonequivalence [23] and cystine oxidation occurs when it increases beyond the limit, resulting in inefficiency in the accurate estimate of the requirement of individuals. The replacement value for cysteine in broilers that are 3–6 weeks old is 52% [24], however, Wheeler and Latshaw [25] reported 43% and NRC [18] reported 47% as the recommended value. It has been estimated that about 10% of dietary protein is diverted by the broiler in the first 6 weeks to the formation of feathers [26]; this process is high in cysteine [27]. Engler et al. [28] reported that less cysteine is required by male broiler genotypes that are low feathering (L/k^+) after the age of 3 weeks, and this results in a 15% advantage in the feather weight of the k^+/k^+ bird at the age of 48 days [29]. If the nutrients are stored in the feathers then it will not be available for other purposes; while the muscle of breast nourishment rate is reduced by the continuous production of keratin, which limits the supply of nutrients [30]. Therefore, the cystine deficiency results in the reduction in the recovery of breast meat and also decreases the extent of feathering. Our finding of no effects of the sources of methionine on the starter phase of broilers is supported by other researchers [22,31] who observed that L-Met and DL-Met did not affect growth performance due to conversion of DL-Met into L-Met. It seems that lower metabolization of D–amino acid due to the lower amount of D–amino acid oxidase in young broilers may reduce the utilization of higher amounts of DL-Met, which leads to metabolic stress and inhibition of body weight gain in the starter phase. Our findings regarding unaltered feed intake, weight gain and FCR of broilers in the starter period due to different levels of methionine are similar to those of other researchers [32,33], which might be attributed to the satisfaction of methionine requirements at the lower standard level.

The results regarding feed intake of broilers in the finisher period indicated significant differences ($p < 0.05$) between L-Met and DL-Met. This finding confirmed the reports of earlier researchers [34] who observed better feed efficiency of chicks fed an L-Met diet as compared to DL-Met because the supplementation of either L-Met or DL-Met have beneficial effects on villus development in association with increased glutathione production and levels of total antioxidant capacity, and reduced protein oxidation in the duodenum. Supplementation of L-Met has a better effect on redox status and development of the gut of young chicks as compared with DL-Met.

Our finding of no changes in the weight gain of broilers in the finisher period by the combined effect of L-Met and DL-Met is the similar to other researchers [33,35,36] who observed that L-Met or DL-Met did not significantly influence the weight gain of broilers in the finisher period. This was because when large quantities of methionine are added in the feed, excess methionine is converted into homocysteine and higher amounts of homocysteine in the body reduce the body weight of broilers [37]. No significant differences ($p > 0.05$) were observed by different type of methionine on feed intake, weight gain, and FCR of broilers in the finisher period. This finding confirms the reports of earlier researchers [32,33,38]. Because d-amino acid oxidase, the key enzyme that converts D-Met to L-Met, exists only in the liver and kidney, D-Met is not utilized directly by the cells of the gastrointestinal tract until it is converted to L-Met either in the liver or kidneys. Research has also shown that the expression of this enzyme is very low for young animals. Therefore, L-Met is the only biologically functional form of methionine that is readily utilized by the intestinal cells of young animals. The quantity of methionine had no effect ($p > 0.05$) on the performance of broilers in the finisher period. This finding confirms the reports of earlier researchers [21,22] who observed that levels of methionine had no effect on feed intake, average daily gain, feed efficiency and FCR of broilers because DL-Met is readily converted into the L-isomer by the animal. Also, our finding that L-Met and DL-Met had no combined effect on feed intake, weight gain and FCR during the overall phase of broiler growth is supported by other researchers [33,35,36] who also observed that L-Met or DL-Met did not influence the feed intake, weight gain and overall FCR of broilers. Zhang [7] studied the effect of different dietary methionine source supplementation including L-Met, DL-Met and DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) on growth performance. He observed no differences among L-Met, DL-Met and DL-HMTBA for weight gain and feed efficiency. No effect of methionine sources on overall growth of broiler has also been found by other researchers [21,22,31] who observed that L-Met and DL-Met had no effect on the overall phase of broilers.

The results regarding thigh weight indicated that the highest value ($p < 0.05$) was achieved at the 80% ratio of L-Met, while the values achieved at the 80% ratio of DL-Met were lower than L-Met. The significant effect of L-Met and DL-Met on the thigh weight of broilers is supported by other researchers [39,40] who also observed that methionine sources improved the thigh weight of broilers because methionine has a role in the synthesis of creatinine in thigh muscles. No differences ($p > 0.05$) were observed for different types of amino acid on the carcass characteristics of broilers. This finding confirmed the reports of earlier researchers [31,41] who observed that the type of methionine had no effect on live weight, carcass weight, after skin removal, eviscerated weight and chest weight because L-Met is directly absorbed in the body and DL-Met, MHA is first converted into L-Met and then absorbed in the body. Also, Drazbo et al. [42] found that the source of dietary methionine had no effect on carcass yield or breast muscle quality.

The levels of methionine had a significant ($p < 0.05$) effect on carcass weight, chest weight and thigh weight. This finding confirms previous studies [43,44], where levels of methionine had a significant influence on the thigh weight, chest weight and carcass weight. This is because D-Met is oxidatively converted to α-ketoanalogues of L-Met, 2-keto-4(methylthio) butanoic acid (KMB) by the enzyme D-amino acid oxidase, which is a proximal oxidase containing flavin adenine dinucleotide (FAD) as a cofactor. Then, KMB is converted into L-methionine by the transfer of nitrogen from the donor amino acid, which is catalyzed by ubiquitous transaminases. In chickens, many amino acids like glutamic acid, arginine, isoleucine and alanine are used for transamination of KMB [7,11].

Results regarding liver weight indicated that the highest value ($p < 0.05$) was observed at the 80% ratio of L-Met while the values achieved at the 80% ratio of DL-Met were lower than L-Met. The combined effect of L-Met and DL-Met had a significant ($p < 0.05$) effect on the liver weight of broilers. Our finding of unaltered heart and gizzard weight due to the combined effect of L-Met and DL-Met is supported by other researchers [45,46] who observed that DL-Met and herbal methionine had no significant effect on the carcass yield, breast meat and eviscerated weight of broilers. The significant differences observed between the heart and liver weight of birds fed different types of

amino acid are corroborated by Ahmed and Abbas [45] who observed that dietary supplementation of methionine significantly affected the liver and heart weight. Ribeiro et al. [35] observed that DL-Met had a significant effect on the gizzard weight in heat stress conditions, which is similar to our findings of differences in gizzard weight due to the type of amino acid.

Unaltered homocysteine due to the combined effect of L-Met and DL-Met in the diet of broilers was supported by the findings of Pillai et al. [47]. They observed that dietary methionine had no effect on hepatic homocysteine remethylation. No effect of the source of methionine on serum homocysteine level of broilers confirms the findings of Harter and Baker [48] who observed that methionine was stored in the plasma of birds fed excess methionine, but plasma levels of homocysteine, cystathionine, and cystine remained unchanged. Haulrik et al. [49] found that high methionine and high protein diet did not significantly increase homocysteine concentration as compared to low methionine and low protein diet, which confirms our findings that there was no change in serum homocysteine level due to different amino acid sources.

On the basis of these results, it may be concluded that if DL-Met and L-Met are included at a standard level in feed, they are equally effective as a source of methionine for broilers. However, better carcass traits may be achieved if Met + Cyst is added at the rate of 80% of digestible lysine.

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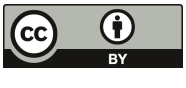

animals

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Article

Dietary Betaine Improves Intestinal Barrier Function and Ameliorates the Impact of Heat Stress in Multiple Vital Organs as Measured by Evans Blue Dye in Broiler Chickens

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Simple Summary: Heat stress alters the normal physiological status, compromising the function of organs such as the small intestine. However, evidence exists of a wider distribution of organ dysfunction, stemming from factors such as a reduction in blood flow due to redistribution to the skin for increased radiant heat loss to the environment. Simultaneously, assessing organ dysfunction at multiple locations presents technical difficulties, and hence studies are lacking. Therefore, the aim of this experiment was to determine the pattern of Evans Blue Dye distribution as a cost-effective indicator of organ dysfunction in HS chickens supplemented with betaine. The results showed that Evans Blue Dye concentration increased in the kidney and muscle during heat stress, while such concentration was reduced with betaine. Therefore, betaine could improve the broiler's tolerance to heat stress, and Evans Blue Dye may be a useful tool for investigating the effects of heat stress on broiler organ dysfunction.

Abstract: In a 2 × 2 factorial design, 60 male Ross-308 broilers were fed either a control or 1 g/kg betaine diet and housed under thermoneutral (TN) or heat stress (HS) conditions. Broilers were acclimated to diets for 1 week under TN (25 °C), then either kept at TN or HS, where the temperature increased 8 h/day at 33 °C and 16 h/day at 25 °C for up to 10 days. Respiration rate (RR) was measured at four time points, and on each of 1, 2, 3, 7 and 10 days of HS, 12 broilers were injected with 0.5 mg/kg of Evans Blue Dye (EBD) solution to quantify regional changes in tissue damage. Betaine was quantified in tissues, and ileal damage was assessed via morphometry and transepithelial resistance (TER). Heat stress elevated RR ($p < 0.001$) and resulted in reduced villous height ($p = 0.009$) and TER ($p < 0.001$), while dietary betaine lowered RR during HS ($p < 0.001$), increased betaine distribution into tissues, and improved ileal villous height ($p < 0.001$) and TER ($p = 0.006$). Heat stress increased EBD in the muscle and kidney of chickens fed the control diet but not in those receiving betaine. Overall, these data indicate that supplemented betaine is distributed to vital organs and the gastrointestinal tract, where it is associated with improved tolerance of HS. Furthermore, EBD markers help reveal the effects of HS on organs dysfunction.

Keywords: heat stress; betaine; Evans blue dye; physiological responses; broiler chickens

1. Introduction

Due to the impacts of a changing climate, heat stress (HS) is of increasing concern for animal production. Broilers are sensitive to HS due to the presence of feather coverage, increased selection for muscling, and because they lack sweat glands. Heat stress compromises efficient broiler production in part by reducing voluntary feed intake. However, some studies have shown that the reduction in feed intake does not fully explain the reduction in growth rate [1]. The reasons for this include factors such as altered endocrine status [2,3]. Furthermore, increased evaporative heat loss by panting and radiant heat loss by blood flow redistribution also compromise efficient growth. As the distribution of blood flow to the skin to facilitate radiant heat loss relies on a commensurate reduction in blood flow elsewhere, continued HS can precipitate dysfunction in affected organs due to reduced nutrient delivery and removal of metabolic by-products. Heat stress compromises intestinal barrier integrity in broiler chickens and other species [4–6], presumably by splanchnic blood restriction, and that can lead to bacterial translocation and a systemic inflammatory response [7,8].

While investigations into the etiology of heat stroke have confirmed the central role of gastrointestinal tract (GIT) damage [9], it is apparent that heat stroke has a wider pattern of organ damage than the GIT alone, with increased incidences of renal and liver injury [10,11]. It is likely that compromised hepatic and renal function also contribute to reduced growth efficiency in broilers and other production species during heat exposure. While quantifying systemic changes in organ damage is technically difficult, this may in part be overcome by using blood-borne markers such as Evans Blue Dye (EBD). Within the blood, EBD binds tightly to plasma albumin and is used as an exogenous marker of plasma volume [12]. Following inflammation or tissue injury, EBD extravasates into the surrounding tissue where it may be quantified as a marker of tissue damage [13,14]. Identifying localised sites of stress is a useful strategy for the development of amelioration strategies, as has been demonstrated with the supplementation of antioxidants in mitigating the impact of HS on intestinal permeability [4]. Therefore, the aims of this experiment were to investigate changes in EBD extravasation in the broiler during HS. Furthermore, the organic osmolyte betaine has been demonstrated to be an effective supplement for ameliorating the effects of HS. It protects cells against osmotic inactivation, improves water retention of cells [15], reduces core body temperature by reducing the activity of the ion pumps required for osmoregulation, allowing more energy for growth [16–18], and acts as a methyl donor for homocysteine remethylation [19]. Therefore, the secondary aim of the experiment was to determine whether supplementation of betaine ameliorated the effects of HS and altered the pattern of EBD extravasation.

2. Materials and Methods

2.1. Animal Ethics

The experiment was approved by The University of Melbourne, Australia (Protocol no. 1814704.1).

2.2. Animals, Diets and Experimental Design

Four week-old male Ross-308 chickens (n = 60) were obtained from a local commercial farm (Turosi, Bannockburn, Victoria, Australia) located within 2 h driving distance from The University of Melbourne. Chickens were randomly allocated to 4 equally sized pens (1.9 × 3.4 m) in two environmentally controlled rooms. All pens were covered with wood shavings (8–10 cm deep) with 4 drinkers and 4 feeders for each pen. The chickens were allowed to acclimate to the pens and facility for 7 days at a constant 25 °C (thermoneutral, TN). From the arrival in the facility, chickens were given either a standard finisher control diet (CON, n = 30), which was formulated as a commercial finisher diet (Feedworks, BESTMIX, CP 21.3% and 12.65 MJ ME/kg) and exceeded nutrient requirements [20],

or a CON plus 1 g/kg betaine (Betafin S1, DuPont, Marlborough, UK) diet (BET, $n = 30$). After 7 days acclimation, the temperature in one room increased to 33 °C for 8 h/day (9 a.m.–5 p.m., 16 h/day 25 °C) to induce heat stress (HS) for 10 days while the alternate room was maintained under TN conditions. The relative humidity for both rooms was between 40–55% during the experiment. Light was provided 20 h/day, and chickens had ad libitum access to feed and water during the period of experiment. On each of days 1, 2, 3, 7 and 10 of environmental treatment, 3 chickens from each pen (each diet × temperature group) were assessed for EBD extravasation.

2.3. Physiological Responses

Respiration rate (RR) was measured at 11:00 a.m. after chickens had been exposed to 1, 3, 7 and 10 days HS (corresponding to 8, 10, 14 and 17 days in the facility and consuming the experimental diets). Chickens were filmed with a cell phone (iPhone 7, Apple Inc., Cupertino, CA, USA) and then the number of breaths taken over a 20-s period was quantified and then expressed as breaths per min.

2.4. Evans Blue Dye Injection, Slaughter and Tissue Collection

Chickens were injected with 0.5 mg/kg of an EBD solution (1.5% *w/w* EBD, Sigma, Aldrich, MO, USA in 0.9% saline solution) into the brachial vein on days 1, 2, 3, 7 and 10 of the environmental treatment. Three chickens were briefly removed from each pen for the injection, then returned to their designated rooms for 2 h. Chickens were then removed from the rooms, electrically stunned (Mitchell Engineering Food Equipment Pty Ltd., Queensland, Australia), placed in an inverted restraining funnel, slaughtered by severing the major blood vessels in the neck and then exsanguinated. Tissue samples were collected from the ileum, jejunum, muscle, liver, spleen and kidney for measuring EBD concentration. Furthermore, about 5 cm of ileum tissue and a piece of *psoas major* (breast muscle) were collected for morphometric analysis.

2.5. Evans Blue Dye Extraction and Qualification

Evans Blue Dye concentration was measured in the collected tissues according to a published method [13]. Tissue samples were dried in an oven at 70 °C for 48 h, then EBD extracted from 100 mg of dried and pulverised tissue with 500 µL formamide (Sigma, Aldrich, St Louis, MO, USA) and incubated at 55 °C for 24 h. Samples were then centrifuged for 15 min at 14,000× *g* and 4 °C, and the A_{610} of 200 µL of supernatant quantified in duplicate against standards using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc, Waltham, MA, USA). The obtained results were expressed as ng EBD per mg tissue dry weight.

2.6. Intestinal Transepithelial Electrical Resistance

Intestinal transepithelial electrical resistance (TER) was measured according to a previously published method [4]. Sections of ileum were collected immediately after euthanasia on days 3, 7 and 10 of the environmental challenge. After collection, sections were placed in chilled phosphate buffered saline, then transferred to Krebs solution (pH 7.4). The ileal sample was then opened along the mesenteric border and the external muscle was removed. The remaining layers were mounted onto a round slider (0.71 cm^2) and placed into a two-part Ussing chamber (EasyMount Diffusion Chambers, Physiologic Instruments) and 5 mL Krebs' solution was added to each side. On the mucosal side, the 11.1 mM glucose was replaced with mannitol. Voltage and I*sc* readings were acquired using a PowerLab amplifier and recorded using LabChart®5 (AdInstruments Pty Ltd., Lexington, New South Wale, Australia). Tissue was left to equilibrate for 20 min before clamping the voltage to 0 V, and epithelial resistance was determined by administering five 2-s pulses of 2 mV. The TER was calculated by Ohm's law and multiplied by the exposed area.

2.7. High-Performance Liquid Chromatography Analysis

Betaine was quantified in ileum, kidney and spleen following derivatisation with bromophenacyl bromide catalysed with 18-crown-6 and the bromophenacyl esters quantified by High-Performance Liquid Chromatography [21]. Briefly, 100 mg of pulverised tissue samples were homogenised with a bead beater (AnytimeLabTrader LLC, Fallbrook, CA, USA) in 1 mL tris buffer (1 M, pH 7) then centrifuged at 10,000× *g* for 15 min. to obtain supernatant. The obtained supernatant was added to monopotassium phosphate (100 mmol/L) and derivatisation solution containing 4-bromophenacyl bromide (50 mmol/L) and 18-crown-6 (2.5 mmol/L) in acetonitrile and vortex mixed. The samples were heated at 80 °C in a block heater for 1 h, cooled to room temperature before filtering into a glass High-Performance Liquid Chromatography. The A_{254} of the bromophenacyl esters of betaine were then quantified versus standards using a High-Performance Liquid Chromatography following a 10 µL injection (Model 2998, Waters, Milford, MA, USA).

2.8. Morphometric Analysis

Tissue samples were collected on days 1, 2, 3, 7 and 10 of the environmental challenge. The midpoint of the ileal section, and *psoas major* were excised and transferred in 10% formalin (Sigma, Aldrich, St Louis, MO, USA) and fixed in paraffin wax [22]. Slides were prepared using 8 µm sections, stained by hematoxylin and eosin, and the villous height, crypt depth, ileum seromuscular layer and *psoas major* fibre diameter (width) were quantified using a light microscope equipped with a camera (Leica, ICC50 W, Wetzlar, Germany), and analysed with ImageJ software [23]. The distance from the tip of the villous to the villous crypt junction represents the villous height, crypt depth was defined as the depth of the invagination between adjacent villous, and seromuscular layer was the smooth muscular layer located under the crypt. A total of 10 samples per section were quantified.

2.9. Statistics Analysis

All data were analysed using ANOVA for the main and interactive effects of temperature and diet (CON vs. BET) and time (1, 2, 3, 7 and 10 days) using Genstat version 18 (VSNi Ltd., Hemel Hempstead, UK). Statistical significance was considered at $p \leq 0.05$, and when achieved, a Duncan's multiple range post-hoc test was performed to differentiate between treatment groups, which were then labelled with differing alphabetic superscripts. Where skewed data occurred, normality was restored following a Log_{10} transformation and analysed as above. The predicted means were then back-transformed (10×) and presented in tables in parentheses. The replication for the main effects of temperature and diet were 30 chickens, respectively. The replication for the interaction between temperature and diet was 20 chickens per group and for temperature × diet × time was 3 chickens per treatment/time.

3. Results

3.1. Respiration Rate

Heat stress increased RR at each time point measured ($p < 0.001$, Figure 1). Dietary BET supplementation reduced RR during HS ($p < 0.001$) at each time point (Figure 1), but there was no effect of dietary BET under TN conditions. No main or interactive effects of time on RR were observed.

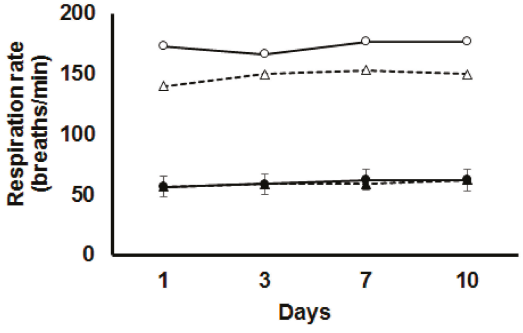

Figure 1. Respiration rate in broilers fed either a control diet (CON, round symbols) or betaine supplemented diet (BET, triangle symbols) after 1, 3, 7 and 10 days of being exposed to either thermoneutral (TN, filled symbols) or heat stress (HS, open symbols) conditions. The standard error of the difference for Temperature × Diet × Day is displayed on the data for the chickens fed the CON diet under TN conditions.

3.2. Evans Blue Dye Distribution

Evans Blue Dye concentration was quantified in muscle, liver, ileum, jejunum, spleen and kidney (Table 1). Heat stress increased EBD concentrations in the kidney (74 vs. 99 ng/mg, $p = 0.007$) but reduced concentrations in the spleen (213 vs. 162 ng/mg, $p = 0.024$). Dietary BET decreased EBD concentrations in the jejunum (94 vs. 76 ng/mg, $p = 0.043$), ileum (100 vs. 76 ng/mg, $p = 0.028$) and kidney (113 vs. 61 ng/mg, $p < 0.001$). There were significant interactions between diet and temperature in muscle and kidney such that HS increased EBD concentrations in chickens consuming the CON diet but not in those on the BET supplemented diet (Figure 2A,B). There was also an interaction between temperature and diet in the spleen where BET increased EBD concentrations under TN conditions, but not during HS (Figure 2C). There were no main or interactive effects of temperature, diet or time for EBD concentration in the liver, while interactions were observed for diet, temperature and time in the kidney, jejunum and ileum (Table 1). These interactions typically reflected that BET under HS conditions decreased tissue EBD concentrations. In the kidney, EBD concentration was higher under HS CON than all other groups after 1 day of HS. In the jejunum, this was observed after 7 days and in the ileum at day 10.

Table 1. Effects of a control diet (CON) or dietary betaine (BET) on Evans Blue Dye distribution in broilers housed under thermoneutral (TN) or heat stress (HS) conditions for 1, 2, 3, 7 and 10 days.

Tissues	Diet (D)	Temp (T)	Day of Thermal Challenge					SED	Significance [1]
			1	2	3	7	10		
Psoas Major (ng/mg)	CON	TN	8.3	16.0	8.0	14.3	29.4	11.3	D × T *
		HS	8.3	42.1	24.5	29.1	39.1		
	BET	TN	6.7	30.8	16.4	18.8	25.2		
		HS	9.5	6.2	16.7	14.3	31.8		
Liver (ng/mg)	CON	TN	37	91	96	110	94	32.8	
		HS	65	107	111	83	80		
	BET	TN	128	111	106	90	80		
		HS	58	141	65	60	87		
Kidney (ng/mg)	CON	TN	96 [a]	93 [ab]	62	61	119	28.2	T **, D ***, D × T **
		HS	158 [b]	117 [b]	98	94	161		D × T × Day +
	BET	TN	90 [a]	31 [a]	41	53	93		
		HS	62 [a]	61 [ab]	36	45	91		

Table 1. *Cont.*

Tissues	Diet (D)	Temp (T)	Day of Thermal Challenge					SED	Significance [1]
			1	2	3	7	10		
Spleen [2] (ng/mg)	CON	TN	2.05 (112)	2.08 (120)	2.20 (157)	2.30 (199)	2.36 (229)	0.15	T *, D × *
		HS	2.04 (110)	1.89 (78)	2.31 (206)	2.31 (206)	2.41 (258)		
	BET	TN	2.28 (191)	2.19 (153)	2.51 (321)	2.43 (267)	2.49 (309)		
		HS	2.04 (110)	2.03 (108)	2.36 (231)	2.14 (175)	2.22 (167)		
Jejunum [2] (ng/mg)	CON	TN	2.02 (104)	2.29 ^{a} (194)	1.90 (79)	1.86 ^{ab} (73)	2.08 (121)	0.14	D *, D × T × Day **
		HS	1.85 (71)	1.84 ^{b} (69)	1.95 (89)	2.08 ^{b} (119)	1.90 (79)		
	BET	TN	1.84 (69)	1.76 ^{b} (58)	1.86 (73)	1.90 ^{ab} (80)	1.98 (100)		
		HS	1.99 (97)	2.10 ^{ab} (125)	1.87 (75)	1.69 ^{a} (49)	1.85 (70)		
Ileum (ng/mg)	CON	TN	151	100	77	75	54 ^{a}	34.2	D *, D × T × Day **
		HS	115	68	69	85	209 ^{b}		
	BET	TN	70	146	49	32	108 ^{a}		
		HS	83	106	55	34	75 ^{a}		

[1] + $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Other main and interactive effects $p > 0.10$. Differing superscripts within a column denotes significant ($p < 0.05$) differences for D × T × Day on a single day of the experiment. [2] Due to skewed data the values were Log_{10} transformed before statistical analysis. Back transformed means are presented in parentheses.

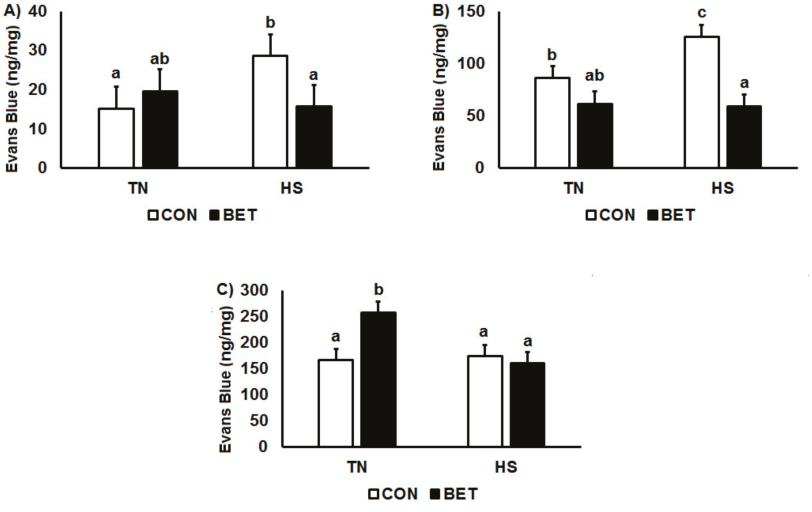

Figure 2. Changes in the distribution of Evans Blue Dye in (**A**) psoas major, (**B**) kidney, and (**C**) spleen in broilers during a thermoneutral (TN) vs. heat stress (HS) environmental challenge. Broilers were fed either a control diet (CON) or betaine diet (BET), and the mean represents the main effect of 5 time points (1, 2, 3, 7 and 10 day challenge). Means with differing superscripts denote $p < 0.05$. Refer to Table 1 for full interactive effects.

3.3. Transepithelial Electrical Resistance

Ileal TER was quantified after 3, 7 and 10 days of environmental challenge (Table 2; Figure 3). Overall, TER increased by BET (182 vs. 235 Ω.cm^2, $p = 0.006$) and reduced by HS (256 vs. 161 Ω.cm^2, $p < 0.001$). Ileal TER declined with time over the course of the experiment ($p < 0.001$). An interaction between HS and time occurred such that the TER of TN chickens at day 3 was almost double that of HS chickens (346 vs. 186 Ω.cm^2, $p = 0.049$). An interaction between diet, temperature and day was observed, such than a reduction in ileal TER had taken place by day 10. Alternatively, the TER from HS BET chickens was nearly double than HS CON at this time (93 vs. 161 Ω.cm^2) (Table 2).

Table 2. Effects of a control diet (CON) or dietary betaine (BET) on ileal transepithelial electrical resistance and ileal morphology in broilers under thermoneutral (TN) or heat stress (HS) conditions for 1, 2, 3, 7 and 10 days.

Ileal	Diet (D)	Temp (T)	Day of Thermal Challenge					SED	Significance [1]
			1	2	3	7	10		
Transepithelial resistance [2] (Ω.cm^2)	CON	TN	-	-	268 ^{a}	211	215 ^{a}	35.7	T ***, D **, Day *** D × T × Day $^{+}$
		HS	-	-	159 ^{a}	148	93.0 ^{b}		
	BET	TN	-	-	424 ^{b}	246	175 ^{ab}		
		HS	-	-	212 ^{a}	192	161 ^{ab}		
Villous height (µm)	CON	TN	754 ^{a}	763 ^{a}	727 ^{a}	741 ^{a}	775 ^{a}	55.5	T ***, D ***, D × T $^{+}$ D × T × D ***
		HS	749 ^{a}	722 ^{a}	718 ^{a}	763 ^{a}	696 ^{b}		
	BET	TN	885 ^{b}	827 ^{b}	894 ^{b}	947 ^{b}	939 ^{c}		
		HS	921 ^{b}	852 ^{b}	775 ^{ab}	796 ^{a}	797 ^{a}		
Villous area [3] (µm^2)	CON	TN	2.10 ^{a} (130)	1.90 ^{a} (82)	2.00 ^{b} (104)	2.06 ^{a} (118)	2.14 ^{c} (140)	0.22	D ***, Day **, D × T ** T × Day *, D × T × Day *
		HS	1.98 ^{a} (100)	1.96 ^{a} (95)	1.87 ^{a} (76)	1.96 ^{a} (92)	1.92 ^{a} (85)		
	BET	TN	2.08 ^{a} (134)	2.14 ^{b} (142)	2.04 ^{b} (112)	2.19 ^{b} (165)	2.14 ^{bc} (141)		
		HS	2.35 ^{b} (228)	2.09 ^{b} (127)	2.07 ^{b} (126)	2.17 ^{b} (156)	2.08 ^{b} (123)		
Crypt depth (µm)	CON	TN	172 ^{a}	183 ^{b}	152	132 ^{a}	144 ^{b}	8.9	D ***, Day *** D × Day ***, T × Day **
		HS	141 ^{a}	197 ^{bc}	162	126 ^{a}	112 ^{a}		
	BET	TN	250 ^{b}	153 ^{a}	170	211 ^{c}	186 ^{c}		
		HS	253 ^{c}	217 ^{c}	158	179 ^{b}	151 ^{b}		
Seromuscular layer (µm)	CON	TN	160 ^{a}	175 ^{a}	159 ^{a}	160 ^{a}	149 ^{a}	11.4	T ***, D ***, D × T *** D × Day **, T × Day *
		HS	161 ^{a}	185 ^{a}	167 ^{a}	145 ^{a}	150 ^{a}		
	BET	TN	282 ^{b}	235 ^{b}	259 ^{b}	315 ^{b}	375 ^{c}		D × T × Day **
		HS	233 ^{c}	209 ^{a}	151 ^{a}	172 ^{a}	171 ^{b}		

[1] * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Other main and interactive effects $p > 0.10$. Differing superscripts within a column denotes significant ($p < 0.05$) differences for D × T × Day on a single day of the experiment. [2] Due to logistical constraints, ileal transepithelial electrical resistance was only measured on days 3, 7 and 10. [3] Due to skewed data, the values were Log_{10} transformed before statistical analysis. Back transformed means are presented in parentheses.

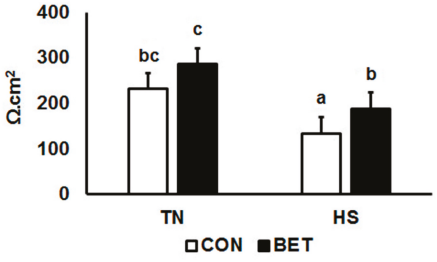

Figure 3. Transepithelial electrical resistance (TER) changes in broilers during a thermoneutral (TN) vs. heat stress (HS) environmental challenge. Broilers were fed either a control diet (CON) or betaine diet (BET), and the mean represents the main effect of 3 time points (3, 7 and 10 day challenge). Refer to Table 2 for full interactive effects.

3.4. Morphometric Analysis

Ileal villous height decreased by HS (826 vs.779 μm, $p = 0.009$) and increased by dietary BET (741 vs.864 μm, $p < 0.001$) (Table 2; Figure 4). There was no main effect of HS on villous area, whereas dietary BET increased villous surface area (97 vs. 136 μm^2, $p < 0.001$) (Table 2; Figure 5A). However, there were interactions such that villous surface area reduced over time particularly in those chickens exposed to HS. There was no main effect of HS on crypt depth, whereas it increased by dietary BET (152 vs. 192 μm, $p < 0.001$) (Table 2). However, there were interactions such that crypt depth decreased over time particularly in those chickens exposed to HS. The seromuscular layer depth decreased by HS (227 vs. 174 μm, $p < 0.001$) and increased by dietary BET (161 vs. 240 μm, $p < 0.001$) (Table 2; Figure 5B). However, there were interactions such that seromuscular layer depth increased over time in those chickens that were consuming the BET diet and housed under TN conditions, whereas it declined in those chickens exposed to HS (Table 2). Heat stress reduced psoas major fibre diameter (227 vs. 174 μm, $p < 0.001$), whereas it increased by dietary BET (161 vs. 240 μm, $p < 0.001$) (Figure 6). However, there were interactions such that psoas major fibre diameter was initially higher in chickens fed dietary BET and then increased over time in those chickens consuming the BET diet and housed under TN conditions, whereas it declined in those chickens exposed to HS. Psoas major fibre diameter remained constant in those chickens consuming the CON diet.

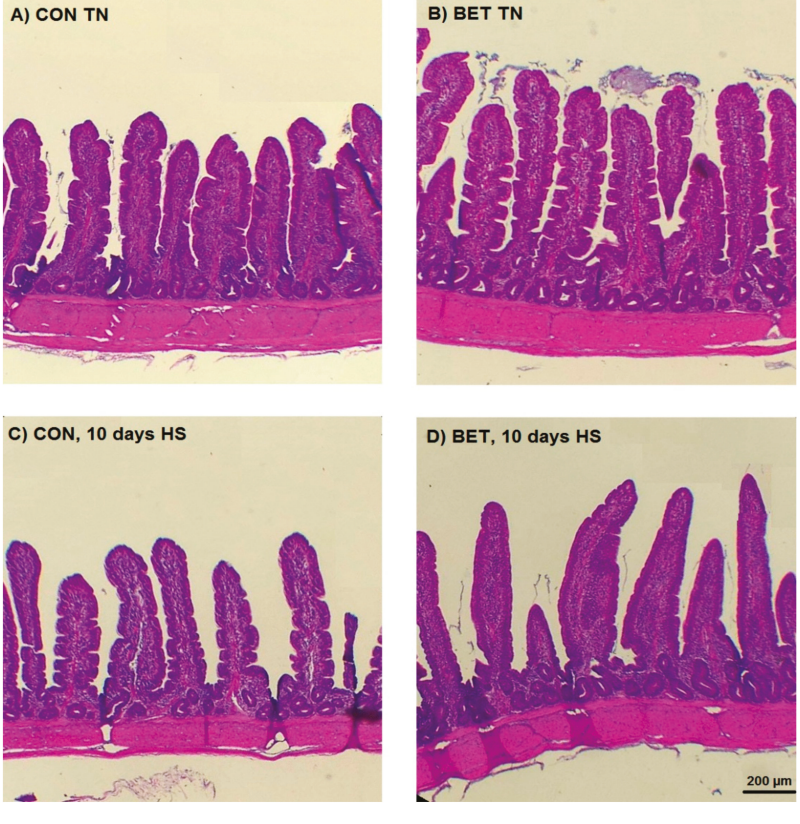

Figure 4. Representative photomicrographs of the ileum after 10 days of the experiment from broilers fed a control diet (CON, **A** and **C**) and betaine (BET, **B** and **D**) on villous height under thermoneutral (TN, **A** and **B**) or after 10 days being exposed to heat stress (HS, **C** and **D**).

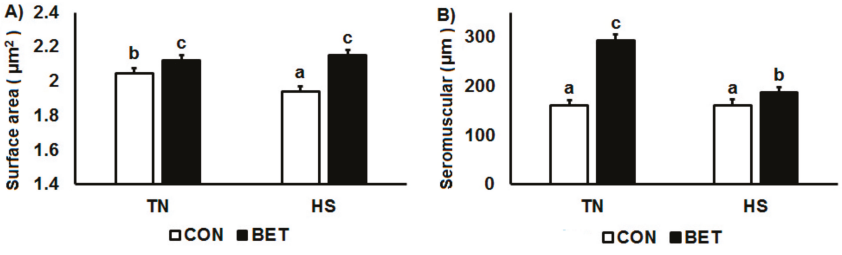

Figure 5. Ileal morphology: (**A**) villous surface area and (**B**) seromuscular layer in broilers during a thermoneutral (TN) vs. heat stress (HS) environmental challenge. Broilers were fed either a control diet (CON) or betaine diet (BET) and the mean represents the main effect at 5 time points (1, 2, 3, 7 and 10 day challenge). Means with differing superscripts denote $p < 0.05$. Refer to Table 2 for full interactive effects.

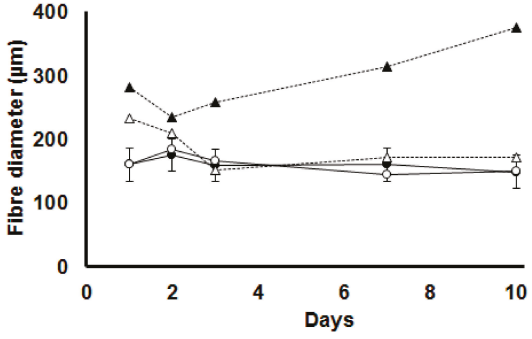

Figure 6. Psoas major fibre diameter in broilers fed either a control diet (CON, round symbols) or betaine supplemented diet (BET, triangle symbols) after 1, 3, 7 and 10 days of being exposed to either thermoneutral (TN, filled symbols) or heat stress (HS, open symbols) conditions. Psoas major fibre diameter increased over the course of experiment in TN BET ($p < 0.001$) while HS groups reduced HS CON. The standard error of the difference for Temperature × Diet × Day is displayed on the data for the chickens fed the CON diet under TN conditions.

3.5. Betaine Distribution

Heat stress decreased betaine concentrations in the ileum (117.1 vs.84.7 µmol/g, $p < 0.001$), whereas they increased in the kidney (59.6 vs.74.2 µmol/g, $p < 0.001$) and spleen (59.9 vs. 64.2 µmol/g, $p = 0.02$) (Table 3; Figure 7). Overall, chickens supplemented with BET had higher betaine concentrations in the ileum (83.2 vs. 118.6 µmol/g, $p < 0.001$), kidney (60.5 vs. 73.4 µmol/g, $p < 0.001$) and spleen (57.7 vs. 66.4 µmol/g, $p < 0.001$) than their control counterparts. However, there were interactions such that ileal betaine concentrations increased over time under TN conditions particularly in those chickens consuming the BET diet. Conversely, for the kidney and spleen, the increase in tissue betaine concentrations in response to dietary BET were greater during HS than under TN conditions (Figure 7).

Table 3. Effects of a control diet (CON) or dietary betaine (BET) on tissue betaine concentration in broilers housed under thermoneutral (TN) or heat stress (HS) conditions for 1, 3, 7 and 10 days.

Tissue	Diet (D)	Temp (T)	Day of Thermal Challenge					SED	Significance [1]
			1	2	3	7	10		
Ileum (μmol/g)	CON	TN	77.7 ^{a}	90.5 ^{b}	95.7 ^{a}	113 ^{ab}	77.7 ^{a}	21.3	T ***, D ***, T × Day **
		HS	68.1 ^{a}	48.7 ^{a}	106 ^{a}	66 ^{a}	68.1 ^{a}		D × T × Day *
	BET	TN	91 ^{ab}	153 ^{c}	152 ^{b}	165 ^{b}	91 ^{ab}		
		HS	126 ^{b}	90.1 ^{b}	84.1 ^{a}	89.4 ^{ab}	126 ^{b}		
Kidney (μmol/g)	CON	TN	41.2 ^{a}	85.1 ^{b}	54.1	47.8 ^{a}	41.2 ^{a}	8.3	T ***, D ***, T × D *
		HS	48.1 ^{a}	87 ^{b}	64.1	56.7 ^{ab}	48.1 ^{a}		Day ***, D × Day ***
	BET	TN	55.9 ^{b}	53.9 ^{a}	75.6	63.6 ^{b}	55.9 ^{b}		
		HS	90.4 ^{c}	81.2 ^{b}	77.6	88.8 ^{c}	90.4 ^{c}		
Spleen (μmol/g)	CON	TN	57.1	50.5 ^{ab}	62.7 ^{a}	60.1 ^{a}	57.1	5.1	T *, D ***, Day ***
		HS	62.5	42.5 ^{a}	75.1 ^{b}	50.9 ^{a}	62.5		D × T *, D × Day ***
	BET	TN	63.5	59.3 ^{bc}	69.6 ^{ab}	56.5 ^{a}	63.5		T × Day *, D × T × Day ***
		HS	52.5	68.5 ^{c}	72.1 ^{ab}	89.2 ^{b}	52.5		

[1] * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Other main and interactive effects $p > 0.10$. Differing superscripts within a column denotes significant ($p < 0.05$) differences for D × T × Day on a single day of the experiment.

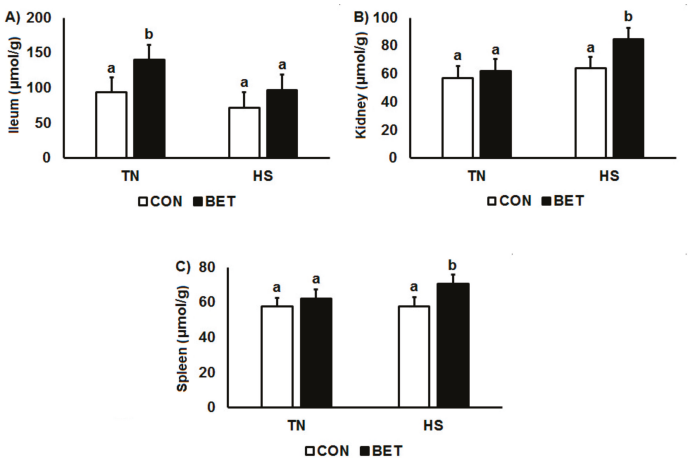

Figure 7. Changes in the distribution of betaine: (**A**) ileum, (**B**) kidney, and (**C**) spleen in broilers during thermoneutral (TN) vs. heat stress (HS) environmental challenge. Broilers were fed either a control diet (CON) or betaine diet (BET), and the mean represents the main effect of 5 time points (1, 2, 3, 7 and 10 day challenge). Means with differing superscripts denote $p < 0.05$. Refer to Table 3 for full interactive effects.

4. Discussion

Consistent with other experiments in this area [24], HS compromised the small intestinal mucosa, as evidenced by reduced villous height and crypt depth. Furthermore, as in other experiments HS reduced the TER of the small intestinal mucosa [6]. The current results indicated that HS did not influence EBD concentration in the jejunum or ileum suggesting there no discernible extravasation in the small intestine during HS. Taken together, these data support that the reduction in villous height observed was not due to an ablation of the villous, as observed in some other investigations into the effect of HS on the intestinal mucosa of pigs [25]. Alternatively, the reduction in crypt depth and absence of increases in EBD indicates that the reduction in villous height was likely due to reduced crypt cell proliferation and would be consistent with reductions in splanchnic blood flow observed in HS layers and other species.

Blood flow redistribution between organs in HS animals were quantified using radioactive microspheres to determine localised changes in capillary blood flow (CBF). In layer hens, it was found that that HS increased CBF to the skin, comb, wattles and upper respiratory tract while CBF in the digestive and reproductive tracts reduced by approximately one half [26]. In baboons, HS increased skin CBF by approximately 10% and was compensated by reductions in splanchnic and renal CBF of 35% and 27%, respectively [27]. The splanchnic bed and kidneys receive approximately 25% and 30% of cardiac output in resting animals. In addition, it has been postulated that this makes them sensitive to disruptions in reductions in blood flow, which in turn can precipitate oxidative stress and hypoxic damage [28], and is a prelude to loss of intestinal barrier function, inflammatory damage and bacterial translocation [29,30]. However, there are exceptions, and under milder forms of HS the consequences are likely reduced protein synthesis and crypt cell proliferation, resulting in a gradual decline in intestinal barrier function [6], which appears to be supported by the present findings.

The organs where differences in EBD distribution due to HS were observed were the kidney and spleen. The results from these organs were quite different, with HS increasing kidney EBD concentration, but alternatively reducing EBD concentration content in the spleen. That the kidney is a site of impairment during HS has been indicated in clinical studies [10,11]. Compared to the GIT, less is known about the etiology of HS mediated kidney damage; parallels may exist, as the kidneys also receive a large proportion of cardiac output, which can be disrupted by HS [27,31]. The interruption in blood flow may be more closely linked to decompensation and heat stroke and may not be applicable under HS [32]. Elsewhere, increased incidences of nephropathy have been recorded in rural communities in rural tropical communities, and this has been attributed to a warming climate, but it has also been postulated to be in part due to dehydration [33]. Although not quantified in this experiment, we previously quantified reductions in haematocrit in broilers [2,5] and in pigs, and this was accompanied by an apparent reduction in plasma volume, even with ad libitum water intake in all experiments [34]. There are fewer reports into the effects of HS on the spleen, and the result from this experiment was primarily driven by an increase in EBD concentration in the TN BET group, and TN and HS CON groups were not significantly different. Recently, it was observed in ducks that HS reduced spleen size, complementing production [35]. Additionally, a study observed that HS altered spleen lymphocyte populations [36]; collectively, these results might indicate that HS may compromise immune function.

Consistent with earlier experiments by our research group and others [2,5,37], betaine supplementation reduced respiration rate and rectal temperature, indicating partial amelioration of the effects of HS. Furthermore, betaine improved ileal villous height, area, crypt depth and seromuscular thickness. This result is in agreement with the experiments in young broilers up to 3 weeks of age [38,39], but differs to the results of [40], who also investigated the effects of betaine on finisher broilers. The experiment by [38] observed that the improved morphology was associated with improved resistance to coccidiosis infection, while morphology was not quantified. A study by [41] showed that dietary betaine reduced coccidiosis intestinal damage scores. As per our earlier work, betaine was shown to improve growth digestive function in HS broilers [5]. Still, it should be noted that in our earlier experiment, no improvement in ileal TER was observed. In summary, betaine was observed to improve productivity in broilers and other species, in part by improving intestinal morphology.

Although the fractional oral bioavailability of betaine has not been quantified, it has been reported to be readily available [42], and in broilers has been reported to be absorbed in the jejunum [38]. In this experiment, betaine concentrations increased with supplementation in the ileum, kidney and spleen, and previously we observed that supplementation increases plasma, liver and muscle concentrations [2]. Elevated ileal betaine concentrations may be an indication of betaine absorption across a wider area of the GIT than previously thought, while the contribution of arterial second-pass betaine metabolism cannot be excluded. Regardless, localised increases in betaine support a direct role for betaine in the ileum, as the ileum is not a site of betaine homocysteine methyltransferase expression [43], it is unlikely that the ileum is utilising betaine. Despite lower concentrations of betaine being quantified in the HS

ileum, as the ileum is not recognised as a site of betaine utilisation, the most likely role for betaine in the HS broiler ileum is as an osmolyte.

The effects of betaine on EBD distribution were that increases in EBD concentration in the HS CON group were not evident with BET, possibly indicating reduced extravasation and muscle damage. This may support the role of betaine in improving growth rates and feed conversion ratio in HS broilers [5], improved meat tenderness and reduced drip loss [2]. Likewise, increased EBD concentrations observed in the kidney of the HS CON were not evident in the HS BET group, also indicating amelioration of HS-mediated damage. As the kidney is a site of betaine homocysteine methyltransferase expression, it is possible that betaine protects the kidney through roles as a methyl donor or as an osmolyte. However, the effect of BET in the spleen was perplexing, increasing under TN but not HS conditions. Furthermore, overall reductions (independent of HS) in EBD were observed in the jejunum and ileum, which is consistent with the improvements in intestinal morphometry. Perhaps surprisingly, no effect of HS or betaine was observed on liver EBD concentration. Elsewhere, HS was observed to induce oxidative stress in the liver [28] and has been reported to be a major site of betaine distribution [38].

5. Conclusions

Supplementation of betaine partially ameliorated the physical symptoms of HS in finishing Ross-308 broilers and when supplemented betaine was widely distributed. In particular, betaine benefitted the small intestine, improving ileal resistance and villous height while reducing EBD concentrations, indicating an improvement in intestinal barrier function and gut health. It has been widely reported that HS compromises intestinal barrier function, a result that was supported by this experiment. However, by using EBD as a marker of organ dysfunction, it was apparent that a wider pattern of compromised function exists. This was evidenced in the muscle, kidney, jejunum, ileum and spleen having elevated EBD concentrations, likely reflecting underlying inflammation or damage. Importantly beneficial interactive effects with betaine were observed in muscle, jejunum, ileum and the kidneys, where elevated EBD concentrations were ameliorated by betaine. This indicates that the wide tissue distribution of betaine enables it to have multiple protective effects against HS, contributing to improved productivity and meat quality.

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animals

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Article

Effect of Feed Additives as Alternatives to In-feed Antimicrobials on Production Performance and Intestinal *Clostridium perfringens* Counts in Broiler Chickens

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Simple Summary: For many years, antibiotics were added to chicken feed to prevent disease and promote growth. This practice has been banned or voluntarily abolished in many countries. However, most countries still allow the use of in-feed ionophorous coccidiostats, which are drugs that possess both antiparasitic and antibacterial properties. Concerns related to antimicrobial resistance have led to increased focus on broiler chickens raised without the use of any antimicrobial agents, and the interest in non-antibiotic feed additives with beneficial effects on gastrointestinal health and productivity is growing. In this study, feed additives with active components belonging to the product classes probiotics, prebiotics, phytogenics and/or organic acids were assessed for their effect on intestinal health and production performance in broiler chickens. Collectively, the group of non-antibiotic feed additives improved gut health and performance, but not to the same extent as the ionophorous coccidiostat narasin. Probiotics and prebiotics had the overall best performances during coccidia challenge, phytogenics improved overall feed conversion and reduced counts of the intestinal bacterium *Clostridium perfringens*, and organic acids increased weight gain independent of age. This study provides comparable and unbiased results from testing of alternatives to antibiotics in a uniform experimental model highly relevant to commercial conditions.

Abstract: Numerous non-antibiotic feed additives (alternatives to antibiotics, ATAs) have been marketed, but few have been evaluated under uniform testing conditions modelling commercial flocks. We compared 24 ATA treatments and the ionophorous coccidiostat narasin against a diet without any feed additives. Feed conversion ratio and body weight gain were registered from day 0 to 28 in Ross 308 chickens housed on litter floor. The chickens were challenged with *Eimeria* spp., and cecal *Clostridium perfringens* (CP) counts were investigated. Active components from all ATA classes had a positive impact on intestinal health or production performance. Whereas narasin had a strong CP-reducing effect in combination with performance-promoting impact, only two ATA treatments achieved significantly beneficial effects on CP counts as well as feed conversion during the time span following *Eimeria* challenge. Active components present in these two treatments include a *Bacillus subtilis* probiotic strain, short- and medium-chain fatty acids and *Saccharomyces cerevisiae* components. Different ATA classes had beneficial impact during distinct rearing phases and on

specific performance targets, suggesting that optimizing combinations and use of active components can make ATAs even more useful tools in broiler rearing without the use of in-feed antimicrobials. Further studies of promising ATAs and ATA combinations are required.

Keywords: broilers; feed additives; probiotics; prebiotics; phytogenics; organic acids; anticoccidials; necrotic enteritis; *Clostridium perfringens*; production performance

1. Introduction

The use of antimicrobial growth promoters (AGPs) was abolished in Sweden, Norway and Denmark in 1986, 1995 and 1998–1999, respectively [1]. As a response to this development, the use of ionophorous coccidiostats (e.g., narasin) in broiler feeds increased and became more important than before [2]. In 2006, the European Union implemented a total ban of AGPs, meaning that antimicrobials other than coccidiostats and histomonostats were no longer allowed as feed additives in the poultry industry [3,4]. Coccidiostats like narasin and other ionophores are still approved in the European Union for control of coccidiosis caused by the parasitic protozoans *Eimeria* spp. in poultry.

Ionophores are primarily approved for control of coccidiosis but may also have antibacterial and antiviral properties [5]. Narasin has a well-known inhibitory effect on the potential pathogen *Clostridium perfringens* (CP), which is associated with the intestinal disease necrotic enteritis (NE) in broiler chickens [6,7]. Selected ionophores have been suggested as novel antimicrobial agents to control infectious diseases in animals as alternatives to antimicrobial classes used to treat human disease [8]. Furthermore, concerns have been raised regarding the possibility that the use of narasin and other ionophores could be associated with bacterial resistance against antimicrobials used in human medicine, and that resistant bacteria could spread to humans both by direct contact with animals and through food supply [2,9]. These considerations have led to increased focus on conventional broilers raised without the use of any in-feed antimicrobial agents, including AGPs as well as ionophores and other coccidiostats. In 2015/2016, the Norwegian broiler industry abolished the routine use of in-feed coccidiostats, including narasin [10].

The former widespread practice of supplementing broiler feeds with AGPs was mainly based on the favorable influence of these compounds on production performance [2]. Impaired production performance leading to increased production costs is a main concern associated with rearing broilers without in-feed antimicrobials. The traditionally most commonly used AGPs are predominantly active against gram-positive bacteria [11], and many of these antimicrobials have been shown to suppress the proliferation of CP in vivo [12,13] and in vitro [14–16]. Several studies report an association between increased numbers of intestinal CP and growth depression in chickens [12,17,18], and collectively these findings suggest that antibacterial activity against CP may be involved in the 'antibiotic growth effect'. Development of NE and a subclinical form of this disease is associated with impaired production performance, cholangiohepatitis and high numbers of intestinal and fecal CP [19–21]. Infection with *Eimeria* spp. is considered an important predisposing factor for CP proliferation and development of NE in chickens [22,23].

The interest in non-antibiotic feed additives (hereafter: alternatives to antibiotics, ATAs) that might facilitate the abolishment of continuous use of in-feed AGPs and coccidiostats has increased during the recent years. Numerous new feed additives have reached the global poultry feed market. Different ATAs, including products based on probiotics, prebiotics, phytogenics and/or organic acids, claim to exert beneficial effects related to productivity, intestinal functions and intestinal health in broiler chickens.

Probiotics are based on non-pathogenic and non-toxigenic live microorganisms (e.g., bacteria or yeasts) supposed to provide health benefits to the host. Possible modes of action of probiotics include colonization of the intestine, competitive exclusion of other microorganisms, production of

specific metabolites and stimulation of the immune system [24]. Two categories of probiotics are non-spore forming bacteria (e.g., *Lactobacillus* spp., *Enterococcus* spp., *Bifidobacterium* spp.) and bacterial spore formers (e.g., *Bacillus* spp.) [25]. Regulatory agencies have been reluctant to approve undefined microbial products due to the uncertainty of a consistent composition of the products. This concern has paved the way for defined probiotic products based on one or a few known strains.

Prebiotics are non-digestible feed ingredients assumed to stimulate proliferation and/or activity of intestinal microorganisms, which leads to beneficial physiological responses in the host [26]. Intake of prebiotics may increase the number of specific microbes and change the composition of the intestinal microbiota [27]. Examples of prebiotic compounds are complex carbohydrates derived from plants or yeasts, such as fructooligosaccharides (FOS), mannanoligosaccharides (MOS) and β-glucans [28,29]. In addition to selective promotion of beneficial bacteria, suggested modes of action of prebiotics are blocking pathogen adhesion, altering gene expression, affecting gut morphological structure and immunomodulation [29].

Phytogenic feed additives are based on bioactive compounds derived from plants, and a multitude of such plant products can broadly be classified as herbs or spices [28]. Examples of biologically active components and substances from plants are essential oils, oleoresins, tannins, saponins, flavonoids, alkaloids and resin acids. Various functions among plant-based products have been suggested, including antimicrobial, antiviral, antioxidative, anti-inflammatory and flavoring effects [30]. The compositional variation is considerable due to biological factors such as plant species, growing conditions, climate, harvest and manufacturing processes, and it is thus challenging to identify and evaluate the functional basis of this broad group of active components [31].

Organic acids of various lengths and their corresponding salts or esters are widely used as feed additives in livestock production and can be used individually or as blends of multiple acids. They may vary considerably in functionality due to number of carbon atoms and may be aliphatic or aromatic. Many organic acids consist of carboxylic acids and are natural constituents of animal or plant tissue or products of microbial fermentation. Industrially produced organic acids often come as salts or esters and in a coated or encapsulated form [31]. Carboxylic acids with an aliphatic chain are designated fatty acids. The subgroup short-chain fatty acids (SCFAs, 1–5 carbon atoms; C1–C5) are aliphatic compounds produced in nature by microbial fermentation of carbohydrates in the hindgut of humans and animals. The subgroup medium-chain fatty acids (MCFAs, 6–12 carbon atoms; C6–C12) are aliphatic compounds formed in nature predominantly in plants and extra-intestinal animal tissues. Suggested effects of organic acids are antibacterial activity through pH-regulation and changes in microbiota composition, immunomodulatory action and stimulation of the gut mucosa [28,29,31]. The heterogeneity of this feed additive category makes it difficult to define common properties and function, and the effects of different organic acids may vary considerably. It has been proposed that SCFAs can act directly upon the cell wall of gram-negative bacteria, and that fatty acids with longer chains can incorporate themselves into the cell membrane of gram-positive bacteria and promote leakage [32].

A multitude of studies on the impact of alternative feed additives in broiler chickens have been published. However, most studies focus on only one or a few additives within one or two ATA classes. Furthermore, these studies often differ with regard to a number of factors that may influence the results (e.g., housing of chickens, number of replicates and challenge), which makes it difficult or impossible to compare results across studies. Another problematic issue is publication bias that occurs when only results that show significant findings are reported [33]. These considerations make it relevant to study the effect of ATAs under uniform testing conditions.

The present study was conducted in order to examine the effect of commercially available ATAs from four different product classes on production performance and cecal CP counts. Feed additives were selected on the basis of being marketed with claimed beneficial effects on production performance, intestinal function and/or intestinal health in poultry. Production performance was recorded during two separate age levels; days 0–14 and days 14–28. CP counts were recorded during the fourth week of rearing, four to six days after challenge with *Eimeria* spp.

The aims of the study were to (a) evaluate the performance of the collective ATA group, (b) compare effects of classes of ATAs (probiotics/prebiotics/phytogenics/organic acids) and (c) identify active components or component combinations with beneficial effects on production performance and CP counts, with emphasis on the time span following *Eimeria* challenge.

2. Materials and Methods

2.1. Animals and Housing

Six trials were carried out at Scandinavian Poultry Research in Våler, Hedmark, Norway, using one-day-old Ross 308 broiler chickens obtained from a commercial hatchery (Nortura Samvirkekylling, Våler, Norway). The chickens were housed in floor pens of 5.6 m^2 on new wood shavings in a climate-controlled poultry research facility, with a 50/50 female-to-male ratio per pen. Water and pelleted feed were given *ad libitum*. The chickens were exposed to light for 23 h a day on the first two days. For the rest of the experimental period, the chickens were exposed to light during 2 × 8 h a day, interrupted by 4 h periods of darkness. Apart from a 10-fold dose of Paracox-5 vet. on day 17 or 18, no vaccines were administered throughout the study. The study period lasted from day of hatch until day 28. Animal experiments were approved by the national animal research authority (Norwegian Food Safety Authority, approval ID 8179), and performed in accordance with national and international guidelines for the care and use of experimental animals.

2.2. Experimental Design

In each of the six trials, a total of 5280 one-day-old Ross 308 broiler chickens were randomly allocated into six experimental groups, each group comprising 11 replicate pens with 80 chickens per pen. All trials had similar design, and included four treatment groups receiving feed with a specific ATA product or a combination of two ATA products, a positive control group (NAR) receiving feed with the polyether ionophore and coccidiostat narasin (Monteban, Elanco Animal Health, Greenfield, IN, USA), and a negative control group (NEG) receiving feed with neither antimicrobial feed additives (AGPs or coccidiostats) nor ATA products. Feed additives were added to the feeds at an inclusion rate recommended by the manufacturers. No AGP products were included in this study, and narasin was used as a sole coccidiostat in the NAR group. The chickens were fed wheat-based starter and grower diets based on Ross Broiler Nutrition Specifications adapted to Norwegian broiler production from 0 to 14 and 14 to 28 days of age, respectively (Table 1).

In the five initial trials, 20 commercially available ATA products were evaluated individually for their effect on production performance and cecal CP counts. In the sixth trial, combinations of two ATA products per treatment group were evaluated using the same outcome variables. Products included in the sixth experiment were selected for testing due to promising impact on either production performance or CP counts in the five initial experiments. Products with positive effects on production performance were combined with products with CP reducing effect in order to study potential synergy effects. Descriptions of active ingredients and dose levels of the feed additives and feed additive combinations tested are listed in Table 2. Composition of the products and dosage levels are based on information given by the feed additive manufacturers on their web sites or as a response to our request.

On day 17 (one trial) or 18 (five trials) post hatch, all treatment groups in all six trials were challenged with a 10-fold dose of Paracox-5 vet. (MSD Animal Health, Boxmeer, the Netherlands) containing live, sporulated oocysts from five attenuated strains of *Eimeria* spp. (one precocious line each of *Eimeria acervulina* [approximately 5750 oocysts per broiler], *Eimeria mitis* [approximately 11,500 oocysts], and *Eimeria tenella* [approximately 5750 oocysts], and two precocious lines of *Eimeria maxima* [approximately 3450 oocysts]) in the drinking water.

Table 1. Diet composition [1].

Chemical Composition	Starter Diet [2]	Grower Diet [3]
(g/kg feed)	0–14 days	14–28 days
Dry matter	887.2	881.3
Crude protein	239.6	222.0
Crude fat	67.8	99.6
Crude fiber	30.3	29.0
Nitrogen-free extracts	493.7	479.0
Ash	55.8	51.7
Lysine	14.0	12.9
Methionine + Cysteine	11.6	11.1
Threonine	9.4	9.0
Tryptophan	2.7	2.5
Arginine	13.8	12.7
Calcium (Ca)	9.2	7.4
Phosphorus (P)	6.3	5.9
Sodium (Na)	1.4	1.6
Potassium (K)	7.7	7.4
Chloride (Cl)	2.3	2.2
Magnesium (Mg)	1.6	1.6
NSP enzymes [4] and phytase	0.15	0.15
Metabolizable energy (MJ/kg)	12.13	12.78

[1] Mean values from diets in six trials. [2] Vitamins and minerals: Cu 15 mg/kg; Zn 82 mg/kg; Mn 126 mg/kg; Se 0.27 mg/kg; I 1.04 mg/kg; Fe 52 mg/kg; Vit.A 9575 IU; Vit.E 96 IU; Vit.D3 4994 IU; Vit.K 7.0 mg/kg; Vit.B1 4.2 mg/kg; Vit.B2 7.3 mg/kg; Vit.B3 59.7 mg/kg; Vit.B5 20.0 mg/kg; Vit.B6 12.0 mg/kg; Vit.B12 0.02 mg/kg; biotin 2.1 mg/kg; folic acid 2.9 mg/kg; choline chloride 1726 mg/kg. [3] Vitamins and minerals: Cu 15 mg/kg; Zn 82 mg/kg; Mn 128 mg/kg; Se 0.27 mg/kg; I 1.05 mg/kg; Fe 53 mg/kg; Vit.A 9488 IU; Vit.E 81 IU; Vit.D3 4983 IU; Vit.K 5.6 mg/kg; Vit.B1 3.6 mg/kg; Vit.B2 6.8 mg/kg; Vit.B3 54.0 mg/kg; Vit.B5 18.0 mg/kg; Vit.B6 11.0 mg/kg; Vit.B12 0.02 mg/kg; biotin 2.4 mg/kg; folic acid 2.7 mg/kg; choline chloride 1500 mg/kg. [4] Non-starch polysaccharide enzymes.

Table 2. Treatment ID, class of feed additives, active components and inclusion rate of feed additive products.

ID [1]	Class [2]	Active Components and Product Description [3]	Dosage [4] (Starter/Grower)
0	NEG	None	–
1	NAR	Narasin (100 g narasin/kg additive)	700/700
2	PRO	*Lactobacillus farciminis* CNMA 67/4R strain (1×10^9 cfu/gram additive)	500/500
3	PRO	*Bacillus subtilis* PB6 strain (2×10^8 cfu/gram additive)	500/500
4	PRO	One *Bacillus subtilis* strain, material no. 671265 (1.6×10^9 cfu/gram additive)	500/500
5	PRO/PRE	*Enterococcus faecium* DSM 16211 (jejunum isolate), *Bifidobacterium animalis* DSM 16284 (ileum isolate), *Lactobacillus salivarus* DSM 16351 (caeca isolate) with mix ratio 3:1:6 (total cfu/gram: 2×10^8), plant-derived fructooligosaccharides from inulin	1000/1000
6	PRE	*Saccharomyces cerevisiae* cell wall extracts (including typ. 25% β-1,3/1,6 glucans and min. 24% mannanoligosaccharides)	1000/1000
7	PRE	*Saccharomyces cerevisiae* cell wall extracts (including min. 60% purified β-1,3/1,6 glucans)	250/250
8	OA/PFA	Benzoic acid (80%–83%) and a blend of essential oils (including thymol 1.0%–1.9%, eugenol 0.5%–1.0%, and piperine 0.05%–0.1%)	300/300
9	PFA	Essential oil blend (min. 31.9%, including carvacrol, thymol, anethol and limonene)	150/150
10	OA	Medium-chain fatty acids (C6, C8 and C10)	1600/1600

Table 2. *Cont.*

ID [1]	Class [2]	Active Components and Product Description [3]	Dosage [4] (Starter/Grower)
11	OA/PFA	Short- and medium chain fatty acids (including C4 and C12), phenolic compound and organic acids	1500/1500
12	OA	Tri- and diglycerides of butyric acid (C4)	1000/1000
13	OA	Diformate derived from C1 (57% Na-formate, 39% formic acid)	3000/3000
14	OA	Lactylates (C12 and C14 esterified with lactic acid)	750/750
15	OA/PFA	Short- and medium-chain fatty acids (including monoglycerides of C3, C4, C8 and C10) and essential oils (mainly cinnamon aldehyde)	3000/2500
16	PRE	Dehydrated *Saccharomyces cerevisiae* culture with whole cells, metabolites and medium nutrients	1250/620
17	OA/PFA	Glycerol-esterified short- and medium-chain fatty acids (including C3, C4, C8 and C10) and 6% phytogenics (including essential oils, saponins and bitter and pungent substances)	750/750
18	PFA	Phytogenics including alkaloids, saponins, thymol and glyco-components derived from Yucca plants	2000/1000
19	PRE	*Saccharomyces cerevisiae* cell wall extracts (primarily mannanoligosaccharides)	800/400
20	PFA	Tall oil fatty acids from coniferous trees, including resin acids (8%–9%)	1000/1000
21	PFA	Oleoresins from turmeric (*Curcuma longa*) (4.4%) and chili peppers (genus Capsicum) (4.4%)	100/100
22	PRO/PRE +PRE	Active components of ID 5 and ID 7	1000/1000 250/250
23	PRE +PFA	Active components of ID 7 and ID 21	250/250 100/100
24	OA/PFA +PRE	Active components of ID 11 and ID 16	1500/1000 1250/625
25	OA +PRE	Active components of ID 13 and ID 16	3000/2000 1250/625

[1] Treatment ID number. [2] NEG = negative control, NAR = positive control, PRO = probiotics, PRE = prebiotics, PFA = phytogenics, and OA = organic acids. [3] Based on available information from the product manufacturers. [4] Amount added product given as grams/ton feed in starter and grower diets.

2.3. Clostridium Perfringens Quantification

On days 4, 5 and 6 after *Eimeria* challenge, 11 chickens per treatment group (1 chicken from each replicate pen) were randomly selected and humanely euthanized by cranial stunning immediately followed by cervical dislocation before necropsy. Samples of cecal contents were collected in sterile stomacher bags and directly subjected to cultivation in order to quantify CP. In brief, the samples were diluted 1:100 in peptone saline water (0.1% peptone, Difco Laboratories Inc., Detroit, US and 0.85% NaCl) and homogenized for 30 s in a stomacher (Bagmixer 400 CC, Interscience, Saint Nom, France). Serial dilutions were made with non-buffered peptone water until a dilution of 10^{-6} was reached. Aliquots of 100 µL from the dilutions 10^{-2}, 10^{-4} and 10^{-6} were plated onto sheep blood agar plates (Oxoid Blood Agar Base No.2 and 5% sheep blood, manufactured by the Norwegian Veterinary Institute, Oslo, Norway). The plates were incubated anaerobically at 37 °C for 24 h (Genbox anaer, Biomérieux, Marcy-l'Étoile, France). Single colonies with double hemolysis were counted, and colony-forming units per gram (cfu/g) cecal contents were calculated based on the given dilution. Typical colonies were selected for pure cultivation and later confirmed as CP by a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Bruker Corp., Billerica, MA, USA).

2.4. Post Mortem Examination

The small intestine of all chickens that were sampled for CP quantification was opened longitudinally and examined for pathological changes indicating NE, and scored as follows (modified from [34]): necrotic enteritis negative with no macroscopic mucosal ulcers or pseudomembranes, or necrotic enteritis positive with minimum one mucosal ulcer or pseudomembrane.

2.5. Production Performance Measurements

The amount of feed per pen was weighed when allocated and remaining feed was weighed before being discarded at feed change and at the end of the experiment. Accumulated feed intake per pen from days 0 to 14, 14 to 28 and 0 to 28 was calculated. Total live chicken weights per pen were recorded on days 0, 14 and 28, and mean body weight gain (BWG, g/chicken) and mean feed conversion ratio (FCR, g feed intake/g weight gain) per pen were calculated.

2.6. Statistical Analysis

Data on production performance and CP counts were examined on three different levels; (a) the impact of ATAs as one collective group (group level), (b) the impact of classes of ATAs (class level), and (c) the impact of individual ATA treatments (treatment level). On all levels, ATAs and the positive control with narasin-supplemented feed (NAR) were compared against the negative control with no feed additive (NEG). Frequencies of broilers with NE lesions were analyzed only on group level using Pearson's chi-squared test in Stata version 14.2 (StataCorp LLC, College Station, TX, USA). Production performance and CP count data were analyzed using regression analyses in R version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria).

Production performance data were analyzed with pen as the unit of concern. Body weight gain and feed conversion ratio was obtained in the periods 0–14 days, 14–28 days and 0–28 days for groups, classes and treatments tested in six trials. The outcome from the six different trials could not be compared directly due to intertrial variability. In order to validly compare results from six different trials, it was necessary to control for the effect of trial in the statistical analysis. The principle approach to achieve such control was to use the results from NEG in each of the six trials as indicators of trial effect. A mixed-effects model (1) with only intercept (a) was used to obtain a trial-specific random effect (ε_{Trial}) for each outcome variable (y_{Neg}) per trial based on results from NEG using the package *lme4* in R [35].

$$y_{Neg} = a + \varepsilon_{Trial} \quad (1)$$

For each of the outcome variables (y), results achieved in the different trials were adjusted with a value equal to the random effect obtained for the respective trial. Results across trials were compared using regression analysis (2) with ATA group/class/treatment (x) as fixed-effect variable and trial-specific random-effects from NEG as offset variable (ε_{Trial}). b represents the estimated parameters in the model.

$$y = \varepsilon_{Trial} + b \cdot x \quad (2)$$

The necessity of adjustment for trial effect was calculated by the intraclass correlation coefficient (ICC), which is variance explained by the random effect divided by total variance of the residuals for the model based on all observations from NEG. Extreme outlier pens that were highly influential on the estimated regression results were identified using the function *outlierTest* from the package *car* in R [36]. Residuals from the regression models were visually inspected using the functions *qqnorm* and *qqline* in R and found to follow a normal distribution. The production performance results were reported in tables as means with standard deviation. Differences from NEG with $p < 0.05$ were accepted as statistically significant differences.

CP counts in cecal samples were analyzed with individual chicken samples as unit of concern. Since the residuals from the regression model did not follow a normal distribution, the CP count

numbers were log transformed in order to fulfil this requirement. The effect of trial was controlled by adjusting for obtained random effect as described above, and subsequently regression analysis with ATA group/class/treatment as fixed-effect variable and trial-specific random-effects from NEG as offset variable was conducted. The results were reported in tables as mean $\log_{10}$ colony forming units per gram cecal content. Estimated mean $\log_{10}$ CP counts with 95% confidence interval for each treatment were presented in a graph where feed additive classes are indicated with different colors.

3. Results

3.1. Impact of the Collective ATA Group on Necrotic Enteritis, Intestinal CP Counts and Production Performance

Broilers with necrotic enteritis lesions during days 4–6 after *Eimeria* challenge constituted 8.1% among chickens from the NEG group (no feed additive, n = 198 chickens), 4.4% in the collective ATA group (24 ATA treatments, n = 792 chickens) and 0.5% in the NAR group (in-feed narasin, n = 198 chickens). Statistical analyses indicated significant difference in NE occurrence between the NEG group and the ATA group ($p < 0.05$), and between the ATA group and the NAR group ($p < 0.01$).

The ATA group reduced CP counts in intestinal contents from $\log_{10}$ 6.09 to $\log_{10}$ 5.63 cfu/g ($p = 0.005$), corresponding to a 65% reduction in non-transformed counts (Table 3). This substantial reduction was, however, moderate as compared to the very strong effect of narasin (from $\log_{10}$ 6.09 to $\log_{10}$ 2.92 cfu/g ($p < 0.001$), corresponding to a 99.9% reduction in non-transformed counts).

Table 3. Body weight gain, feed conversion ratio and *Clostridium perfringens* counts for negative control, narasin and alternatives to antibiotics [1].

Group	Days 0–14		Days 14–28		Days 0–28		CP Counts $\log_{10}$ cfu/g
	BWG g	FCR g/g	BWG g	FCR g/g	BWG g	FCR g/g	
NEG [2]	474 ± 4	1.098 ± 0.006	1240 ± 9	1.338 ± 0.005	1714 ± 11	1.248 ± 0.003	6.09 ± 0.14
NAR [3]	488 ± 6 $p = 0.032$	1.064 ± 0.008 $p < 0.001$	1337 ± 12 $p < 0.001$	1.273 ± 0.007 $p < 0.001$	1825 ± 16 $p < 0.001$	1.192 ± 0.005 $p < 0.001$	2.92 ± 0.20 $p < 0.001$
ATAs [4]	478 ± 5 $p = 0.419$	1.087 ± 0.006 $p = 0.079$	1275 ± 10 $p < 0.001$	1.317 ± 0.006 $p < 0.001$	1753 ± 12 $p = 0.002$	1.232 ± 0.004 $p < 0.001$	5.63 ± 0.16 $p = 0.005$
ICC [5]	0.35	0.61	0.43	0.35	0.42	0.28	0.08

[1] Results are reported as means ± standard deviation. Body weight gain (BWG) in grams/chicken, feed conversion ratio (FCR) in grams feed intake/grams weight gain and *Clostridium perfringens* (CP) counts as $\log_{10}$ colony forming units/gram cecal content. [2] Negative control (no feed additive); production performance data based on n = 66 pens, and CP data based on n = 198 individual chicken samples. [3] Narasin; production performance data based on n = 66 pens, and CP data based on n = 198 individual chicken samples. [4] Alternatives to antibiotics treatments; production performance data based on n = 264 pens, and CP data based on n = 792 individual chicken samples. [5] Intraclass correlation coefficient.

Both the ATA group and the NAR group had strongest beneficial impact on production performance during days 14–28, i.e., the age interval characterized by intestinal stress induced by *Eimeria* challenge on day 17 or 18. The collective ATA group demonstrated a 1.6% improvement ($p < 0.001$) in FCR during days 14 to 28 ($FCR_{14–28}$) and a 2.8% increase ($p < 0.001$) in BWG during days 14 to 28 ($BWG_{14–28}$) compared to the NEG group (Table 3). The beneficial effect of the ATA group on production performance was not as pronounced as the positive effect of narasin (4.9% improved $FCR_{14–28}$ and 7.8% increased $BWG_{14–28}$).

3.2. Impact of ATA Classes on Intestinal CP Counts and Production Performance

Four ATA classes (probiotics, PRO; prebiotics, PRE; phytogenics, PFA; organic acids, OA), a set of treatments each based on more than one ATA class (mixed products, MIX) and NAR (i.e., narasin) were compared with NEG (i.e., no feed additive) (Table 4). Although all ATA classes demonstrated

a reducing effect on numbers of CP per gram intestinal contents, only two classes (PFA and PRO) showed statistically significant reduction ($p < 0.05$). The estimated reducing impacts of PFA and PRO were 87% and 75% in non-transformed CP counts, respectively, when compared to NEG.

Table 4. Body weight gain, feed conversion ratio and *Clostridium perfringens* counts for negative control, narasin and classes of alternatives to antibiotics [1].

Class	Days 0–14		Days 14–28		Days 0–28		CP Counts $\log_{10}$ cfu/g
	BWG g	FCR g/g	BWG g	FCR g/g	BWG g	FCR g/g	
NEG [2]	474 ± 4	1.098 ± 0.006	1240 ± 9	1.338 ± 0.005	1714 ± 11	1.248 ± 0.003	6.09 ± 0.14
NAR [3]	488 ± 6 $p = 0.032$	1.064 ± 0.008 $p < 0.001$	1337 ± 12 $p < 0.001$	1.273 ± 0.007 $p < 0.001$	1825 ± 16 $p < 0.001$	1.192 ± 0.005 $p < 0.001$	2.92 ± 0.20 $p < 0.001$
PRO [4]	455 ± 8 $p = 0.012$	1.113 ± 0.009 $p = 0.118$	1283 ± 15 $p = 0.004$	1.302 ± 0.009 $p < 0.001$	1736 ± 19 $p = 0.239$	1.232 ± 0.006 $p = 0.004$	5.49 ± 0.25 $p = 0.017$
PRE [5]	479 ± 7 $p = 0.496$	1.095 ± 0.009 $p = 0.761$	1288 ± 14 $p < 0.001$	1.305 ± 0.008 $p < 0.001$	1767 ± 18 $p = 0.003$	1.229 ± 0.005 $p < 0.001$	5.70 ± 0.23 $p = 0.092$
PFA [5]	480 ± 7 $p = 0.375$	1.086 ± 0.009 $p = 0.152$	1247 ± 14 $p = 0.610$	1.323 ± 0.008 $p = 0.062$	1727 ± 17 $p = 0.457$	1.233 ± 0.005 $p = 0.004$	5.18 ± 0.23 $p < 0.001$
OA [5]	490 ± 7 $p = 0.025$	1.062 ± 0.009 $p < 0.001$	1288 ± 14 $p < 0.001$	1.325 ± 0.008 $p = 0.114$	1778 ± 17 $p < 0.001$	1.232 ± 0.005 $p = 0.002$	5.74 ± 0.23 $p = 0.130$
MIX [6]	479 ± 6 $p = 0.339$	1.087 ± 0.007 $p = 0.103$	1275 ± 11 $p = 0.002$	1.320 ± 0.007 $p = 0.007$	1754 ± 14 $p = 0.005$	1.234 ± 0.004 $p = 0.001$	5.79 ± 0.19 $p = 0.113$
ICC [7]	0.35	0.61	0.43	0.35	0.42	0.28	0.08

[1] Results are reported as means ± standard deviation. Body weight gain (BWG) in grams/chicken, feed conversion ratio (FCR) in grams feed intake/grams weight gain and *Clostridium perfringens* (CP) counts as $\log_{10}$ colony forming units/gram cecal content. [2] Negative control (no feed additive); production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [3] Narasin; production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [4] Probiotics (PRO); production performance data based on $n = 33$ pens, and CP data based on $n = 99$ individual chicken samples. [5] Prebiotics (PRE), phytogenics (PFA), organic acids (OA); production performance data based on $n = 44$ pens, and CP data based on $n = 132$ individual chicken samples. [6] Mixed products (MIX), i.e., treatments based on more than one ATA class; production performance data based on $n = 99$ pens, and CP data based on $n = 297$ individual chicken samples.[7] Intraclass correlation coefficient.

Three ATA classes (PRO, PRE and MIX) improved $FCR_{14–28}$ (1.3%–2.7% improvement, $p < 0.01$), and four classes (PRO, PRE, OA and MIX) increased $BWG_{14–28}$ (2.8%–3.9% increase, $p < 0.01$). Accumulated feed conversion during days 0 to 28 ($FCR_{0–28}$) was improved by all ATA classes (1.1%–1.5%, $p < 0.01$). However, only the OA class improved feed conversion during days 0 to 14 ($FCR_{0–14}$) significantly (3.3%, $p < 0.001$). Narasin outperformed the ATA classes at all age intervals, except for body weight gain during days 0 to 14 ($BWG_{0–14}$) and $FCR_{0–14}$, where the OA class performed similarly.

3.3. Impact of Treatments on Intestinal CP Counts and Production Performance

Intestinal CP counts were significantly reduced ($p < 0.05$) by 8 out of 24 ATA treatments (ID 3, 5, 15, 16, 18, 20, 21 and 24) as shown in Table 5. Estimated reduction in non-transformed CP counts among these eight treatments ranged from 84% to 97% when compared to NEG. Phytogenic components were present in 5/8 treatments (ID 15, 18, 20, 21 and 24), prebiotic components in 3/8 treatments (ID 5, 16 and 24), probiotic components in 2/8 treatments (ID 3 and 5) and OA components were present in 2/8 treatments (ID 15 and 24). Mean $\log_{10}$ CP counts with 95% confidence interval for each ATA treatment are shown in Figure 1.

Table 5. Body weight gain, feed conversion ratio and *Clostridium perfringens* counts for negative control, narasin and alternatives to antibiotics treatments [1].

ID-Class	Days 0–14		Days 14–28		Days 0–28		CP Counts log_{10} cfu/g
	BWG g	FCR g/g	BWG g	FCR g/g	BWG g	FCR g/g	
0-NEG [2]	474 ± 4	1.098 ± 0.006	1240 ± 9	1.338 ± 0.005	1714 ± 11	1.248 ± 0.003	6.09 ± 0.14
1-NAR [3]	488 ± 6	1.064 ± 0.008	1337 ± 12	1.273 ± 0.007	1825 ± 16	1.192 ± 0.005	2.92 ± 0.20
	$p = 0.032$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
2-PRO [4]	452 ± 11	1.118 ± 0.014	1285 ± 22	1.305 ± 0.012	1735 ± 29	1.236 ± 0.008	5.46 ± 0.38
	$p = 0.049$	$p = 0.153$	$p = 0.044$	$p = 0.007$	$p = 0.455$	$p = 0.120$	$p = 0.097$
3-PRO [4]	451 ± 11	1.110 ± 0.014	1273 ± 22	1.307 ± 0.012	1723 ± 29	1.235 ± 0.008	5.11 ± 0.38
	$p = 0.044$	$p = 0.383$	$p = 0.132$	$p = 0.012$	$p = 0.740$	$p = 0.094$	$p = 0.010$
4-PRO [4]	462 ± 11	1.111 ± 0.014	1290 ± 22	1.295 ± 0.012	1751 ± 29	1.224 ± 0.008	5.90 ± 0.38
	$p = 0.274$	$p = 0.357$	$p = 0.024$	$p < 0.001$	$p = 0.198$	$p = 0.002$	$p = 0.623$
5-MIX [4]	472 ± 11	1.084 ± 0.014	1268 ± 22	1.329 ± 0.012	1739 ± 29	1.244 ± 0.008	5.21 ± 0.38
	$p = 0.872$	$p = 0.304$	$p = 0.207$	$p = 0.459$	$p = 0.378$	$p = 0.579$	$p = 0.021$
6-PRE [4]	476 ± 11	1.112 ± 0.014	1305 ± 22	1.280 ± 0.012	1782 ± 29	1.216 ± 0.008	5.98 ± 0.38
	$p = 0.854$	$p = 0.324$	$p = 0.004$	$p < 0.001$	$p = 0.023$	$p < 0.001$	$p = 0.782$
7-PRE [4]	470 ± 11	1.106 ± 0.014	1311 ± 22	1.269 ± 0.012	1781 ± 29	1.211 ± 0.008	5.91 ± 0.38
	$p = 0.731$	$p = 0.544$	$p = 0.002$	$p < 0.001$	$p = 0.018$	$p < 0.001$	$p = 0.637$
8-MIX [4]	469 ± 11	1.093 ± 0.014	1293 ± 22	1.280 ± 0.012	1763 ± 29	1.208 ± 0.008	6.05 ± 0.38
	$p = 0.672$	$p = 0.708$	$p = 0.016$	$p < 0.001$	$p = 0.086$	$p < 0.001$	$p = 0.928$
9-PFA [4]	459 ± 11	1.108 ± 0.014	1288 ± 22	1.284 ± 0.012	1747 ± 29	1.221 ± 0.008	5.89 ± 0.38
	$p = 0.178$	$p = 0.480$	$p = 0.030$	$p < 0.001$	$p = 0.243$	$p < 0.001$	$p = 0.600$
10-OA [4]	499 ± 11	1.073 ± 0.014	1280 ± 22	1.327 ± 0.012	1780 ± 29	1.233 ± 0.008	5.76 ± 0.38
	$p = 0.029$	$p = 0.070$	$p = 0.072$	$p = 0.368$	$p = 0.021$	$p = 0.051$	$p = 0.395$
11-MIX [4]	511 ± 11	1.037 ± 0.014	1335 ± 22	1.317 ± 0.012	1847 ± 29	1.215 ± 0.008	6.22 ± 0.38
	$p = 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.092$	$p < 0.001$	$p < 0.001$	$p = 0.720$
12-OA [4]	494 ± 11	1.038 ± 0.014	1287 ± 22	1.324 ± 0.012	1782 ± 29	1.223 ± 0.008	5.62 ± 0.38
	$p = 0.078$	$p < 0.001$	$p = 0.034$	$p = 0.252$	$p = 0.017$	$p = 0.001$	$p = 0.222$
13-OA [4]	501 ± 11	1.028 ± 0.014	1318 ± 22	1.311 ± 0.012	1820 ± 29	1.208 ± 0.008	6.05 ± 0.38
	$p = 0.019$	$p < 0.001$	$p < 0.001$	$p = 0.031$	$p < 0.001$	$p < 0.001$	$p = 0.918$
14-OA [4]	465 ± 11	1.108 ± 0.014	1266 ± 22	1.340 ± 0.012	1730 ± 29	1.263 ± 0.008	5.54 ± 0.38
	$p = 0.423$	$p = 0.469$	$p = 0.237$	$p = 0.884$	$p = 0.567$	$p = 0.058$	$p = 0.147$
15-MIX [4]	476 ± 11	1.097 ± 0.014	1278 ± 22	1.338 ± 0.012	1754 ± 29	1.255 ± 0.008	5.18 ± 0.38
	$p = 0.845$	$p = 0.939$	$p = 0.085$	$p = 0.977$	$p = 0.165$	$p = 0.344$	$p = 0.017$
16-PRE [4]	485 ± 11	1.085 ± 0.014	1304 ± 22	1.335 ± 0.012	1788 ± 29	1.251 ± 0.008	4.76 ± 0.38
	$p = 0.352$	$p = 0.346$	$p = 0.004$	$p = 0.822$	$p = 0.009$	$p = 0.669$	$p < 0.001$
17-MIX [4]	458 ± 11	1.105 ± 0.014	1228 ± 22	1.354 ± 0.012	1685 ± 29	1.270 ± 0.008	6.33 ± 0.38
	$p = 0.157$	$p = 0.588$	$p = 0.593$	$p = 0.185$	$p = 0.316$	$p = 0.004$	$p = 0.518$
18-PFA [4]	491 ± 11	1.067 ± 0.014	1226 ± 22	1.353 ± 0.012	1717 ± 29	1.243 ± 0.008	5.31 ± 0.38
	$p = 0.132$	$p = 0.025$	$p = 0.524$	$p = 0.216$	$p = 0.926$	$p = 0.552$	$p = 0.040$
19-PRE [4]	485 ± 11	1.078 ± 0.014	1229 ± 22	1.336 ± 0.012	1713 ± 29	1.237 ± 0.008	6.12 ± 0.38
	$p = 0.371$	$p = 0.158$	$p = 0.624$	$p = 0.850$	$p = 0.971$	$p = 0.156$	$p = 0.918$
20-PFA [4]	486 ± 11	1.091 ± 0.014	1228 ± 22	1.334 ± 0.012	1713 ± 29	1.242 ± 0.008	4.95 ± 0.38
	$p = 0.301$	$p = 0.590$	$p = 0.592$	$p = 0.748$	$p = 0.987$	$p = 0.428$	$p = 0.003$
21-PFA [4]	485 ± 11	1.077 ± 0.014	1246 ± 22	1.321 ± 0.012	1730 ± 29	1.226 ± 0.008	4.59 ± 0.38
	$p = 0.330$	$p = 0.128$	$p = 0.799$	$p = 0.170$	$p = 0.566$	$p = 0.004$	$p < 0.001$
22-MIX [4]	486 ± 11	1.089 ± 0.014	1270 ± 22	1.320 ± 0.012	1755 ± 29	1.231 ± 0.008	5.56 ± 0.38
	$p = 0.301$	$p = 0.502$	$p = 0.179$	$p = 0.146$	$p = 0.147$	$p = 0.028$	$p = 0.168$
23-MIX [4]	484 ± 11	1.083 ± 0.014	1251 ± 22	1.327 ± 0.012	1736 ± 29	1.235 ± 0.008	5.94 ± 0.38
	$p = 0.355$	$p = 0.273$	$p = 0.612$	$p = 0.378$	$p = 0.448$	$p = 0.090$	$p = 0.694$
24-MIX [4]	494 ± 11	1.086 ± 0.014	1292 ± 22	1.307 ± 0.012	1786 ± 29	1.222 ± 0.008	5.30 ± 0.38
	$p = 0.078$	$p = 0.386$	$p = 0.018$	$p = 0.014$	$p = 0.011$	$p < 0.001$	$p = 0.037$
25-MIX [4]	464 ± 11	1.105 ± 0.014	1255 ± 22	1.311 ± 0.012	1720 ± 29	1.229 ± 0.008	6.33 ± 0.38
	$p = 0.394$	$p = 0.610$	$p = 0.489$	$p = 0.028$	$p = 0.844$	$p = 0.013$	$p = 0.518$
ICC [5]	0.35	0.61	0.43	0.35	0.42	0.28	0.08

[1] Results are reported as means ± standard deviation. Body weight gain (BWG) in grams/chicken, feed conversion ratio (FCR) in grams feed intake/grams weight gain and *Clostridium perfringens* (CP) counts as log_{10} colony forming units/gram cecal content. [2] Negative control (no feed additive); production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [3] Narasin; production performance data based on $n = 66$ pens, and CP data based on $n = 198$ individual chicken samples. [4] Probiotics (PRO), prebiotics (PRE), phytogenics (PFA), organic acids (OA), mixed products (MIX); production performance data based on $n = 11$ pens, and CP data based on $n = 33$ individual chicken samples. [5] Intraclass correlation coefficient.

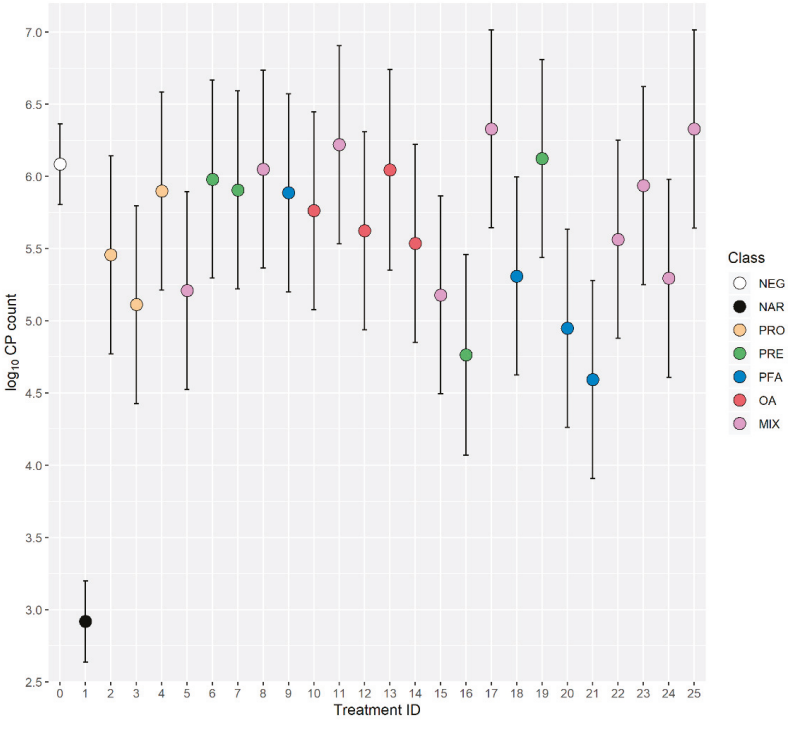

Figure 1. Cecal *Clostridium perfringens* (CP) counts with 95% confidence intervals. Negative control (NEG) is treatment 0, narasin (NAR) is treatment 1, probiotics (PRO) are treatments 2–4, prebiotics (PRE) are treatments 6, 7, 16 and 19, phytogenics (PFA) are treatments 9, 18, 20 and 21, organic acids (OA) are treatments 10, 12, 13 and 14, and mixed products (MIX) are treatments 5, 8, 11, 15, 17 and 22–25.

$FCR_{14–28}$ was improved ($p < 0.05$) by 10/24 tested ATA treatments (Table 5). Five of these treatments (ID 4, 6, 7, 8 and 9) achieved $FCR_{14–28}$ improvements (3.2% to 5.2%, $p < 0.001$) that returned the same significance level as narasin (4.9% improvement, $p < 0.001$). These five treatments had active components classified as probiotics (ID 4), prebiotics (ID 6 and 7), phytogenics (ID 8 and 9) or organic acids (ID 8). In total, 13/24 ATA treatments improved $FCR_{0–28}$ (1.4% to 3.2% improvement, $p < 0.05$). Seven of these treatments (ID 6, 7, 8, 9, 11, 13 and 24) achieved improvements in FCR $_{0–28}$ that returned the same significance level (2.1% to 3.2% improvement, $p < 0.001$) as narasin (4.5% improvement, $p < 0.001$).

$BWG_{14–28}$ and body weight gain during days 0 to 28 ($BWG_{0–28}$) were increased by 10/24 and 8/24 ATA treatments, respectively. Two treatments (ID 11 and 13) excelled in increasing both these parameters, with a significance level similar to narasin ($p < 0.001$).

In the sixth trial, two-product combinations of treatments with predominantly CP-reducing impact (ID 5, 16 and 21) and treatments with predominantly production performance-promoting impact (ID 7, 11 and 13) were evaluated (comprising treatment ID 22–25 in Table 2). Treatment 16 did not appear to reduce the FCR-improving effect of treatments 11 and 13 (Table 5) but tended to diminish the growth promoting impact of these treatments. Treatment 5 seemed to diminish the FCR-improving effect and remove the growth-promoting effect of treatment 7. Treatment 21 appeared to reduce or remove the improvement in FCR and to remove the growth-promoting effect of treatment 7. On the other hand, treatment 7 seemed to remove the CP-reducing impact of treatments 5 and 21, and treatment 13 appeared to remove the CP-reducing impact of treatment 16. In contrast to these

results, treatment 11 did not appear to impair the CP-reducing impact of treatment 16. As a result of these interactions between predominantly CP-reducing and production performance-promoting single treatments, treatment 24 was the only one of four tested product combinations (ID 22–25, Table 5) with beneficial effects on production performance variables as well as CP counts.

3.4. Active Components with Combined Beneficial Effects on $FCR_{14–28}$ and CP Counts

In total, 10/24 and 8/24 tested treatments improved ($p < 0.05$) $FCR_{14–28}$ and CP counts, respectively. Collectively these treatments comprised a group of 16 treatments; 14 treatments either improved $FCR_{14–28}$ or reduced CP counts, and only two treatments (ID 3 and 24) influenced both $FCR_{14–28}$ and CP counts in a beneficial way. One of the two superior treatments according to these criteria was a probiotic (ID 3) with the *Bacillus subtilis* PB6 strain as the only active component. The other treatment (ID 24) was a combination of three ATA classes (OA/PFA/PRE) and two products; one product (ID 16) containing whole cells of *Saccharomyces cerevisiae* and its metabolites, and another (ID 11) being a mixture of SCFAs (including C4), MCFAs (including C12) and a phenolic compound.

4. Discussion

The collective group of 24 ATA treatments tested in this study reduced the occurrence of NE and reduced intestinal CP counts after *Eimeria* challenge. Production performance measured as BWG and FCR was improved by the collective ATA group, but not significantly during the phase prior to *Eimeria* challenge. These results indicate a beneficial effect of several ATAs when chickens are exposed to mild to moderate intestinal stress. The favorable effects of the polyether ionophore narasin on CP counts and production performance were numerically and in part significantly stronger than the effect of the collective group of ATA treatments.

In this study, the chickens were challenged orally with five precocious lines of *Eimeria* spp. as a predisposing factor for NE. NE is expected to appear during oocyst excretion, which begins between three and four days following inoculation with precocious *Eimeria acervulina* [37] and *Eimeria mitis* lines, and presumably later with precocious *Eimeria maxima* and *Eimeria tenella* lines [38]. In a previous study with a similar type of *Eimeria* challenge, most gut damage was detected four to six days after inoculation [39]. Postmortem examinations in this study confirmed the presence of NE during this time span after *Eimeria* challenge. Intestinal CP counts are strongly associated with NE [19,34,40]. Furthermore, increased occurrence of NE in commercial broiler flocks has been associated with impaired accumulated FCR at slaughter [20]. Weakened production performance is likely to be most pronounced during the part of the rearing phase that is affected by NE. Based on the considerations mentioned above we chose to emphasize CP counts on days four to six after *Eimeria* challenge and $FCR_{14–28}$ in our analyses of effect of ATAs on intestinal health.

The evaluation of ATAs can rest on different criteria, depending on point of view, practical circumstances and current health problems. Disease conditions associated with increased metabolism and rapid growth in broilers (in particular cardiovascular and musculoskeletal disorders) are generally important, and it has been claimed that such conditions cause greater economic loss than infectious agents [41]. In a recent study, it was found that chickens with higher body weight and BWG were predisposed to develop more severe NE lesions when challenged with CP [42]. Although attractive in the short run, increased weight gain may therefore come at a cost not only to chicken health and welfare but also to the farmers' economy and a sustainable use of feed resources. In light of this consideration, we have emphasized the effect on FCR as production performance parameter, because it is an indicator of intestinal health as well as resource efficiency.

The only two ATA treatments (ID 3 and 24) with a combined beneficial effect on CP counts (84 to 89% reduction, $p < 0.05$) and $FCR_{14–28}$ (2.3% improvement, $p < 0.05$) were based on different types of active components. One of the treatments (ID 3) was a mono-strain (*Bacillus subtilis* strain PB6) spore-forming bacterial probiotic. This probiotic strain has been reported to inhibit CP in vitro [43] and improve FCR [44], which is in agreement with our findings.

The other treatment (ID 24) was based on a heterogeneous collection of active components including short- and medium-chain fatty acids, a phenolic compound and dehydrated whole cells and metabolites of the yeast *Saccharomyces cerevisiae* (SC). This treatment comprised two commercial products that were also tested individually (ID 11 and 16). Whereas the yeast product (ID 16) alone demonstrated a 95% reduction ($p < 0.001$) in non-transformed CP counts, the product containing a blend of organic acids and a phenolic compound (ID 11) had no reducing impact on CP. Viewed against this background it seems probable that the CP reducing effect of treatment 24 was mainly associated with one or several yeast components found in treatment 16. In addition to treatment 24, three other ATA treatments based on the yeast SC were tested. These treatments, which were based on SC cell wall extracts (ID 6, 7 and 19), did not reduce CP counts to an extent that was significant with the sample size and/or feed additive dosage used in our study, whilst the treatments based on SC whole cells and metabolites (ID 16 and 24) did. No previous reports on the effect of SC metabolites and SC whole cells on CP counts in broilers have been found. Regarding yeast cell wall extracts, previously published literature has indicated both significant [45] and non-significant [46] CP-reducing impact. Our results indicate that whole cells and/or metabolites of SC inhibited intestinal CP growth more efficiently than SC cell wall extracts with the product inclusion levels used in this study.

One of the active components in treatment 11 was lauric acid (C12), a MCFA that has been demonstrated to inhibit CP in vitro [47]. Treatment 11 did, as mentioned above, not reduce CP counts when used as sole feed additive in this study. Possible explanations include too low concentration of lauric acid and/or interfering effects by other treatment components.

Regarding production performance, the combination of treatments 11 and 16 (i.e., treatment 24) had a significantly beneficial effect on $FCR_{14–28}$. However, neither of these two treatments improved $FCR_{14–28}$ when tested individually. This finding suggests a synergy effect with regard to $FCR_{14–28}$ between active components present in the two products. Beneficial effects of dietary supplementation of whole cells and metabolites of SC on production performance in broilers have been reported by others [48].

The combination of SCFAs (including butyric acid-C4), MCFAs (including lauric acid-C12) and a phenolic compound in treatment 11 generated the numerically highest weight gain ($BWG_{0–14}$ and $BWG_{14–28}$) of all ATA treatments in this study but had no apparent impact on CP counts. This result suggests that rapid growth is possible in the presence of relatively high cecal CP counts. A possible explanation could be that this treatment reduced the counts of virulent CP strains (e.g., strains harbouring the *netB* gene) or the expression of virulence factors (e.g., the NetB toxin), but not the total CP counts. Treatment 11 might also have influenced the intestinal microbiota in a way that neutralized the negative impact of high CP counts.

Six of 24 ATA treatments (ID 5, 15, 16, 18, 20 and 21) were associated with reduced CP counts (at least 83% reduction in non-transformed counts, $p < 0.05$) without improving $FCR_{14–28}$ significantly. Treatment 21 had a very strong reducing impact on CP counts (a 97% reduction, $p < 0.001$) and improved $FCR_{0–28}$ (1.8%, $p < 0.01$), but had only a numerically (1.3%, non-significant) beneficial impact on $FCR_{14–28}$. Active components of treatment 21 included oleoresins from turmeric (*Curcuma longa*) and chili peppers (genus *Capsicum*). These results are in agreement with reports on inhibitory activity against CP of turmeric extracts [49], reduced gut lesion scores in CP-challenged broilers treated with *Capsicum* and *Curcuma* oleoresins [50,51] and improved cumulative FCR of turmeric powder [52]. Treatment 20 was based on tall oil fatty acids from coniferous trees including resin acids. Resin acids have been reported to inhibit CP in vitro [53], and our results suggest similar effects in vivo.

Treatments 22–25 were tested in a final trial intended to evaluate two-product combinations of treatments improving production performance and treatments with CP-reducing impact. Treatment 24 was the only combination with beneficial impact on both CP counts and production results. These results suggest that the interaction between predominantly CP-reducing and production-promoting components vary substantially. Among three tested CP-reducing treatments in the final trial (ID 5, 16 and 21), a treatment based on dehydrated SC culture with whole cells and metabolites (ID 16)

was the least impairing with regard to the production-promoting effects of its combination treatment. Among the three tested production performance-improving treatments (ID 7, 11 and 13), treatment 11 based on short- and medium-chain fatty acids and other components was the only one that did not impair the CP-reducing impact of its combination treatment. More work is needed to identify the role of the different components in treatments 11 and 16, and whether the beneficial interaction of these components also can be extended to include other CP-reducing and production-promoting components.

Our findings indicate that a reduction of CP counts induced by ATAs was not always associated with improved production performance. Lack of a positive impact on feed efficiency and growth rate has also been documented with regard to ionophores under certain conditions [54], in spite of these compounds' suppressing effect on CP counts. However, when used at recommended concentrations in broiler flocks exposed to coccidia, the net effect of ionophores is usually improved performance. In our study, considerably improved performance combined with a strong CP-reducing effect of the ionophore narasin was present. These results confirm that our challenge model worked as expected, and that the in-feed concentration of narasin was within the optimal range. The reason why some of the ATAs with CP-reducing effect in our study did not induce a significantly positive net impact on production performance under the same test conditions remains unclear. Possible explanations may be that the inhibiting effect on CP was accompanied by reduced ability to utilize feed efficiently and/or establishment of another performance-impairing intestinal microbiota.

Eight of 24 ATA treatments (ID 2, 4, 6, 7, 8, 9, 13 and 25) improved $FCR_{14–28}$ (at least 2.0% improvement, $p < 0.05$) without reducing CP counts significantly. One of these treatments (ID 4) was a mono-strain *Bacillus subtilis* probiotic. Data from other studies demonstrate the capacity of *Bacillus subtilis* strains to suppress the growth of CP and improve production performance and intestinal morphology [55–57]. However, the favorable impact on $FCR_{14–28}$ in this study might have been caused by other mechanisms than inhibition of CP growth. Suggested modes of action associated with probiotics are maintenance of balanced microbial populations, modulation of the host immune system, promotion of epithelial barrier integrity and alteration of villus length and crypt depth [44,58–61].

Two (ID 6 and 7) of the treatments improving $FCR_{14–28}$ but not CP counts contained cell wall extracts from the yeast SC. Both treatments 6 and 7 had a considerably beneficial impact on $FCR_{14–28}$ (estimated 4.3 and 5.2% reduction, respectively). Of the SC cell wall-based treatments, the products with the apparently highest content of β-glucans (ID 6 and 7) had the best effect on $FCR_{14–28}$ as compared with the other yeast cell wall-based treatment in our study (ID 19). Treatment 7, containing minimum 60% purified β-1.3/1.6 glucans, even outperformed narasin numerically with regard to $FCR_{14–28}$. These findings suggest that SC-derived β-glucans are potent when it comes to improvement of FCR in broilers exposed to *Eimeria* spp. Beneficial effects of yeast β-glucans on performance in broilers are supported by some [62,63] and in contradiction with results from other previous reports [64,65]. Possible explanations for the $FCR_{14–28}$-promoting effect of feed additives containing β-glucans are modulations of the immune response [62,64].

Two other treatments (ID 8 and 9) with favorable effect on $FCR_{14–28}$ without significant reduction of CP counts contained essential plant oils. Essential oil components in treatments 8 and 9 included thymol (in both treatments), eugenol and piperine (in treatment 8) and carvacrol, anethol and limonene (in treatment 9). In treatment 8, essential oils were combined with benzoic acid. Published results on effects and mode of action of essential oils suggest that several of these compounds inhibit the growth of CP [66–68], although the findings are not always clear cut [69], or they show no effect on CP counts [70]. Reports on mitigation of gut lesions in chickens challenged with CP [67,71] underpin the view that at least thymol and carvacrol suppress the pathogenic action of CP. Studies on the effect of essential oil components on production performance reveal variable results. One study reports a negative effect on broiler performance using a blend of thymol, eugenol, curcumin and piperine [70], another describe a non-significant tendency of improved $FCR_{14–28}$ using a blend of carvacrol and thymol [71], and a third study presents significant improvement of $FCR_{0–28}$ by carvacrol but not by thymol [72]. The lack of standardization of studies, including variable feed additive dosage and different combinations of

active compounds, makes comparison of results from different studies difficult. The interpretation of results is further complicated by the multiple suggested effects of different essential oils, including antibacterial and antioxidant properties, enhancement of the immune system, and stimulation of digestive secretions and blood circulation [73,74]. Regardless of mechanism, these two predominantly phytogenic feed additives (ID 8 and 9) had a pronounced beneficial effect on $FCR_{14–28}$ on par with narasin in the current study.

The apparent lack of a CP-reducing effect of yeast cell wall extracts, essential oils and other active components associated with improved $FCR_{14–28}$ in this study may in part be related to experimental design. As observed from our results (Table 5), estimated CP count reductions of 76% or less (e.g., treatment ID 2 and 14) returned non-significant (>0.05) *p*-values. The main reason for this low statistical power was high variance of CP counts in individual observations within each treatment, leading to imprecise estimates. Our experiments were designed with 33 replicates of individual CP counts per ATA treatment, and this sample size returned relatively wide confidence intervals (as shown in Figure 1). The statistical analysis involving the whole ATA group (Table 3) indicated that when 792 individual samples with a $\log_{10}$ 5.63 CP estimate were compared with the NEG group with 198 individual samples and a $\log_{10}$ 6.09 estimate (corresponding to a 65% difference), this difference was significant ($p = 0.005$).

The ATAs did not suppress CP counts to the same extent as the ionophorous coccidiostat narasin. The superior results of narasin in this respect were most likely due to the strong antibacterial effect of this compound. Narasin has been reported with inhibitory effect on CP growth similar to or better than antibiotics used as drinking water medication for poultry [75].

Different ATAs can add value to the broiler chicken industry in several ways. Some improve BWG and/or FCR, others inhibit growth of CP or have a beneficial effect on both production performance parameters and intestinal CP counts. The use of specific ATAs could possibly be targeted to specific age intervals or current health status in the flock. Future studies of the impact of ATAs on intestinal CP counts would most likely benefit from modified sampling protocols and quantification methods. Study designs that were useful for investigating the effect of AGPs and ionophorous coccidiostats should not be copied without reservation when studying non-antibiotic alternative feed additives. Finally, a less pronounced effect than narasin of selected ATAs on production performance and/or CP counts in this study does not necessarily mean that the impact is of no importance to broiler health and production economy.

In this study, ATA classes displayed distinct performance profiles. The probiotic class reduced CP counts and improved production performance during the time period with intestinal stress (days 14–28), but impaired weight gain during days 0–14. The prebiotic class improved production performance during days 14–28 and had a non-significantly reducing impact on CP counts. The phytogenic class had a markedly reducing impact on CP counts and improved $FCR_{0–28}$. The organic acid class increased weight gain throughout the study period and improved $FCR_{0–14}$ but did not reduce CP counts significantly. These findings suggest that employing ATA classes for specific purposes may be useful. As an example, combining probiotic and organic acid treatments might boost production performance throughout the grow-out period and at the same time reduce CP counts during intestinal stress. In this study, we tested other ATA class combinations with variable results, indicating the need for testing of specific combinations of active components within the ATA classes.

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Effect of Housing System and Rosemary and Cinnamon Essential Oils on Layers Performance, Egg Quality, Haematological Traits, Blood Chemistry, Immunity, and Antioxidant

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Simple Summary: The current study aimed to investigate the effects of a housing system, and dietary supplementation of rosemary and cinnamon essential oils on layers performance and egg quality. A factorial arrangement (2 × 3) was performed including two housing systems (floor and cage) and three different types of essential oils (0, 300 mg/kg diet of rosemary and 300 mg/kg diet of cinnamon essential oils) to study their effects on the productive performance, egg quality, immunity, oxidative stress and haematology of laying hens during the production stages. The data suggested that the supplementation of rosemary and cinnamon essential oils in laying hen diet showed significant positive effects on hen performance and egg production. Additionally, the different housing systems did not result in any positive or negative impact on these traits.

Abstract: Housing system and nutrition are non-genetic factors that can improve the well-being of animals to obtain higher quality products. A better understanding of how different housing systems and essential oils can influence the performance of layers is very important at the research and commercial levels. The current study aimed to investigate the effects of a housing system and dietary supplementation of rosemary and cinnamon essential oils on layers' performance and egg quality. A factorial arrangement (2 × 3) was performed include two housing systems (floor and cage) and three different types of essential oils (0, 300 mg/kg diet of rosemary and 300 mg/kg diet of cinnamon essential oils) to study their effects on the productive performance, egg quality, immunity, oxidative stress and haematology of ISA brown laying hens during the production stages (from 28 to 76 weeks of age). Birds were randomly divided into two groups each comprising of 1500 birds; the first group was moved from the litter to reared laying cages while the second group was floor reared. Each group was randomly divided into three groups, the first was considered as a control group, the second treated with rosemary essential oil, and the third with cinnamon essential oil. The differences in egg production and weight, egg quality, feed intake and conversion, blood picture and chemistry, immunity, and antioxidant parameters between the different housing systems (floor and cage) were not significant at ($p < 0.05$ or 0.01). On the other hand, the egg production and weight, Haugh

unit, feed intake and conversion, blood cholesterol, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), urea, Ca, P, immunity, and antioxidant parameters were significantly ($p < 0.05$ or 0.01) better in rosemary and cinnamon groups than in the control group. Furthermore, the results of dietary supplementation with rosemary and cinnamon were very close. Regarding egg production and weight, there were no significant differences due to the interactions. The differences in egg mass among the interactions were also not significant except at 68–76 weeks, where the cage × cinnamon group was the highest. Under the floor rearing system, birds that were fed a diet supplemented with or without essential oils (EOs) consumed more feed than those raised under the cage system. Regarding feed conversion rate (FCR), the differences among the interactions were not significant except at 44–52, 52–60 and 68–76 weeks, where the cage × cinnamon group was the lowest. Excluding glutathione peroxidase (GPx) activity ($p < 0.001$), all immunity and antioxidant indices were not statistically different as a consequence of the interaction among EOs and housing systems. Additionally, the highest levels of phosphorus were observed for layers fed diets enriched with cinnamon oil with the cage or floor system. In conclusion, the data suggested that supplementation of rosemary and cinnamon essential oils in laying hen diet showed significantly positive effects on hen performance and egg production. Cholesterol, liver and kidney functions, immunity, and antioxidant parameters improved with rosemary and cinnamon supplementation when compared to the control. Additionally, the different housing systems did not result in any positive or negative impact on these traits.

Keywords: antioxidant; essential oils; housing system; immunity; ISA brown; production

1. Introduction

Housing systems have always had an impact on animal welfare and performance [1–3]. Housing systems, as a non-genetic factor, can improve the well-being of animals to obtain higher quality products. A better understanding of how different housing systems can influence the performance of layers is required. The housing system could influence both the laying hen's performance and egg quality traits [4]. Englmaierová et al. [4] found that the highest egg production, lowest daily feed consumption, and feed conversion ratio were measured in cages compared to litter. Moreover, higher eggshell, yolk index, and albumen qualities were observed in cages. El-Deek and El-Sabrout [5] concluded that the maintenance of hens in enriched cages and with outdoor access would make it easier for the layers to express their natural behaviour, which has a favourable effect on their welfare and production. Additionally, consumers are recently interested in poultry products originating from alternative housing systems [6], which are natural, organic and have less content of substances that can endanger human health.

In recent decades, plants' oils have been used routinely in chicken farms for keeping chickens healthy and enhancing their productive performance because they contain active components which exert positive effects on physiological processes and have medicinal effects such as antibacterial, anti-inflammatory, and antioxidant [7–10]. There is a global trend in restricting the use of antibiotic growth promoters in the animal diet [11] and finding alternative solutions to maintain current animal production efficiency. Essential oils (EOs) have great potential among the alternatives and are generally regarded as safer and residue-free [12]. Due to their preventive and curative properties, species of the Labiatae family have enjoyed a rich tradition of flavoring and pharmaceutical use. Rosemary (*Rosmarinus officinalis*) is an herb that belongs to this family and has been recognized as the plant with the highest antioxidative activity [13]. Rosmarinic acid, camphor, and the antioxidants carnosic acid and carnosol are the most important organic chemicals, which have been already extracted from rosemary [14,15]. The supplementation of rosemary oil (200 mg/kg) in the laying hen's diet led to a significant improvement in feed conversion and an increase in the Haugh unit (key indicators of

internal egg quality) of the egg as well as a larger egg weight [16]. It was also determined that rosemary oil exhibited higher antimicrobial activity than the control (commercial diet) by reducing the *E. coli* concentration in feces. Additionally, using rosemary as a natural antioxidant can decrease plasma total lipids when compared to the control, while LDL-cholesterol and total cholesterol can be insignificantly decreased [7]. Supplementation of 1% rosemary can also improve feed conversion and decreased malonaldehyede (MDA) formation in egg yolk, and has been shown to have a positive impact on oxidative stability of eggshell storage [7]. Moreover, Alagawany and Abd El-Hack [17] concluded that rosemary supplemented up to 6 g/kg diet can be used as an effective feed additive to improve performance, immunity and antioxidant status in laying hens.

On the other hand, cinnamon (*Cinnamomum zeylanicum*) is a common herb and is produced from the bark of the cinnamon tree. Cinnamon herb or its derivatives can serve as a hepatic stimulant by increasing bile secretion, removing toxins, and regulating hydration and can be used as a growth enhancer. Additionally, nutritional aspects of cinnamon powder or its derivatives include positive impacts regarding growth curve, digestion, absorption, activity of gut microbiota, immunity, as well as improved feed utilization and public health of poultry [18]. As a conclusion of Şimşek et al.'s [9] study, working on laying quails, cinnamon essential oil supplementation into a diet with a 200 ppm level increased egg production, eggshell quality, and improved the feed conversion ratio. On the other hand, rosemary supplementation with the same amount did not result in any positive or negative effects on egg production traits while the mixture of both of them had a negative effect on egg weight.

The data concerning the effect of different essential oils (rosemary and cinnamon) under different housing systems (floor and cage) on production, egg quality, immunity, haematology, blood biochemical and antioxidant parameters of layers are rare. Therefore, the objective of this research was to investigate the effects of a housing system, and the supplementation of rosemary and cinnamon essential oils on layers' performance, haematological traits, blood chemistry, immunity, egg quality, and antioxidants.

2. Materials and Methods

All procedures were implemented according to the Local Experimental Animal Care Committee and approved by the ethics of the institutional committee of Damanhour University, Egypt.

2.1. Birds and Experimental Design

Three thousand 27-week old ISA brown laying hens were obtained from Al Waha poultry industry (Damo—El Basyounia—El Fayoum—Egypt). A factorial arrangement (2×3) was performed, which included two housing systems and three different types of essential oils (0, 300 mg/kg diet of rosemary and 300 mg/kg diet of cinnamon essential oils) to study their effects on the productive performance, egg quality, immunity, oxidative stress and haematology of laying hens during the production stages (from 28 to 76 weeks of age). Birds were randomly divided into two groups each comprising 1500 birds; the first group was moved to laying cages, while the second group was floor reared. Each group was randomly divided into three groups, the first were considered as a control group, the second treated with rosemary essential oil, and the third with cinnamon essential oil. Each group was divided into five equal replicates each of 100 birds. Rosemary essential oil was obtained from Quanao, Shaanxi, China; and cinnamon essential oil was obtained from YiSenYuan, Jiangxi, China (the purity of both oils was 100%). The birds were housed in an open sided farm and each replicate of floor reared layers were housed in a separate pen of about 10 m^2 space. The pens were separated by nets of 2 m height to avoid group mixing and to avoid interference with air movement, while caged birds in each replicate were housed in separate cages divided into pens, where each pen had dimensions of 40 cm × 50 cm × 40 cm suitable for five birds. The cage consisted of two levels, with each level containing 10 pens (5 on each side).

2.2. Dietary Treatments

Composition of basal diet and their calculated analysis is presented in Table 1. The hens were fed diets in mash form during the experiment (28–76 weeks). The diets were formulated to meet or exceed NRC [19] recommendations.

Table 1. Ingredients and calculated analysis of layer basal diet.

Item	%
Ingredients	
Yellow corn	61.23
Soybean meal (44% protein)	19.02
Corn gluten meal (60% protein)	7.02
Vitamins and minerals premix *	0.30
Wheat bran	0.46
Calcium carbonate	1.36
Di-calcium phosphate	8.96
DL-methionine	0.05
NaCl	0.40
Lysine	1.20
Chemical analysis (%) **	
Crude protein	18.01
Metabolic energy (Kcal/kg)	2800
Crude fiber	2.85
Calcium	3.81
Phosphorus	0.63

* Each diet was supplied with 3 kg/ton Vitamins and Minerals Mix (commercial source B. p. Max) Each 3 kg contains, Vit. A 10,000,000 MIU, Vit. D 2,000,000 MIU, Vit. E 10,000 mg, Vit. K3 1000 mg, Vit. B1 1000 mg, Vit. B2 5000 mg, Vit. B6 1500 mg, Biotin 50 mg, BHT 10,000 mg, Pantothenic 10,000 mg, folic acid 1000 mg, Nicotinic acid 30,000 mg Mn 60 g, Zinc 50 g, Fe 30 g, Cu 4 g, I 3 g, Selenium 0.1 g and Co 0.1 g. ** The diets were formulated to meet or exceed NRC [19] recommendations.

2.3. Estimation of Laying Performance Parameters and Egg Quality

Hen-day egg production (HDEP), feed consumption and egg weight was recorded daily on a replicate basis. Feed intake was calculated by subtracting the remaining feed daily from the offered feed. Feed conversion ratio was calculated as grams of feed intake per gram of egg mass produced. Average egg mass (per hen per day in grams) = % HDEP × Average egg weight in grams. The parameters relative to egg quality were evaluated at 72 weeks of age. Fifteen eggs were randomly collected per treatment to determine these parameters. The collected eggs were weighed using a digital balance. On breaking, the egg contents were poured. The Haugh unit (HU) was measured for the internal quality of the eggs [20]. The height, correlated with the weight, determined the HU. The higher the number, the better the quality of the egg (fresher, higher quality eggs have thicker whites). Eggshell, albumin, and yolk percentages were also measured. Eggshell thickness (without the shell membrane) was measured from the middle part of the egg using a micrometer. Yolk index was calculated by formula: Yolk height/Yolk width × 100; while the egg index was calculating using the following formula: (Egg width/egg length) × 100.

2.4. Estimation of Blood Haematological and Biochemical Parameters

Blood samples (n = 25) were collected from each group as five samples from each replicate from the wing vein at 56 weeks of age. After collecting the blood samples, the tubes were left in the slope position until serum samples were separated through centrifugation at 3000 rpm for 15 min. Red blood cells (RBCs) and white blood cells (WBCs) counts were determined according to Stoskopf [21] using haemocytometer. Blood hemoglobin (HB) was assessed by cyanomta-hemoglobin method [22]. Packed cell volume (PCV) was carried out by using microhaematocrit capillary tubes centrifuged at 12,000 rpm for 5 min. The reading was made with the aid of a microhaematocrit reader and expressed as the

volume of erythrocytes per 100 cm^3 according to Blaxhall and Daisley [23]. Differential leucocytic counts were defined using a blood film that was prepared according to the method described by Lucky [24]. Ten drops from May-Grunwald stain stock solution on a dry, unfixed smear were added to an equal amount of distilled water, then mixed and left for 1 min for staining. The dye was decanted without rinsing. Diluted Giemsa's solution (10 drops of the dye were added to 10 mL of distilled water) was poured over the film as a counter stain and left for 20 min, then rinsed in water current and examined by oil immersion lens. The percentage and absolute value for each of the type of cells were calculated according to Schalm et al. [25]. The sera were collected and preserved in a deep freezer at (−20 °C) until the time of analysis. All the following studied parameters were calorimetrically evaluated. Estimation of blood cholesterol content was determined by cholesterol kit of Bio-diagnostic according to Richmond [26] and Allain et al. [27]. Total protein was determined by kits of Bio-diagnostic according to the method of Gornal et al. [28]. Alanine Aminotransferase (ALT) was determined by the ALT kit of Bio-diagnostic according to the method of Reitman and Frankel [29], while Aspartate Aminotransferase (AST) was determined by the AST kit of Bio-diagnostic according to the method of Retiman and Frankel [29]. Creatinine was defined according to the method of Bartles et al. [30], while urea was defined according to the method of Fawcett and Scott [31]. Calcium (Ca) and Phosphorus (P) were measured spectrophotometrically by using commercial kits (Spectrum chemical company, PO. Box 30, Obour City—Cairo, Egypt).

2.4.1. Estimation of Malondialdehyde, Glutathione Peroxidase and Super Oxide Dismutase

Estimation of blood Malondialdehyde (MDA) concentration was measured by the method of Jo and Ahn [32]. Determination of Glutathione peroxidase (GPx) activity measured using the Paglia and Valentine [33] spectrophotometric method based on the Northwest Life Science Specialties (NWLSS™) Glutathione peroxidase assay kits protocol NWK-GPX01. Determination of Super Oxide Dismutase (SOD) activity was assessed using the NWLSS™ Superoxide dismutase activity assay, which provided a simple, rate method for determining SOD activity. This method is based on monitoring the auto-oxidation rate of haematoxylin as originally described by Martin Jr. et al. [34].

2.4.2. Estimation of Phagocytic Index, Phagocytic Activity and Cellular Immunity

Blood and serum samples were collected at 56 days of age (five samples per replicate and total 25 samples per each group) as mentioned above according to Stott and Fellah [35] and used for the determination of Phagocytic activity and phagocytic index was determined according to Kawahara et al. [36]. Fifty micrograms of Candida albicans culture was added to 1 mL of citrated blood from each sample and incubated in a water bath at 25 °C for five hours, and then blood smears from each tube were stained with Giemsa stain. Phagocytosis was estimated by determining the proportion of macrophages, which contained intracellular yeast cells in a random count of 300 macrophages and expressed as percentage of phagocytic activity (PA). The number of phagocytized organisms was counted in the phagocytic cells and called the phagocytic index (PI).

Phagocytic activity (PA) = Percentage of phagocytic cells containing yeast cells.

Phagocytic index (PI) = Number of yeast cells phagocytized/Number of phagocytic cells.

2.5. Statistical Analysis

Data were analyzed by the statistical analysis system SAS [37]. A 2 × 3 factorial design was used to analyze data of performance as a response to two housing systems and three different types of essential oils. Differences among means were detected using two-way analysis of variance (ANOVA). The differences among means were determined using the Duncan test ($p < 0.05$). The model used was:

$$Y_{ij} = \mu + D_i + A_j + DA_{ij} + e_{ij}$$

where: Y_{ij} = an observation, μ = the overall mean, D_i = fixed effect of housing system, A_j = fixed effect of essential oils, DA_{ij} = fixed effect of interaction between housing system and essential oils and e_{ij} = random error associated to each observation.

3. Results and Discussion

The effects of different housing systems, essential oils supplementations and their interaction on the egg production of laying hens is shown in Table 2. The differences in egg production percentages between the different housing systems (floor and cage) were not significant at $p < 0.05$ or 0.01. This finding was in agreement with the results of Zita et al. [38]. They indicated no effect of housing system on the egg production of hens. Egg production percentages were significantly ($p < 0.05$ or 0.01) higher in the rosemary and cinnamon groups than in the control group, whereas there were no significant interactions. Şimşek et al. [9] found that cinnamon supplementation helped to increase the egg production. Moreover, Ding et al. [39] revealed that hen-day egg production was significantly improved ($p < 0.05$) at 58 to 61 weeks with the diet supplemented with essential oils Enviva commercial product (50, 100, and 150 mg/kg) including thymol 13.5% and cinnamaldehyde 4.5%.

Table 2. Egg production of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items	Egg Production % During					
	28–36 week	**36–44 week**	**44–52 week**	**52–60 week**	**60–68 week**	**68–76 week**
Housing system						
Cage	87.86	88.89	85.12	79.76	71.99	62.60
Floor	85.11	85.36	81.20	75.44	68.45	58.83
Essential oils (EOs) [1]						
0	83.87 [b]	84.68 [b]	80.18 [b]	73.06 [b]	65.56 [b]	56.00 [b]
Rosemary EO	88.30 [a]	88.95 [a]	85.40 [a]	80.15 [a]	72.25 [a]	63.00 [a]
Cinnamon EO	87.30 [a]	87.75 [a]	83.90 [a]	79.60 [a]	72.85 [a]	63.15 [a]
SEM [2]	0.705	1.142	1.412	1.373	1.287	0.927
Probability						
Housing system	<0.001	0.001	0.004	0.001	0.004	<0.001
EOs	<0.001	0.002	0.007	<0.001	<0.001	<0.001

Means in the same column within each classification bearing different letters are significantly different. ($p < 0.05$ or 0.01). [1] EOs: Essential oils, EO: Essential oil. [2] SEM: standard error mean.

The effects of different housing systems, essential oils supplementations and their interaction on egg weight of laying hens are shown in Table 3. The differences in egg weight between the different housing systems (floor and cage) were not significant at ($p < 0.05$ or 0.01). This finding was also in agreement with the results of Zita et al. [38]. The egg weights were significantly ($p < 0.05$ or 0.01) higher in rosemary and cinnamon groups than in the control group at 28–36 and 52–60 weeks while there were no significant interactions. The highest egg weight was found in the rosemary group at 44–52 weeks and 60–68 weeks. In agreement, Şimşek et al. [9] reported that the highest egg weight in quail was found in the rosemary group. On the other hand, Alagawany and Abd El-Hack [17] reported that there were no differences in egg weight due to adding rosemary to laying hens. Furthermore, Ding et al. [39] and Cufadar [40] reported that egg weight was not affected by the diet supplemented with essential oils.

Table 3. Egg weight of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items	Egg Weight (g) During					
	28–36 week	**36–44 week**	**44–52 week**	**52–60 week**	**60–68 week**	**68–76 week**
Housing system						
Cage	45.52	51.03	55.27	57.93	59.24	60.35
Floor	43.90	50.69	55.15	57.11	58.65	59.82
Essential oils (EOs) [1]						
0	41.93 [b]	49.93	54.75 [b]	56.12 [b]	57.93 [b]	59.06
Rosemary EO	46.00 [a]	51.95	56.80 [a]	58.70 [a]	59.70 [a]	60.80
Cinnamon EO	46.20 [a]	50.70	54.10 [b]	57.75 [a]	59.20 [ab]	60.40
SEM [2]	0.827	0.529	0.529	0.561	0.502	0.722
Probability						
Housing system	0.015	0.632	0.844	0.083	0.180	0.349
EOs	<0.001	0.086	0.002	0.001	0.010	0.050

Means in the same column within each classification bearing different letters are significantly different. ($p < 0.05$ or 0.01). [1] EOs: Essential oils, EO: Essential oil. [2] SEM: standard error mean.

The effects of different housing systems, essential oils supplementations and their interaction on egg mass of laying hens are shown in Table 4. The differences in egg mass between the different housing systems (floor and cage) were not significant at ($p < 0.05$ or 0.01). The egg masses were significantly ($p < 0.05$ or 0.01) higher in rosemary and cinnamon groups than in the control group at 28–36, 44–52, 52–60, 60–68 and 68–76 weeks, while the differences among the interactions were not significant except at 68–76 weeks, and the cage × cinnamon group was the highest. In agreement, Alagawany and Abd El-Hack [17] reported that egg mass linearly increased with rosemary supplementation, while Cufadar [40] indicated that there were no differences in egg mass due to the addition of rosemary in laying hen diet.

Table 4. Egg mass of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items		Egg Mass (g) During					
		28–36 week	**36–44 week**	**44–52 week**	**52–60 week**	**60–68 week**	**68–76 week**
Housing system							
	Cage	43.60	46.26	47.14	44.69	40.13	18.35
	Floor	40.61	44.74	43.90	41.83	37.87	16.94
Essential oils (EOs) [1]							
	0	38.56 [b]	44.31 [b]	42.15 [b]	39.66 [b]	35.68 [b]	15.89 [b]
	Rosemary EO	44.35 [a]	47.32 [a]	48.32 [a]	45.30 [a]	40.52 [a]	18.69 [a]
	Cinnamon EO	43.41[a]	44.86 [b]	46.08 [a]	44.82 [a]	40.80 [a]	18.36 [a]
Housing × EOs							
Cage	0	39.76	45.78	44.60	41.31	36.69	16.88[c]
	Rosemary EO	45.69	47.36	49.52	46.04	41.20	18.78 [ab]
	Cinnamon EO	45.37	45.64	47.30	46.72	42.49	19.40 [a]
Floor	0	37.35	42.85	39.71	38.02	34.66	14.90 [d]
	Rosemary EO	43.02	47.27	47.12	44.56	39.85	18.59 [ab]
	Cinnamon EO	41.46	44.09	44.87	42.92	39.10	17.32 [b]
SEM [2]		0.829	0.856	0.980	0.837	0.760	0.300
Probability							
Housing system		<0.001	0.048	0.001	0.001	0.002	<0.001
EOs		<0.001	<0.006	<0.001	<0.001	<0.001	<0.001
Housing × EOs		0.650	0.310	0.416	0.368	0.407	0.008

Means in the same column within each classification bearing different letters are significantly different. ($p < 0.05$ or 0.01). [1] EOs: Essential oils, EO: Essential oil. [2] SEM: standard error mean.

The effects of different housing systems, essential oils supplementations and their interaction on feed intake of laying hens are shown in Table 5. The differences in feed intake between the different housing systems (floor and cage) were not significant at $p < 0.05$ or 0.01. The feed intakes were significantly ($p < 0.05$ or 0.01) lower in the rosemary and cinnamon groups than in the control group at 60–68 and 68–76 weeks. Apart from feed intake at 44–52 weeks, there were no significant differences due to the interaction effect between EOs and housing system. Under the floor system, birds fed diet supplemented with or without EOs consumed more feed (127.40 to 128.62 g) than those raised under the cage system (119.50 to 124.60 g). Feed intake was found to be similar between the rosemary and cinnamon groups as mentioned before by Şimşek et al. [9]. Although, essential oils are perceived as growth promoters in poultry diets [41], recent studies on poultry feeding intakes [42,43] have indicated that dietary incorporation of essential oils did not significantly affect the bird feed intake or they could decrease it insignificantly. However, there is a possible explanation for the reduced intake of feed is the irritating scent of essential oils, which makes the diet unpleasant to birds.

Table 5. Feed intake (g) of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items		Feed Intake (g) During					
		28–36 week	36–44 week	44–52 week	52–60 week	60–68 week	68–76 week
Housing system							
	Cage	88.89	108.58	122.30	126.83	121.06	116.04
	Floor	97.15	114.41	127.84	136.41	129.44	117.80
Essential oils (EOs) [1]							
	0	93.81	112.75	124.06	132.37	127.31 [a]	117.87 [a]
	Rosemary EO	92.60	110.95	126.00	130.85	123.95 [b]	116.30 [b]
	Cinnamon EO	92.65	110.80	125.15	131.65	124.50 [b]	116.60 [ab]
Housing × EOs							
Cage	0	89.87	110.75	119.50 [d]	127.00	122.50	116.62
	Rosemary EO	88.60	107.70	124.60 [b]	127.00	120.30	116.20
	Cinnamon EO	88.20	107.30	122.80 [c]	126.50	120.40	115.30
Floor	0	97.75	114.75	128.62 [a]	137.75	132.12	119.12
	Rosemary EO	96.60	114.20	127.40 [a]	134.70	127.60	116.40
	Cinnamon EO	97.10	114.300	127.50 [a]	136.80	128.60	117.90
SEM [2]		0.825	0.840	0.713	0.728	0.769	0.547
Probability							
Housing system		<0.001	<0.001	<0.001	<0.001	<0.001	0.001
EOs		0.324	0.079	0.056	0.164	0.001	0.032
Housing × EOs		0.806	0.230	0.001	0.112	0.377	0.071

Means in the same column within each classification bearing different letters are significantly different. ($p < 0.05$ or 0.01).[1] EOs: Essential oils, EO: Essential oil. [2] SEM: standard error mean.

The effects of different housing systems, essential oils supplementations and their interaction on feed conversion of laying hens are shown in Table 6. The differences in feed conversion between the different housing systems (floor and cage) were not significant at $p < 0.05$ or 0.01. The feed conversions were significantly ($p < 0.05$ or 0.01) lower in the rosemary and cinnamon groups than in the control group at 28–36, 36–44, 52–60, 60–68 and 68–76 weeks, while the differences among the interactions were not significant at ($p < 0.05$ or 0.01) except at 44–52, 52–60, and 68–76 weeks, with the lowest rates being observed for layers fed diets enriched with EOs in the cage. The cage × rosemary group achieved the best values of FCR at 44–52 and 52–60 weeks; while at 68–76 weeks, the best value was recorded by the cage × cinnamon group. It can be concluded that the interactions among cage and EOs system achieved the good results for the FCR in comparison with the floor system. Şimşek et al. [9] found that the best feed conversion was obtained in the cinnamon group. Ding et al. [39] reported that the hen feed conversion ratio was significantly improved ($p < 0.05$) at 58 to 61 weeks with the diet supplemented with essential oils. On the other hand, Alagawany and Abd El-Hack [17] reported that

there were no differences in the feed consumption and feed conversion ratio due to adding rosemary to laying hens. However, there are two acceptable mechanisms to understand the effect of these essential oils. The first one considers the promotion of digestive enzyme secretion, and the second deals with the gut microflora ecosystem stabilization, leading to enhanced utilization of food and decreased exposure to growth-depressing disorders that could be related to the metabolism and the digestion processes [44–46]. Several chicken studies have documented positive effects of essential oils on the digestive enzyme (pancreatic α-amylase and intestinal maltase) secretion and intestinal mucosa [47,48]. In broilers, the ileal activity of trypsin and chymotrypsin was significantly increased in the thymol group at day 21 compared with the control group [49]. In the in vitro study, Mathlouthi et al. [50] stated that rosemary essential oils had different antimicrobial impacts against pathogenic microbes however, had the same effect on avilamycin as a growth promoter when added to broiler rations. Furthermore, the latter authors found that in vivo growth promotion effects were due to the alterations in the gut microbiota rather than antimicrobial activities against a single bacterial species and genus. Decreased microbes in the gastrointestinal tract may enhance the proliferation ability of epithelial cells and thus improve intestinal absorptive capacity [51]. These effects have been confirmed by increased nutrient digestibility, however, this has not resulted in improved growth performance [52,53].

Table 6. Feed conversion of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items		Feed Conversion (g Feed/g Egg) During					
		28–36 week	36–44 week	44–52 week	52–60 week	60–68 week	68–76 week
Housing system							
	Cage	2.23	2.39	2.60	2.75	2.85	3.08
	Floor	2.60	2.65	2.87	3.18	3.24	3.37
Essential oils (EOs) [1]							
	0	2.67 ^{a}	2.67 ^{a}	2.84 ^{a}	3.24 ^{a}	3.36 ^{a}	3.58 ^{a}
	Rosemary EO	2.28 ^{b}	2.40 ^{b}	2.60 ^{b}	2.79 ^{b}	2.88 ^{b}	3.04 ^{b}
	Cinnamon EO	2.30 ^{b}	2.49 ^{b}	2.76 ^{a}	2.87 ^{b}	2.89 ^{b}	3.07 ^{b}
Housing × EOs							
Cage	0	2.48	2.52	2.61 ^{b}	2.94 ^{a}	3.13	3.37 ^{b}
	Rosemary EO	2.12	2.30	2.54 ^{c}	2.65 ^{b}	2.76	3.00 ^{cd}
	Cinnamon EO	2.09	2.36	2.65 ^{bc}	2.66 ^{b}	2.67	2.88 ^{d}
Floor	0	2.86	2.82	3.06 ^{a}	3.55 ^{a}	3.59	3.79 ^{a}
	Rosemary EO	2.44	2.50	2.67 ^{b}	2.93 ^{a}	3.01	3.07 ^{c}
	Cinnamon EO	2.52	2.63	2.87 ^{b}	3.07 ^{a}	3.12	3.25 ^{b}
SEM [2]		0.035	0.046	0.055	0.058	0.056	0.058
Probability							
Housing system		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
EOs		<0.001	<0.001	0.002	<0.001	<0.001	<0.001
Housing × EOs		0.291	0.569	0.036	0.040	0.147	0.014

Means in the same column within each classification bearing different letters are significantly different. ($p < 0.05$ or 0.01). [1] EOs: Essential oils, EO: Essential oil. [2] SEM: standard error mean.

The effects of different housing systems, essential oils supplementations and their interaction on egg quality of laying hens are shown in Table 7. The differences in egg quality traits between the different housing systems (floor and cage) were not significant at $p < 0.05$ or 0.01. In contrast, Tumova and Ebeid [54] found that the egg quality characteristics were better in eggs produced in cages when compared to alternative systems, and eggs produced from cage systems had higher values of Haugh units, albumen and yolk indices. Furthermore, higher eggshell and albumen qualities were observed in conventional cages by Englmaierová et al. [4]. The differences between the study's results could be due to differences in the strain used or the environmental conditions surrounding it (such as cages indoors or outdoors), as well as differences in the cages design. However, the differences in shell thickness among the different essential oil groups (including the control group) were also not significant

($p < 0.05$ or 0.01). On the other hand, Cufadar [40] reported that eggshell thickness was significantly increased with rosemary essential oil supplementation (250 mg/kg) in the diet of NOVOgen White laying hens. In general, Ding et al. [39] reported that eggshell thickness was significantly increased at 65 weeks with the diet supplemented with essential oils. Haugh unit scores were higher in rosemary and cinnamon groups than in the control group, while the control group was the highest in yolk index among the interactions at $p < 0.05$ or 0.01. It means that rosemary and cinnamon supplementation (0.3 g/kg) decreased the egg yolk index for Isa Brown laying hens. On the other hand, Alagawany and Abd El-Hack [17] found that adding rosemary (up 6 g/kg) to Hi-sex Brown laying hen' diets resulted in a linear increase in yolk percent and yolk-to-albumen ratio. While, Botsoglou et al. [55] indicated that diets supplemented with rosemary oil (5 g/kg) for Lohmann laying hens had no effects on the yolk index neither Haugh units. These conflicting results may be due to the different supplementation ratio or/and the strain used. Furthermore, Ding et al. [39] reported that Haugh units, generally, were not affected by the diet supplemented with essential oils.

Table 7. Egg quality of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items		Shell Thickness (μm)	Eggshell %	Yolk %	Albumin %	Egg Index	Yolk Index	Haugh Unit
Housing system								
	Cage	0.36	8.79	28.80	62.39	77.34	22.54	82.53
	Floor	0.35	8.50	28.62	62.86	76.49	22.65	80.99
Essential oils (EOs) [1]								
	0	0.35	8.54	28.49 ^{b}	62.96 ^{a}	75.56 ^{c}	23.55 ^{a}	79.55 ^{c}
	Rosemary EO	0.35	8.70	28.90 ^{a}	62.39 ^{b}	77.34 ^{b}	22.17 ^{b}	82.40 ^{a}
	Cinnamon EO	0.36	8.71	28.75 ^{ab}	62.52 ^{b}	77.84 ^{a}	22.07 ^{b}	83.33 ^{a}
Housing × EOs								
Cage	0	0.36	8.77	28.56	62.66	76.10	23.33 ^{a}	80.50
	Rosemary EO	0.37	8.83	29.07	62.09	77.84	22.29 ^{b}	83.27
	Cinnamon EO	0.37	8.78	28.79	62.42	78.08	22.02 ^{b}	83.82
Floor	0	0.35	8.30	28.41	63.27	75.03	23.78 ^{a}	78.61
	Rosemary EO	0.34	8.56	28.73	62.70	76.84	22.05 ^{b}	81.52
	Cinnamon EO	0.36	8.64	28.72	62.63	77.60	22.14^{b}	82.85
SEM [2]		0.091	0.801	0.110	0.134	0.177	0.121	0.416
Probability								
Housing system		0.025	<0.001	0.059	<0.001	<0.001	0.275	<0.001
EOs		0.480	0.099	0.007	0.002	<0.001	<0.001	<0.001
Housing × EOs		0.378	0.178	0.465	0.252	0.233	0.041	0.519

Means in the same column within each classification bearing different letters are significantly different ($p < 0.05$ or 0.01). [1] EOs: Essential oils, EO: Essential oil. [2] SEM: standard error mean.

The effects of different housing systems, essential oils supplementations and their interaction on the blood picture (haematological traits) of laying hens are shown in Table 8. The differences in blood picture between the different housing systems (floor and cage) and among the different essential oils groups were not significant at $p < 0.05$ or 0.01 except in some differential WBCs (basophils; lymphocytes and monocytes %).

Table 8. Blood picture of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items [1]		WBC (× 10^3/mm^3)	RBC (× 10^6/mm^3)	PCV %	HB %	Eosino %	Lympho %	Hetero %	Baso %	Mono %
Housing system										
Cage		23.78	3.21	29.17	14.14	8.33	35.71	23.43	1.08	5.36
Floor		23.86	3.22	29.16	14.19	8.38	35.88	23.54	1.08	5.35
Essential oils (EOs) [2]										
0		23.77	3.19	29.12	14.11	8.28	35.13 ^{b}	23.38	1.05 ^{b}	5.21 ^{b}
Ros EO		23.93	3.22	29.08	14.22	8.34	35.93 ^{ab}	23.63	1.10 ^{a}	5.41 ^{ab}
Cinn EO		23.76	3.22	29.29	14.17	8.44	36.33 ^{a}	23.45	1.09 ^{a}	5.46 ^{a}
Housing × EOs										
Cage	0	23.77	3.22 ^{a}	29.07	14.10	8.35	35.60	23.37	1.07 ^{b}	5.30
	Ros EO	23.88	3.21 ^{a}	29.16	14.24	8.26	35.58	23.50	1.08 ^{b}	5.38
	Cinn EO	23.70	3.20 ^{a}	29.28	14.08	8.38	35.96	23.42	1.08 ^{b}	5.42
Floor	0	23.77	3.17 ^{b}	29.17	14.12	8.22	34.67	23.40	1.02 ^{b}	5.12
	Ros EO	23.99	3.23 ^{a}	29.00	14.20	8.42	36.28	23.76	1.11 ^{a}	5.44
	Cinn EO	23.81	3.25 ^{a}	29.314	14.26	8.50	36.70	23.48	1.10 ^{a}	5.50
SEM [3]		0.108	0.016	0.145	0.078	0.085	0.364	0.140	0.013	0.088
Probability										
Housing system		0.377	0.432	0.932	0.437	0.479	0.585	0.293	0.966	0.853
EOs		0.173	0.222	0.237	0.422	0.236	0.018	0.178	0.006	0.018
Housing × EOs		0.840	0.029	0.605	0.360	0.260	0.075	0.624	0.030	0.260

Means in the same column within each classification bearing different letters are significantly different ($p < 0.05$ or 0.01). [1] WBC: White blood cells, RBC: Red blood cells, PCV: Packed cell volume, HB: Hemoglobin, Eosino: Eosinophils, Lympho: Lymphocytes, Heter: Heterophils, Baso: Basophils, Mono: Monocytes. [2] EOs: Essential oils, Ros EO: Rosemary essential oil, Cinn EO: Cinnamon essential oil. [3] SEM: Standard error mean.

The effects of different housing systems, essential oils supplementations and their interaction on immunity and antioxidant parameters of laying hens are shown in Table 9. The differences in immunity and antioxidant parameters between the different housing systems (floor and cage) were not significant at $p < 0.05$ or 0.01 while among the different essential oils groups, they were significant in ND, AI H5, AI H9, MDA, and SOD. Excluding GPx activity ($p < 0.001$), all immunity and antioxidant indices were not statistically different as a consequence of the interaction among housing systems and EOs. The interaction between floor and basal diet gave the highest (26.25 U/gHb) activity of GPx in comparison with the other interactions. However, the lowest value (15.00 U/gHb) was found in birds fed with a control diet with the cage system.

In general, some designs of housing systems can cause some stress on hens. This stress can play an effective role in the bird's immune system resulting in failure of vaccination or increased disease during production [56,57]. However, essential oils are a total of volatile constituents, and therefore the effects of essential oils could be a complete product of all components and their interactions. Two or three components can account for up to 85% of the total mixture, and thereby contribute to the primary property of the essential oil mixture [58]. For example, phenols (thymol and carvacrol) account for more than 70% of plant essential oils and are primarily responsible for their antibacterial and antioxidant functions. Rosemary has been recognized as the plant with the highest anti-oxidative activity [13]. On the other hand, cinnamon or its oil play an important role in improving the growth, production, digestion, absorption, activity of gut microbiota, immunity, as well as feed utilization and public health of poultry [18].

Table 9. Immunity and antioxidant parameters of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items [1]		Phagocytic Index	Phagocytic Activity	ND 60 W	AI H5 60 W	AI H9 60 W	MDA (nmoles/mL)	GPx (U/gHb)	SOD (U/gHb)
Housing system									
Cage		1.62	16.35	2.89	2.75	2.62	2.13	18.26	72.38
Floor		1.63	16.24	2.75	2.61	2.55	2.31	22.63	75.15
Essential oils (EOs) [2]									
0		1.53	15.75	2.63 ^{b}	2.36 ^{b}	2.22 ^{b}	2.50 ^{a}	20.62	81.00 ^{a}
Ros EO		1.66	16.45	2.86 ^{a}	2.78 ^{a}	2.68 ^{a}	2.21 ^{b}	20.80	73.70 ^{b}
Cinn EO		1.69	16.70	2.97 ^{a}	2.89 ^{a}	2.86 ^{a}	1.97 ^{c}	20.00	66.60 ^{c}
Housing × EOs									
Cage	0	1.57	16.37	2.82	2.49	2.33	2.45	15.00 ^{c}	77.75
	Ros EO	1.62	16.20	2.86	2.86	2.68	2.10	20.60 ^{b}	74.00
	Cinn EO	1.68	16.50	2.99	2.89	2.86	1.86	19.20 ^{b}	65.40
Floor	0	1.50	15.12	2.44	2.23	2.12	2.55	26.25 ^{a}	84.25
	Ros EO	1.70	16.70	2.86	2.71	2.68	2.32	21.00 ^{b}	73.40
	Cinn EO	1.70	16.90	2.96	2.89	2.86	2.08	20.80 ^{b}	67.80
SEM [3]		0.072	0.47	0.095	0.085	0.081	0.070	0.882	2.15
Probability									
Housing system		0.892	0.752	0.070	0.072	0.304	0.007	<0.001	0.145
EOs		0.135	0.125	0.004	<0.001	<0.001	<0.001	0.644	<0.001
Housing × EOs		0.600	0.128	0.093	0.366	0.384	0.669	<0.001	0.318

Means in the same column within each classification bearing different letters are significantly different ($p < 0.05$ or 0.01). [1] ND: Newcastle disease, AI H5: Avian influenza H5, AI H9: Avian influenza H9, MDA: Malondialdehyde, GPX: Glutathione peroxidase, SOD: Superoxide dismutase. [2] EOs: Essential oils, Ros EO: Rosemary essential oil, Cinn EO: Cinnamon essential oil. [3] SEM: Standard error mean.

Through supplementing essential oils, a bird's anti-oxidative stability can be enhanced [12]. Placha et al. [59] found that malondialdehyde concentration in the liver and kidney was significantly reduced by the supplementation of essential oils to bird diet. Moreover, Lee et al. [60] and Khan et al. [61] reported that thyme essential oil had a significant effect on avian-derived products (meat and eggs) retarding oxidant degradation. Antioxidant activity could be derived from the phenolic OH group, which acts as a hydrogen donor interacting with peroxy radicals during the initial process of lipid oxidation and thereby inhibiting the formation of hydroxyl peroxide [60]. The antioxidant effect differs from plant essential oil to others depending on the amount of total phenols in it. With its successful antioxidant activities in broiler meat, rosemary has been identified [62,63].

The effects of different housing systems, essential oils supplementations and their interaction on the blood chemistry of laying hens are shown in Table 10.

The differences in blood chemistry including the cholesterol between the different housing systems (floor and cage) were not significant at $p < 0.05$ or 0.01. Contrary to this, Zita et al. [38] found that the blood cholesterol level was higher in birds raised in cages than on litter. However, the cholesterol, ALT, AST, and urea levels were significantly ($p < 0.05$ or 0.01) lower in rosemary and cinnamon groups than in the control group. The effect of interaction between dietary the interaction among EOs and housing systems was not significant ($p < 0.05$) on blood chemistry tests except for the blood phosphorus content, the highest levels were observed for birds fed diets enriched with cinnamon oil with a cage or floor system. Alagawany and Abd El-Hack [17] found that serum constituents were not significantly influenced by rosemary supplementation, except for urea and cholesterol. Moreover, Torki et al. [64] reported that birds given rosemary exhibited lower serum cholesterol and triglycerides concentration. The Ca and P were higher in rosemary and cinnamon groups than in the control group.

Table 10. Blood chemistry of laying hens as affected by different housing systems, essential oils and their interaction during the experiment.

Items		Cholesterol (mg/dl)	Protein (g/dl)	Calcium (m mol/L)	Phosphorus (m mol/L)	Urea (m mol/L)	Creatinine (m mol/L)	ALT [1] (U/L)	AST [2] (U/L)
Housing system									
Cage		190.43	3.49	2.71	2.21	5.31	0.44	20.40	86.10
Floor		193.56	3.50	2.69	2.21	5.54	2.65	21.08	89.41
Essential oils (EOs) [3]									
0		206.50 ^{a}	3.51	2.44 ^{c}	2.08 ^{c}	5.75 ^{a}	0.51	22.62 ^{a}	99.37 ^{a}
Ros EO		187.30 ^{b}	3.49	2.76 ^{b}	2.22 ^{b}	5.35 ^{b}	3.78	20.40 ^{b}	83.70 ^{b}
Cinn EO		182.20 ^{c}	3.48	2.89 ^{a}	2.33 ^{a}	5.18 ^{b}	0.35	19.20 ^{b}	80.20 ^{c}
Housing × EOs									
Cage	0	204.50	3.54	2.43	2.09 ^{c}	5.65	0.51	22.00	96.50
	Ros EO	185.80	3.47	2.74	2.19 ^{b}	5.18	0.44	20.40	81.40
	Cinn EO	181.00	3.45	2.93	2.36 ^{a}	5.10	0.37	18.80	80.40
Floor	0	208.50	3.48	2.45	2.07 ^{c}	5.85	0.50	23.25	102.25
	Ros EO	188.80	3.51	2.77	2.26 ^{a}	5.52	7.12	20.40	86.00
	Cinn EO	183.40	3.51	2.84	2.30 ^{a}	5.26	0.33	19.60	80.00
SEM [4]		1.830	0.093	0.031	0.023	0.065	2.86	0.53	1.28
Probability									
Housing system		0.057	0.883	0.636	0.797	<0.001	0.373	0.145	0.006
EOs		<0.001	0.966	<0.001	<0.001	<0.001	0.427	<0.001	<0.001
Housing × EOs		0.919	0.797	0.144	0.026	0.372	0.429	0.533	0.067

Means in the same column within each classification bearing different letters are significantly different ($p < 0.05$ or 0.01). [1] ALT: Alanine transferase. [2] AST; Aspartate transferase. [3] EOs: Essential oils, Ros EO: Rosemary essential oil, Cinn EO: Cinnamon essential oil. [4] SEM: Standard error mean.

4. Conclusions

The supplementation of rosemary and cinnamon essential oils had great impacts on egg production and weight, some egg quality traits, feed intake and conversion, some haematological traits and blood chemistry, immunity, and antioxidant parameters. On the other hand, the different housing systems did not result in any positive or negative impact on these studied traits. However, the cage × cinnamon group was the highest in egg mass, and the lowest in FCR. Therefore, we have recommended the usage of rosemary or cinnamon essential oils at 300 mg/kg in layer's diet to improve its productive performance and egg production.

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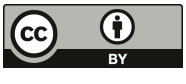

animals

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Communication

Green Tea Powder Decreased Egg Weight Through Increased Liver Lipoprotein Lipase and Decreased Plasma Total Cholesterol in an Indigenous Chicken Breed

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Simple Summary: Tons of green tea powder (GTP) are produced and cast off during green tea processing. It is suggested that GTP could increase immunity and health, and so improve animal production performance. We demonstrated that one percent of GTP supplemented in the diet did not affect egg production. However, long time GTP inclusion resulted in decreased egg weight and increased feed-to-egg ratio. Combined with plasma lipid concentration, the decreased egg weight might be because of lower plasma lipid concentration, increased plasma orexin A, and liver lipoprotein lipase expression in chickens fed a diet containing GTP.

Abstract: Whether or not green tea promotes egg production is unclear. Huainan partridge chickens at 20 weeks of age were divided into two groups, with one group fed a basal diet (control) and one fed a basal diet plus 10 g/kg green tea powder (GTP) for 12 weeks. Egg production (EP) and feed intake (FI) were recorded daily. Plasma lipid parameters, and apolipoprotein-B (Apo-B), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and lipoprotein lipase (LPL) expression were determined every four weeks. Egg production and FI showed no significant difference between the two groups ($p > 0.05$). Egg weight was 47.58 g in the control group, which was higher than that of the GTP group, and the feed-to-egg ratio (FCR) was 4.62 in the control group, which was lower than that of the GTP group after 12 weeks feeding. Compared with the control group, plasma orexin A ($p < 0.05$), high-density lipoprotein (HDL), apolipoprotein A (Apo A), and very high-density lipoprotein (VHDL) ($p < 0.01$, respectively) were increased. Plasma glucose (Glu), free fatty acid (FFA), apolipoprotein B (Apo B), triglyceride (TG), total cholesterol (TC) ($p < 0.01$, respectively), and low density lipoprotein (LDL) ($p < 0.05$) were decreased in the GTP group after 8 weeks feeding. The LPL expression in the liver was increased in the GTP group after 8 to 12 weeks feeding when compared to the control group ($p < 0.05$). Chickens fed GTP did not affect EP, but decreased egg weight, which might be because of lower plasma lipid concentration, increased plasma Orexin A, and liver LPL expression.

Keywords: egg production; green tea powder; huainan partridge chicken; lipoprotein lipase; plasma lipid

1. Introduction

Green tea is one of the most popular beverages worldwide and produces nearly 14,380,000 tons each year in China. Nearly 5% to 10% green tea powder is produced during green tea processing and

cast off. Major components of green tea are polyphenols, including catechins (constitute about 30% of its dry weight), alkaloids, polysaccharide, etc. [1,2]. Tea polyphenols are natural antioxidants that can scavenge free radicals and protect magnum from damage [3]. Green tea could prevent dental caries and reduce cholesterol and lipid absorption in the gastrointestinal tract [4]. Koo and Noh [5] further demonstrate that green tea could lower body fat through interfering with intestinal absorption of dietary fat, cholesterol, and other lipids. Catechins, the major component in tea polyphenols, could decrease plasma and liver malondialdehyde (MDA) concentrations, plasma glucose, and total cholesterol level [6]. A meta-analysis of randomized clinical trials also suggested that green tea decreased plasma total cholesterol and low-density lipoprotein (LDL) cholesterol [1].

The egg is one kind of animal product that is predominantly consumed; it contains many easily absorbed nutrients, and is easy to digest [7]. However, egg production performance could be affected by many factors, including genetics, feed composition, age, etc. [8]. The consumption of eggs from indigenous breeds is widely looked for in the market [8]. The most important problem for an indigenous breed is low egg production performance. Egg production is mostly affected by follicle maturation and ovulation, which is regulated by synthesis and transportation of yolk precursors [9]. The main component for yolk precursors include yolk very low-density lipoprotein (VLDLy, more than 90%) and vitellogenin (VTG); meaning VLDLy and VTG synthesis determine the speed of follicular development [10]. As a rate-limited endogenous cholesterol synthesis enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) determines egg yolk cholesterol content [11]. Apolipoprotein B (ApoB) regulates the synthesis and secretion of VLDLy. Apolipoprotein B could also increase lipoprotein lipase (LPL), binding to cells, and promoting the degradation of Very low density lipoprotein (VLDL) [12].

Wang et al. [3] suggested that adding 200 mg kg^{-1} tea polyphenols improved egg production performance in laying hens. Xia et al. mentioned that 10 g/kg green tea powder did not affect egg laying and feed conversion ratio, but a high amount of green tea powder (>20 g/kg) decreased egg production performance [13]. Our previous study also demonstrated that 10 g/kg green tea inclusion could improve meat color and Lactobacillus proliferation for broiler production [14].

It is well known that green tea could lower body fat in animals. However, lipids are one of the most important components in egg yolks, and determine yolk formation. Biswas et al. [15] reported that 6 g/kg of Japanese green tea powder (GTP) supplemented for laying hens tended to decrease egg weight and increase egg production rate. Thus, whether green tea powder could be used as feed additive in indigenous chicken breed during egg laying is still in doubt. The present study was conducted to evaluate the effect of 10 g/kg of green tea powder on chicken laying performance, plasma lipid content, and lipid synthesis related gene expression levels in Chinese indigenous Huainan partridge chickens.

2. Materials and Methods

All the experimental protocols involving care, handling, and treatment of broilers were approved by the Institutional Animal Care and Use Committee of Anhui Agricultural University, Hefei, Anhui, China. The permission number is No. SYDW-P2018110702.

2.1. Birds and Experimental Design

A total of 1080 Huainan partridge hens at 18 weeks of age, with similar body weights (1.46 ± 0.13 kg), were raised in one row of battery cages. There were 30 battery cages (30 replicates, 6 tiers, and 6 cages per tier) in the row, and one hen per cage. One side of the battery cage was used as the control group and the other side was used as the experimental group. The two sides of the battery cages had equal distances to windows. The hens received 13 h light at 20 weeks old, which was extended to 16 h light at 32 weeks of age. Hens from the control group received a basal diet (without GTP), and hens from the experimental group received a basal diet plus 10 g/kg GTP, instead of bran. The feed ingredient and chemical composition are listed in Table 1. The experiment consisted

of a two-week acclimation period and a 12-week collection period. During the two-week acclimation period, the diet with 10 g/kg GTP was gradually applied to hens, instead of the basal diet in one week, and another week for adaptation. Mean body weight, egg production (EP), egg weight (EW) and feed intake (FI) were calculated every 2 weeks from 20 to 32 weeks of age. The feed conversion ratio (FCR) was calculated by the formula FCR = FI/EP × EW.

Table 1. Feed composition and nutrition level.

Composition %	Group [2]	
	CON	**GTP**
Soybean	22.40	22.40
Corn	66.00	66.00
Wheat bran	4.50	3.50
Lime powder	2.00	2.00
Premix [1]	5.00	5.00
Green tea powder	0.00	1.00
Nutritional level		
Crude fat %	4.67	4.96
Total energy MJ/kg	13.07	12.99
Crude protein %	16.49	16.48
Ca % [3]	2.00—3.20	2.00—3.20

[1] Premix provided per kg of diet: Fe, 65 mg; Cu, 8 mg; Zn, 80 mg; Mn, 105 mg; I, 1 mg; Se, 0.3 mg; vitamin A, 9800IU; vitamin D3, 3100IU; vitamin E, 26 IU; vitamin B1, 2.5 mg; vitamin B2, 7 mg; vitamin B12, 0.018 mg; vitamin K, 2.2 mg; biotin, 0.09 mg; folic acid, 1 mg; pantothenic acid, 11 mg; niacin, 38 mg. [2] CON means chickens fed a basal diet as control group. GTP means chickens fed a basal diet plus 10 g/kg green tea powder. The same as Table 5. [3] The diet calcium was 2.0% at 20 weeks of age, and then gradually increased to 3.2% with the increased egg production.

At 20, 24, 28, and 32 weeks of age, thirty hens from each replicate of each group were randomly selected for blood sampling from the wing vein, and ten hens (one from three replicates) were slaughtered for liver and follicular membrane collection.

2.2. Blood Plasma Orexin A and Lipid Content Determination

Blood samples (2 mL) were separated by centrifugation (2500× *g* for 10 min at 4 °C). Separated plasma were frozen for lipids and orexin A analysis within one week. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used for the measurement of very high density lipoprotein (JL21659), total cholesterol (JL21710), triglyceride (JL21645), low density lipoprotein (JL15965), high density lipoprotein (JL21648), apolipoprotein B (JL45582), apolipoprotein A (JL21703), free fatty acid (JL15893), plasma glucose (JL21700), and orexin A (JL25500) by using an automatic ELISA analyzer (Rayto RT-6100). All of the kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.3. Determination of mRNA Expression Level by Real-time Reverse Transcription

Liver and follicular membrane (stored in −80 °C) were used for total RNA extraction by using a commercial kit (Omega bio-Tek Inc., GA, American) according to the manufacturer's instructions. The quality and quantity of total RNA was determined by using Nanodrop2000 (Thermo Fisher, MA, American). After DNase treatment, 5 μg of total RNA was reverse transcribed by using RNase reverse transcriptase (Easyscript RT/RI Enzyme mix, TransGen Biotech, Beijing, China), random primers (Anchored Oligo[dT]18 Primer [0.5 μg/μL]), and random 6 mers. The mRNA expression level for each gene was determined by real-time reverse transcription according to Chen et al. [16]. β-actin was chosen as reference. The primers used for quantification were listed in Table 2.

Table 2. Primers for quantitative RT-PCR.

Gene	Primer Sequences (5′—3′)	Annealing Temperature °C	Product Size (bp)
ApoB	CACCATCTAAAGCGTAAACCGAAC AAATGGGTGATTTTCAGGGTTTTT	60	196
HMGR	CGTGGAATGGCAATTTTAGGTC CCAAAGCAGCACATGATTTCAAG	60	116
LPL	GGAACAGCCAAGAAATGGAACA GCAGTGGTCTTGAAGAATGAGC	60	174
β-actin	CTGTGCCCATCTATGAAGGCTA ATTTCTCTCTCGGCT-GTGGTG	60	152

2.4. Statistical Analysis

The results from the egg production, egg weight, feed intake, feed conversion ratio, and gene expression were analyzed by two-way ANOVA, with treatment and feeding time as two variables. The results of the lipid parameters and orexin A analyses were subjected to Student *T*-tests by SAS 9.3. Data for gene expression were presented in mean ± SE, and other data were expressed as mean ± SD.

3. Results

3.1. Laying Hens Performance

The chicken performances during the feeding trial period were summarized in Table 3. Egg production, egg weight, and feed intake significantly increased with age ($p < 0.01$, respectively), and feed conversion ratio (FCR) significantly decreased with age ($p < 0.01$). No significant difference was observed in egg production performance between the control and GTP groups during the experimental period. Egg weight showed no significant difference between the two groups before 30 weeks of age. That is, after 10 weeks of the feeding diet, containing 10 g/kg GTP, egg weight was significantly lowered than that of the control group ($p < 0.05$). Feed intake tended to be lower in the GTP group than that of the control group at the first four weeks of feeding, and no significant difference was observed between the two groups ($p > 0.05$). Feed conversion ratio (FCR,) was relatively high before 24 weeks of age because of low egg production. A relatively higher feed intake caused a higher FCR in the control group when compared to the GTP group at 22 and 24 weeks of age ($p < 0.05$). Feed conversion ratio was higher in the GTP group than that of the control group after 10 weeks feeding ($p < 0.05$).

Table 3. Effect of dietary GTP on laying performance of Huainan partridge chickens.

Item	Treatment	Weeks of Age							SEM	*p*-Value		
		20	22	24	26	28	30	32		Age	Treatment	Interaction
EP, %												
	CON	1.91	3.10	6.85	28.99	50.43	56.62	56.49	0.98	<0.01	0.15	0.37
	GTP	1.48	3.70	9.94	29.68	51.63	55.84	55.68				
EW, g												
	CON	32.33 ^{d}	33.93 ^{d}	37.94 ^{c}	42.71 ^{b}	44.94 ^{ab}	46.21 ^{a}	47.58 ^{a}	2.30	<0.01	0.27	<0.01
	GTP	32.82 ^{d}	36.76 ^{cd}	36.41 ^{cd}	42.62 ^{b}	44.89 ^{ab}	43.84 ^{b}	43.64 ^{b}				
FI, g/d												
	CON	-	77.99	93.74	92.65	103.93	122.09	124.18	1.43	<0.01	0.06	0.14
	GTP	-	73.37	89.32	92.69	103.51	124.37	124.96				
FCR												
	CON	-	74.15 ^{a}	36.07 ^{c}	7.48 ^{e}	4.58 ^{g}	4.66 ^{g}	4.62 ^{g}	1.07	<0.01	0.023	<0.01
	GTP	-	53.94 ^{b}	24.68 ^{d}	7.33 ^{e}	4.47 ^{g}	5.08 ^{f}	5.14 ^{f}				

$^{a, b}$ Different lowercase letter in the same row within the same item indicates significant difference ($p < 0.05$). EP, egg production; EW, egg weight; FI, feed intake; FCR, feed conversion ratio.

3.2. Effect of Green Tea Powder on Orexin A and Plasma Lipid Content of Huainan Partridge Chicken

Compared with the control group, orexin A, apolipoprotein A (Apo A), and high-density lipoprotein (HDL) were significantly increased ($p < 0.01$, respectively), and glucose (Glu), free fatty acid (FFA), total cholesterol (TC), triglyceride (TG) ($p < 0.01$, respectively), and apolipoprotein-B (Apo B) ($p < 0.05$) were decreased in chickens fed diets with 10 g/kg GTP for 4 weeks. No significant difference was observed in the low-density lipoprotein (LDL) and very high-density lipoprotein (VHDL) of chickens fed a diet with GTP or not for 4 weeks. Compared with the control group, orexin A, HDL, Apo A, and VHDL significantly increased; Glu, FFA, Apo B, LDL, TG, and TC significantly decreased in chickens fed a diet with GTP for 8 or 12 weeks (Table 4).

Table 4. Effect of Green tea powder on plasma parameters and orexin A of Huainan partridge chickens.

Treatment	Items	Week of Age			
		20	24	28	32
CON	Orexin A, pg/mL	223.3 ± 37.6	274.18 ± 50.99^{B}	296.47 ± 48.68 ^{b}	269.31 ± 52.57 ^{B}
	Glu, mmol/L	6.86 ± 0.98	6.24 ± 0.67^{A}	6.47 ± 0.79 ^{A}	6.54 ± 0.93 ^{A}
	FFA, μmol/L	566.1 ± 83.0	562.49 ± 56.44 ^{A}	513.52 ± 76.11 ^{A}	579.64 ± 74.18 ^{A}
	Apo-B, μg/ml	782.9 ± 89.5	781.55 ± 116.96 ^{a}	908.17 ± 91.34 ^{A}	925.62 ± 112.73 ^{A}
	Apo-A, μg/mL	1238.9 ± 245.3	1370.5 ± 252.12 ^{B}	1197.8 ± 203.53 ^{B}	1239.5 ± 139.10 ^{B}
	HDL, mg/dL	101.8 ± 20.5	112.42 ± 17.49 ^{B}	106.35 ± 20.48 ^{B}	105.97 ± 18.30 ^{B}
	LDL, mmol/L	3.54 ± 0.70	4.88 ± 0.83	4.77 ± 0.80 ^{a}	5.39 ± 0.88 ^{A}
	TG, nmol/L	6.35 ± 1.08	5.70 ± 0.81 ^{A}	6.38 ± 0.95 ^{A}	6.19 ± 0.78 ^{A}
	TC, mmol/L	6.76 ± 0.87	6.62 ± 1.01 ^{A}	6.42 ± 1.08 ^{A}	7.00 ± 1.20 ^{A}
	VHDL, mmol/L	3.15 ± 0.45	2.63 ± 0.44	2.62 ± 0.49 ^{B}	2.63 ± 0.42 ^{B}
GTP	Orexin A, pg/mL	232.1 ± 46.1	321.85 ± 53.15 ^{A}	328.41 ± 35.08 ^{a}	375.98 ± 54.35 ^{A}
	Glu, mmol/L	6.76 ± 0.89	5.03 ± 0.75 ^{B}	5.13 ± 0.93 ^{B}	4.29 ± 0.84 ^{B}
	FFA, μmol/L	559.2 ± 81.5	453.36 ± 93.85 ^{B}	339.57 ± 111.24 ^{B}	356.92 ± 72.63 ^{B}
	Apo-B, μg/ml	735.7 ± 110.0	663.50 ± 103.76 ^{b}	641.74 ± 108.14 ^{B}	644.12 ± 95.95 ^{B}
	Apo-A, μg/mL	1205.6 ± 290.5	1596.8 ± 295.46 ^{A}	1605.2 ± 192.89 ^{A}	1851.6 ± 226.76 ^{A}
	HDL, mg/dL	103.7 ± 13.0	132.43 ± 17.77 ^{A}	150.68 ± 16.66 ^{A}	153.40 ± 12.39 ^{A}
	LDL/mmol/L	388 ± 0.67	4.85 ± 0.89	4.11 ± 0.87 ^{b}	3.86 ± 0.70 ^{B}
	TG/nmol/L	6.53 ± 1.02	4.6 ± 0.92 ^{B}	4.32 ± 0.72 ^{B}	3.98 ± 0.90 ^{B}
	TC/mmol/L	6.39 ± 0.79	5.59 ± 0.82 ^{B}	4.05 ± 0.94 ^{B}	4.32 ± 0.97 ^{B}
	VHDL/mmol/L	3.45 ± 0.56	2.78 ± 0.42	3.24 ± 0.44 ^{A}	3.25 ± 0.3 ^{A}

$^{a, b}$ A different lowercase letter in the same row within the same item indicates significant difference ($p < 0.05$). $^{A, B}$ A different uppercase letter in the same row within the same item indicates significant difference ($p < 0.01$). Glu, glucose; FFA, free fatty acid; Apo-B, apolipoprotein B; Apo-A, apolipoprotein A; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglyceride; TC, total cholesterol; VHDL, very high-density lipoprotein.

3.3. Effect of Green Tea Powder on Lipid Metabolize Related Gene Expression

The expression level of genes related to lipid metabolize was listed in Table 5. The expression of Apo-B in the liver of the control group showed no significant difference at different ages ($p > 0.05$). While the Apo-B expression in the liver of the GTP group significantly increased after 4 weeks of GTP feeding, significantly decreased after 8 weeks of feeding, and kept decreasing after 12 weeks of feeding ($p < 0.05$). The expression of Apo-B in follicular membrane of the control group was significantly higher in chickens at 20 and 24 weeks of age as compared to the chickens at 28 and 32 weeks of age ($p < 0.05$). The expression of Apo-B in the follicular membrane of the GTP group significantly decreased after GTP feeding for 4, 8, or 12 weeks as compared to chickens before GTP feeding ($p < 0.05$). The expression of HMGR in the liver of the control group was higher at 20 and 24 weeks of age as compared to 28 and 32 weeks of age ($p < 0.05$). The expression of HMGR in the liver of the GTP group was decreased after GTP feeding for 4, 8, or 12 weeks as compared to chickens before GTP feeding ($p < 0.05$). The expression of HMGR in follicular membrane showed no significant difference in both control and GTP groups ($p > 0.05$). The LPL expression in the liver showed no significant difference within the control and GTP groups during the experiment ($p > 0.05$). However, the LPL expression in the liver significantly increased in chickens fed GTP for 8 to 12 weeks, as compared to control groups during the same time

($p < 0.05$). The LPL expression in the follicular membrane showed no significant difference in both control and GTP groups ($p > 0.05$).

Table 5. Expression of Apo-B, HMGR, and LPL in the liver and follicular membrane in Huainan partridge chickens fed GTP, or not, for different times.

Treatment	Week of Age	Apo-B		HMGR		LPL	
		Liver	Follicular Membrane	Liver	Follicular Membrane	Liver	Follicular Membrane
CON	20	1.64	5.06	3.24	1.22	1.14	1.05
	24	1.80	5.24	3.67	1.19	1.08	0.88
	28	1.18	1.56	1.02	1.00	0.43	0.56
	32	1.84	1.32	1.34	0.83	0.50	0.64
GTP	20	1.23	4.85	3.06	1.08	0.89	1.01
	24	3.61	1.71	1.48	1.19	1.34	1.17
	28	2.07	1.85	1.28	0.85	2.18	2.20
	32	1.00	1.01	1.00	1.01	2.04	2.28
SEM		0.195	0.147	0.298	0.355	0.315	0.182
				p-value			
treatment		0.504	0.291	0.197	0.346	0.037	0.490
age		0.047	0.021	0.079	0.506	0.754	0.611
treatment × age		0.493	0.328	0.208	0.582	0.192	0.557

[a, b] Means with no common superscripts are different within the same row ($p < 0.05$).

4. Discussion

In our previous research, it has been demonstrated that 10 g/kg of green tea powder as a feed additive could promote intestinal health and meat quality, and did not affect the body weight of broilers [14]. Xia et al. [13] also suggested that 1% green tea powder was beneficial on egg quality from Chinese local chicken breeds, but a high amount of green tea powder (>20 g/kg) inclusion in the diet could decrease the egg weight and increase the feed-to-egg ratio. Thus, 10 g/kg of green tea powder inclusion was selected to analyze its effect on egg production performance, chicken blood parameters, and lipid synthesis related genes (Apo-B, HMGR, and LPL) expression level.

The results of laying performance suggested that 10 g/kg of green tea powder did not affect egg laying in chickens. A digitally higher feed intake in chickens from the GTP group was observed, which might be because of increased orexin A caused by intake of green tea powder. However, Soori et al. [17] stated that green tea could reduce orexin A in overweight and obese women. Chickens before egg laying are usually under strict feed restriction to control body weight. In addition, green tea could trigger feed consumption especially under starvation. Combined with a lower palatability of diet with GTP, a digitally lower feed intake was observed in chickens fed GTP at the first four weeks. However, we also detected increased orexin A in chickens fed diets with GTP for 4 weeks, which further suggests that green tea might trigger feed consumption in laying hens after 8 weeks of feeding.

In this research, a lower level of glucose, FFA, Apo-B, LDL, TG, and TC were detected in chickens fed diets with GTP when compared to chickens from the control group. The main components in green tea powder are tea polyphenol, caffeine, catechins, crude fiber, etc. [13]. Although some intervention research suggested no significant effects on plasma lipids after six cups of green tea per day for 4 weeks [18], there were still many studies that insisted that catechins decreased blood glucose, triglycerides, and total cholesterol content in humans and poultry [6,19]. Tea could suppress the glucose transport activity in the intestinal epithelium to lower the absorption of sugar and then reduce blood sugar levels [20]. Green tea has been demonstrated to reduce cholesterols and lipids absorption in the gastrointestinal tract [5]. It is also widely accepted that green tea consumption could reduce blood LDL and glucose levels [1,21]. A higher Apo-A, HDL, and VHDL were also observed in chickens

fed diets with GTP as compared to chickens fed basal diets. Catechins from green tea could decrease the apolipoprotein B-100 (ApoB) level of human plasma in a radical reaction initiated by Cu^{2+} [22], which explained the decreased level of ApoB in chickens fed diets with GTP. In vitro experiments also demonstrated that green tea could inhibit ApoB secretion via the proteasome-independent pathway [23]. Increased HDL and apolipoprotein A (Apo A) was observed [21] in Portuguese adults who were given 1 liter of green tea per day for 4 weeks, which is in accordance with the data obtained from this experiment. During egg laying, lipogenic, and genes related to egg yolk formation in the liver were highly expressed, which caused high plasma lipid concentration in layers [24]. High plasma VLDL will then pass through the granulose basal lamina and reach the receptors located on the oocyte surface for providing yolk precursors [25]. Reduced plasma VLDL concentration might decrease egg yolk weight. The yolk weight is positively correlated with egg weight [25]. Thus, a significantly lower egg weight detected in chickens from the GTP group might be because of the decreased plasma lipid concentration. The increased feed intake and decreased egg weight caused a higher FCR (feed-to-egg ratio).

HMGR is a key rate-limiting enzyme in the synthesis of endogenous cholesterol. When dietary cholesterol absorption increased, the expression of HMGR decreased and resulted in a reduced endogenous cholesterol synthesis [11]. In this experiment, the expression of HMGR was in large quantity in the liver for the rapid development of oocytes before egg laying, and then decreased after egg laying. Under the stimulation of estrogen, large number of lipoproteins Apo-B and Apo-2 were synthesized in the liver, the secreted apolipoproteins bind with cholesterol to form VLDLy for the rapid growth of oocytes [26]. Before egg laying, plasma VLDL increased rapidly under the regulation of estrogen and the Apo-B accounts for more than 70 percent of lipoproteins [27]. Thus, it is clear that the high expression of Apo-B in liver and follicular membranes before egg laying is to promote the rapid development of follicles. As a key enzyme for regulating lipid metabolism, LPL can catalyze the hydrolysis of triglyceride to glycerol and FFA, and can remove TG-rich lipoproteins [28]. In this experiment, a significant difference was observed in the expression of LPL between the two treatment groups. It is stated that the expression of the high level of VLDL could inhibit the activity of lipoprotein lipase in laying birds; thus, allowing greater lipid transport to the ovary for egg yolk formation [24]. It is further demonstrated that higher LPL expression in chickens from the GTP group inhibited VLDL formation and yolk synthesis.

5. Conclusions

In conclusion, green tea powder inclusion decreased plasma lipid concentration and increased orexin A content in Huainan partridge chickens. Green tea powder might decrease egg production after long-time inclusion through decreasing plasma TG content by increasing liver LPL expression.

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Detoxification Impacts of Ascorbic Acid and Clay on Laying Japanese Quail Fed Diets Polluted by Various Levels of Cadmium

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Simple Summary: The present study aimed to evaluate the impacts of ascorbic acid and clay supplementation on laying Japanese quail fed diets polluted by various levels of cadmium (Cd). Results revealed that consuming polluted diets with Cd causes harmful impacts on the productive performance of laying Japanese quail. The supplementation of ascorbic acid or natural clay to layer diets had beneficial effects on productive performance, improved egg quality and diminished the toxic effect of Cd.

Abstract: A total number of 360 laying Japanese quail (8 weeks of age) were randomly divided into 12 groups. Birds in all groups had nearly the same average initial body weight. A factorial arrangement (4 × 3) was performed including four levels of dietary cadmium (Cd) as cadmium chloride (0, 50, 100, and 150 mg/kg diet) and three levels of feed additives (without, 300 mg/kg ascorbic acid and 1.50% natural clay). Results revealed that Cd contaminated feed caused significant ($p < 0.01$) retardation in body weight, lower egg number and egg mass and worse feed conversion. On the other hand, the addition of ascorbic acid or natural clay to quail diets caused a significant ($p < 0.01$) improvement in all studied traits. With respect to the interaction among Cd and the experimental additives, results showed that within each Cd level, ascorbic acid or clay supplementation recorded the highest body weight, egg number, egg weight and mass in addition to improved feed conversion. Cadmium levels decreased ($p < 0.05$) blood total protein, albumen and A/G ratio. Both 300 mg ascorbic acid and 1.50% clay increased ($p < 0.05$) blood total protein and albumen compared to non-supplemented groups. It could be concluded that the consumption of polluted diets Cd causes deleterious effects on the productive performance of laying Japanese quail. The addition of ascorbic acid or natural clay to the diets causes beneficial effects on productive performance traits, improves egg quality criteria and diminishes the toxic effects of Cd.

Keywords: cadmium; ascorbic acid; clay; performance; blood biochemical parameters

1. Introduction

For commercial use, domestic quails are available in both laying and meat strains [1–3]. The reproductive performance of Japanese quail is important in the overall management of the flock [4]. The contamination of the diets and the environment with heavy metals remains a big problem. It affects food safety and subsequently affects the consumers. The contamination of poultry rations with heavy metals causes a high reduction in feed efficiency and egg production, which finally result in a great economic loss for poultry farmers. All potential feed ingredients contain some kinds of heavy metals. Cadmium is considered one of the major environmental pollutants [5]. It is a highly toxic and reactive element which is distributed sparsely in the most of agricultural ecosystems. Once absorbed by humans or animals, Cd is poorly excreted. Great efforts are being made to protect the human food-chain from the entry of cadmium. The excess of Cd intake more than 2 mg/kg diet resulted in elevation in metallothionein synthesis, disturbance of the metabolism of Zn, Ca, and Fe [6]. Cd toxicity induced altered energy, altered behavioral responses, metabolism, kidney damage, anemia, adrenal hypertrophy and cardiac dysfunction [7]. Some reports found that kidney changes occurred independent of bone disease, while others concluded that Cd induced renal dysfunction is the secondary cause of skeletal deterioration [8]. Recently, Saleemi et al. [9] concluded that Cd leads to hepatotoxic and gonadotoxic effects in the quails with dose of 150 and 300 mg/kg feed. As well, researchers found that Cd affects the biochemical parameters of birds. It may decline blood concentrations of total protein and total albumen, A/G ratio and increase the activity of liver enzymes and levels of blood uric acid, urea, and alkaline phosphatase [8,9].

Ascorbic acid is a potent water soluble antioxidant capable of neutralizing and scavenging an array of reactive oxygen species viz., alkoxyl, hydroxyl, superoxide anion, peroxyl, hydroperoxyl radicals, and radicals of reactive nitrogen such as nitroxide, nitrogen di-oxide, and peroxynitrite at very low concentrations [10–12]. Ascorbic acid is required for the conversion of vitamin D into its metabolite form (calciterole) which is essential for calcium regulation and the calcification process [13]. It is also is an antioxidant and water-soluble vitamin that is found intra- and extra cellular as ascorbate [14]. In addition, it is a natural antioxidant that prevents the increased production of free radicals induced by oxidative damage to lipids and lipoproteins in various cellular compartments and tissues [15]. Ascorbic acid supplementation at 200 ppm is beneficial for enhancing the immunity, performance, and exploiting the broilers' full genetic potential [16]. Sharaf et al. [17] concluded that ascorbic acid has potent antioxidant activity against lead and Cd toxicity. They added that the consumption of foods rich in ascorbic acid is a highly recommended to reduce the damage caused by the toxicity with cadmium.

Supplementing poultry diets with natural clay improves growth performance and egg production rate which is a consequence of the improvement in the nutrient digestibility, feed conversion, ability to make rations more available to the bird and nitrogen retention in bird body, and retarded the absorption of toxic products of digestion that reduce toxicity [18]. Ability of clay to diminish the harmful effects of radiation may have a role in this respect [18]. Abou-Kassem et al. [19] reported that clay supplementation diminished the toxic effects of Cd with levels up to 120 mg/kg of quail diet. The present study aimed to investigate the role of ascorbic acid or natural clay on laying performance, some blood biochemical components and Cd residues in egg components of Japanese quail layers fed diets polluted by Cd at various levels.

2. Materials and Methods

A total number of 360 laying Japanese quail at 8 weeks of age were randomly divided into 12 groups (30 birds/group). Each group was sub-divided into five replicates (6 females each). Each replicate was housed in one cage with area of (2400 cm^2). A factorial arrangement (4×3) was applied including four levels of cadmium chloride (CdCl2); the first group (G0) received zero cadmium level and served as a control group, the other three groups G1, G2, and G3 received 50, 100 and 150 mg/kg diet, respectively). Each group was subdivided into three kinds of feed additives (control, 300 mg ascorbic acid/kg diet

and 1.50% natural clay) to study the effect of cadmium, feed additives and their interactions on the productive performance, egg quality and cadmium residues in eggs of laying Japanese quails.

Basal experimental diet was formulated to meet the laying Japanese quail nutrient requirements as recommended by National Research Council (NRC) [20] as shown in Table 1. Vitamin C (Rovimix® Stay-C 35 (Obour city, Egypt); specifically produced for the use as a stabilized source of vitamin C in feed; L ascorbic acid), according to the manufacturers' guidelines. Cadmium chloride (CdCl2, 2.5 H2O) imported from Chem-Lab NV, Industriezone, De Arend 2, B-8210 Zedelgem, Batch Nr.: 23.5852506, Belgium. Natural clay (from Adwia Company LTD, Obour city, Egypt, Batch No. E09860200) analysis as soluble cations and anions (milligram equivalent (meq)/ 100 gm dry matter soil) were Mg++ 0.25, Ca++ 0.75, K+ 0.10, Na+ 0.05, So4 0.30, Cl 0.55, and HCO3 0.75. Exchangeable cations (meq/100 g dry matter soil) was 2.65 and available nutrients (mg /100 g dry matter soil) were K 1.2, Mn 2.4, P 5.0, Cu 0.30, Zn 0.74, and Fe 0.55 mg [21]. The birds were fed the contaminated diets from 8 to 20 weeks of age. While, at the period from 21 to 24 weeks of age, birds were fed diets without cadmium addition. Birds were reared during the experimental periods under the same management, hygienic and environmental conditions. The bird's health status was monitored throughout the trial. Quails were exposed to 16 hours of light per day; fed ad-libitum and fresh water was available during the experimental periods. Drinkers and feeding troughs were daily cleaned.

Table 1. Composition and chemical analysis of experimental diet (8–24 w).

Ingredients	Percentages
Yellow corn	56.50
Soy bean meal (44%)	30.10
Corn gluten (60%)	3.00
Cotton seed oil	2.50
Limestone	5.00
Dicalcium phosphate	2.30
Nacl	0.30
Premix *	0.30
Calculated analysis **	
C.P%	20.01
ME. Kcal/kg	2894.00
Ca%	2.51
P% Avail, P.	0.54
Lysine	1.04
Met. + Cys. %	0.69

* Layer Vitamin and Mineral premix Each 3 kg consists of: Vit A. 12 Mi.U, Vit E. 15 IU., Vit. D_3 4 Mi.U, Vit, B_1 1g, Vit, B_2 8g, Pantothenic acid 10.87g, Nicotinic acid 30g, Vit. B_6 2g, Vit. B_{12} 10 mg. Folic acid 1 g, Biotin 150 mg, Copper 5g, Iron 5g, Manganese 70 g Iodine 0.5g, Selenium 0.15 g, Zinc 60 g. Antioxidant 10 g; ** Calculated according to NRC [20].

2.1. Laying Performance

Egg number and egg weight were daily recorded per replicate in each pen from 8 weeks of age up to the end of the experiment (24 weeks of age). Egg mass (g) was obtained by multiplying egg number by the average egg weight in each pen per day (8–24 weeks of age). Feed conversion during the experimental period (8–24 weeks of age) was calculated for egg production as follows:

Feed conversion for egg produced = g feed/ g egg produced.

Mortality rate of laying experimental periods was calculated as follows:

$$M.R = N_1 - N_2/N_1 \quad (1)$$

where, M.R—Mortality rate; N_1—The number of birds at the beginning of laying period; N_2—The number of birds at the end of laying period.

2.2. Blood Biochemical Components

At 20 and 24 weeks of age, blood samples were collected from sacrificed quails into heparinized sterile tubes. Samples then centrifuged at 3500 rpm for 15 min and serum was separated, collected and stored at −20 °C till examination. Total albumin, protein, urea-N, creatinine, uric acid, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline-phosphatase activities (ALP) were assessed using biodiagnostic commercial kits provided from Biodiagnostic Company (29 El-Tahrir St. Dokki, Giza, Egypt) Batch No: ALT (cat#AL1031), AST (cat#AS1061) according to the manufacturers' guidelines (REF: 264 001, 264 002) and a spectrophotometer (Shimadzu). The globulin concentrations and A/G ratio were calculated from the difference between the concentrations of total protein and albumin.

2.3. Cadmium Residue in Egg Components

At 20 and 24 weeks of age, Cd residues in egg components were determined. Twenty eggs from each treatment group were randomly taken, broken, and egg components were separated (white, yolk and shell) using a separation funnel and directly put in plates then transferred to an oven at 70 °C for 24 h or till a constant dray mass was achieved [22]. The dried samples were perfectly ground and homogenized to be prepared for digestion and residues determination. Cadmium residues were estimated in eggs collected from each treatment group (μg/g wet weight, ppm) in the Central Laboratory of the Faculty of Veterinary Medicine, Zagazig University, Egypt. One gram of each egg component sample (white, yolk, and shell) was placed in a clean screw capped glass bottle and digested with a 4 mL of digesting solution (nitric/per chloric acids, 1:1). Initial digestion was carried out for 24 h at room temperature followed by heating at 110 °C for 2 h. After cooling, deionized water was added, then the solutions were warmed in water bath for 1 h to expel nitrous gases. Digests were then filtered (Whatman No. 1, Ashless, grade 42, cat# 1442-110), and diluted in water to 25 ml deionized water [23]. The obtained solutions were analyzed by flame atomic absorption spectrophotometer (FAAS), (PerkinElmer, 520 South Main St., Suite 2423, Akron, Ohio 44311, USA, Model 2380, Serial No. 131865) to measuring the level of Cd residues in each egg components for all treatment groups. The laboratory had established a calibration programme, which reviewed and adjusted as necessary in order to maintain confidence in the status of calibration, whereas, the laboratory follows international standard Iso/IEC, 17025.

2.4. Statistics

Data were statistically analyzed on a 4 × 3 factorial arrangement basis according to Snedecor and Cochran [24] using the following model:

$$Y_{ijk} = \mu + A_i + S_j + AS_{ij} + e_{ijk} \quad (2)$$

where Y_{ijk} = an observation, μ = the overall Mean, A_i = effect of cadmium level (i = 1 to 4), S_j = effect of feed additives (j = 1 to 3), AS_{ij} = the interaction between cadmium level and feed additives (ij = 1 to 12) and e_{ijk} = random error. Differences among means within the same factor were tested using Duncan's New Multiple Range test [25].

3. Results

3.1. Productive Performance

Significant ($p < 0.05$ and $p < 0.01$) reduction was shown in live body weight with increasing the dietary Cd level at 12, 20, and 24 weeks of age compared to the control (Table 2). Final body weight of laying Japanese quail fed diet supplemented with ascorbic acid or clay was significantly ($p < 0.05$ or 0.01) increased comparing to those fed the control diet (Table 2).

Table 2. Live body weight changes, egg number, egg weight and mortality rate of Japanese quail layers as affected by dietary cadmium levels, some feed additives during the experiment (8–24 weeks of age).

Items	Body Weight (g)					M.R	Egg Number					Egg Weight (g)				
	8 w	12 w	16 w	20 w	24 w	8–20 w	8–12 w	13–16 w	17–20 w	8–20 w	21–24 w	8–12 w	13–16 w	17–20 w	8–20 w	21–24 w
Cadmium level (mg/kg diet)																
Control	194.78	192.66 ^{a}	190.84	197.12 ^{b}	207.16 ^{ab}	0.00	16.83 ^{b}	17.33 ^{b}	18.17 ^{b}	17.45 ^{b}	17.92 ^{b}	10.61 ^{a}	10.72 ^{a}	10.80 ^{b}	10.71 ^{a}	10.90 ^{a}
50 mg Cd/kg	194.96	190.20 ^{b}	190.67	196.21 ^{b}	205.89 ^{b}	0.00	17.25 a	17.06 ^{bc}	17.69 ^{bc}	17.33 ^{b}	17.42 ^{c}	10.56 ^{a}	10.86 ^{a}	10.99 ^{a}	10.81 ^{a}	11.00 ^{a}
100 mg Cd/kg	194.68	187.57 ^{c}	189.01	196.28 ^{b}	205.54 ^{b}	4.16 ^{b}	16.47 ^{b}	16.72 ^{c}	17.06 ^{c}	16.75 ^{c}	17.22 ^{cd}	10.43 ^{b}	10.67 ^{a}	10.72 ^{b}	10.60 ^{b}	10.86 ^{a}
150 mg Cd/kg	194.53	185.03 ^{d}	188.01	195.15 ^{b}	203.89 ^{c}	12.50 ^{a}	15.42 ^{c}	15.50 ^{d}	15.33 ^{d}	15.42 ^{d}	15.69 ^{d}	10.31 ^{c}	10.36 ^{b}	10.29 ^{c}	10.32 ^{c}	10.51 ^{b}
SEM	0.81	1.56	1.91	1.48	1.89	0.18	1.09	0.55	0.48	0.37	0.54	0.18	0.21	0.32	0.13	0.29
p-value	0.814	0.024	0.568	0.370	0.019	0.001	0.001	0.004	0.007	0.001	0.002	0.001	0.008	0.001	0.006	0.018
Feed additives																
Control	194.13	187.47	188.48	194.20	203.83 ^{b}	0.00	15.96 ^{b}	15.79 ^{c}	15.90 ^{c}	15.88 ^{c}	16.48 ^{c}	10.44	10.61	10.62	10.55	10.72
300 mg.vit C	195.20	190.15	191.19	198.64	207.85 ^{ab}	0.00	17.37 ^{a}	17.73 ^{a}	18.50 ^{a}	17.86 ^{a}	18.73 ^{a}	10.55	10.78	10.84	10.72	10.94
1.50% clay	195.07	189.63	190.71	197.45	208.97 ^{a}	0.00	17.28 ^{a}	17.35 ^{b}	17.90 ^{b}	17.47 ^{b}	17.67 ^{b}	10.51	10.66	10.73	10.63	10.94
SEM	0.63	1.69	1.74	1.17	1.60	0.00	0.35	0.50	0.44	0.52	0.73	0.31	0.24	0.28	0.20	0.34
p-value	0.231	0.367	0.501	0.013	0.009	0.918	0.001	0.005	0.011	0.001	0.001	0.547	0.417	0.842	0.028	0.067

$^{a–d}$ Values followed by different letters in each column within main effect are significantly different ($p < 0.05$); M.R: mortality rate.

3.2. Mortality Rate:

Mortality rate was significantly ($p < 0.01$) affected by Cd polluted diet during the experimental period of 8–20 weeks of age. The highest value was 12.50% with the high Cd contaminated level (150 mg/kg diet). Results in Table 2 showed that mortality rate was not significantly affected by the addition of ascorbic acid or natural clay to the diet during all experimental periods. Mortality rate was not affected by the interaction among Cd level and ascorbic acid or natural clay in the diet at 20 w.

3.3. Egg Number

Egg number was significantly ($p < 0.05$) decreased in quails as the concentration of the Cd increased at all the experimental periods. Ascorbic acid or clay supplementation significantly ($p < 0.05$) increased egg number comparing to hens fed on a diet without supplementation during all the experimental periods (Table 2). The results revealed that egg number was not significantly affected by the interaction between feed additives and Cd pollution during the whole experimental period (Table 3). Within any Cd level, ascorbic acid or clay supplementation increased egg number compared to the other groups.

3.4. Egg Weight

Egg weight was significantly ($p < 0.05$) decreased with increasing Cd level during the experimental period (Table 2). Ascorbic acid or clay supplementation ($p < 0.01$) increased egg weight comparing to the control during the whole period (8–20) and 20–24 weeks of age. Whilst, egg weight values were insignificantly improved by ascorbic acid or clay addition during the other periods (Table 2). Results showed that egg weight was not significantly affected by the interaction between feed additives and Cd contamination during the whole experimental period (Table 3).

3.5. Egg Mass

Quails exposed to Cd had decreased ($p < 0.01$) egg mass as compared to the control throughout the experimental periods and the whole experimental period (Table 4). Results in Table 4 showed that ascorbic acid or clay supplementation significantly ($p < 0.01$) improved egg mass.

While, the interaction between main the two factors insignificantly affected egg mass values. Results indicated that quails fed diet without Cd with 300 mg ascorbic acid/kg diet produced higher egg mass value (8.45 g/day/bird). Hens fed diet contained 150 mg cadmium/kg supplemented with 1.50% clay produced the lowest egg mass value (4.39 gm/day/bird).

3.6. Feed Conversion Ratio

With increasing cadmium level in quail diets, feed conversion became significantly worst per egg unit ($p < 0.05$) throughout the experimental periods and the whole period except the insignificant effect during 13–16 weeks of age (Table 4). A significant improvement ($p < 0.01$) was found in feed conversion as a result to ascorbic acid or clay supplementations during the whole experimental period (8–20 weeks of age) and the period of 21–24 weeks of age. However, the diet had 300 mg ascorbic acid recorded the best ($p < 0.05$) feed conversion (3.27 and 3.15 gm feed/gm/egg) values during 8–20 and 21–24 weeks of age, respectively compared to the other experimental groups.

Table 3. Live body weight, egg number, and egg weight of Japanese quail layers as affected by interaction between dietary cadmium levels and some feed additives during the experiment (8–24 weeks of age).

Items	Body Weight g/bird				M.R	Egg Number/Bird					Egg Weight (g)				
	8 w	12 w	16 w	20 w	24 w	8–20 w	8–12 w	12–16 w	16–20 w	8–20 w	21–24 w	8–12 w	12–16 w	16–20 w	8–20 w	21–24 w
Interaction effect																
Without cadmium																
Control	194.78	192.66	190.84	197.12	207.16	0.00	16.83	17.33	18.17	17.45	17.92	10.61	10.72	10.80	10.71	10.90
300 mg.vit C	195.20	194.42	194.13	201.09	213.75	0.00	19.08	19.67	20.83	19.86	20.92	10.78	10.95	11.07	10.93	11.20
1.50% clay	193.01	193.50	193.43	200.02	210.32	0.00	18.67	18.67	19.83	19.05	19.08	10.65	10.83	10.89	10.79	11.07
50 mg Cadmium																
Control	192.34	188.47	189.67	195.14	202.46	4.17 [b]	15.83	15.42	15.67	15.64	17.17	10.49	10.89	10.95	10.78	10.96
300 mg.vit C	194.98	190.70	190.33	197.19	205.33	0.00	18.08	17.92	18.58	18.20	18.83	10.58	10.88	11.03	10.83	11.01
1.50% clay	196.97	191.44	192.01	196.30	206.30	0.00	17.83	17.83	18.83	18.17	19.25	10.61	10.82	11.00	10.81	11.04
100 mg Cadmium																
Control	192.71	185.23	187.45	193.21	198.20	8.34 [a]	16.00	15.50	15.66	15.56	15.58	10.37	10.53	10.51	10.47	10.64
300 mg.vit C	193.37	189.17	191.58	198.71	206.20	4.17 [b]	16.92	17.83	18.58	17.78	18.83	10.47	10.85	10.91	10.74	10.95
1.50% clay	191.97	188.30	188.01	196.91	209.23	4.17 [b]	16.50	16.83	17.41	16.92	17.25	10.44	10.63	10.74	10.60	10.99
150 mg Cadmium																
Control	192.70	183.51	185.94	191.34	195.52	8.34 [a]	15.17	14.92	14.58	14.89	15.25	10.28	10.29	10.21	10.26	10.39
300 mg.vit C	193.25	186.29	188.70	197.57	204.11	4.17 [b]	15.42	15.50	15.92	15.61	16.33	10.35	10.43	10.36	10.38	10.61
1.50% clay	194.73	185.30	189.40	196.56	206.04	4.16 [b]	15.67	16.08	15.50	15.75	15.50	10.30	10.36	10.28	10.31	10.53
SEM	0.97	3.21	4.07	2.38	2.71	0.17	0.93	0.71	1.03	0.67	1.08	0.27	0.24	0.30	0.19	0.21
p-value	0.910	0.715	0.640	0.553	0.418	0.324	0.298	0.354	0.209	0.117	0.870	0.227	0.324	0.108	0.487	0.144

[a,b] Values followed by different letters within each effect in each column are significantly different ($p < 0.05$); M.R: mortality rate.

Table 4. Egg mass and feed conversion of Japanese quail layers as affected by dietary cadmium levels, some feed additives during the experiment (8–24 weeks of age).

Items	Egg Mass g/day/bird					Feed Conversion g feed/g/egg				
	8–12 w	12–16 w	16–20 w	8–20 w	21–24 w	8–12 w	13–16 w	17–20 w	8–20 w	21–24 w
Cadmium level										
Control	6.38 ^{a}	6.63 ^{a}	7.00 ^{a}	6.67 ^{b}	6.98 ^{b}	3.25 ^{c}	3.26	3.29 ^{c}	3.27 ^{d}	3.20 ^{c}
50 mg Cd/kg	6.51 ^{a}	6.61 ^{a}	6.94 ^{a}	6.69 ^{b}	7.24 ^{a}	3.30 ^{c}	3.27	3.24 ^{c}	3.27 ^{d}	3.23 ^{c}
100 mg Cd/kg	6.13 ^{b}	6.38 ^{b}	6.54 ^{b}	6.35 ^{c}	6.64 ^{c}	3.46 ^{b}	3.41	3.41 ^{b}	3.42 ^{b}	3.29 ^{b}
150 mg Cd/kg	5.68 ^{c}	5.74 ^{c}	5.63 ^{b}	5.68 ^{d}	5.44 ^{d}	3.65 ^{a}	3.51	3.53 ^{a}	3.57 ^{a}	3.45 ^{a}
Feed additives										
Control	5.95 ^{b}	5.98 ^{b}	6.04 ^{b}	5.99 ^{b}	6.28 ^{b}	3.45	3.34	3.45	3.44 ^{a}	3.38 ^{a}
300 mg.vit C	6.55 ^{a}	6.83 ^{a}	7.17 ^{a}	6.85 ^{a}	7.33 ^{a}	3.26	3.37	3.23	3.27 ^{c}	3.15 ^{c}
1.50% clay	6.44 ^{a}	6.61 ^{a}	6.87 ^{ab}	6.64 ^{b}	6.60 ^{b}	3.39	3.32	3.31	3.34 ^{b}	3.29 ^{b}
Interaction effect										
Without cadmium										
Control	6.38	6.63	7.00	6.67	6.98 ^{c}	3.25	3.26	3.29	3.27	3.20
300 mg ascorbic acid	7.35	7.69	8.24	7.76	8.45 ^{a}	2.77	3.43	2.96	3.06	2.99
1.50% Clay	7.09	7.22	7.71	7.34	7.55 ^{b}	3.16	3.20	3.18	3.18	3.32
50 mg Cadmium										
Control	5.94	6.00	6.13	6.02	6.72 ^{c}	3.31	3.31	3.33	3.32	3.31
300 mg ascorbic acid	6.84	6.95	7.31	7.03	7.41 ^{b}	3.28	3.25	3.16	3.23	3.13
1.50% Clay	6.76	6.89	7.38	7.01	7.60 ^{b}	3.30	3.25	3.23	3.26	3.24
100 mg Cadmium										
Control	5.92	5.83	5.69	5.81	5.79 ^{b}	3.52	3.55	3.57	3.54	3.43
300 mg ascorbic acid	6.32	6.90	7.24	6.82	7.36 ^{b}	3.41	3.30	3.30	3.34	3.23
1.50% Clay	6.15	6.40	6.68	6.41	6.77 ^{c}	3.46	3.38	3.34	3.39	3.21
150 mg Cadmium										
Control	5.57	5.48	5.32	5.46	5.66 ^{d}	3.72	3.60	3.62	3.64	3.57
300 mg ascorbic acid	5.70	5.77	5.89	4.79	6.18 ^{d}	3.60	3.50	3.48	3.53	3.39
1.50% Clay	5.77	5.95	5.69	5.81	4.39 ^{e}	3.64	3.44	3.48	3.52	3.38
SEM	0.34	0.39	0.43	0.28	0.51	0.19	0.22	0.29	0.15	0.13
p-value										
Cadmium level	0.006	0.001	0.001	0.002	0.021	0.041	0.137	0.007	0.009	0.029
Feed additives	0.003	0.001	0.001	0.002	0.034	0.119	0.225	0.362	0.048	0.043
Interaction effect	0.320	0.524	0.182	0.417	0.019	0.294	0.381	0.615	0.540	0.343

$^{a–d}$ Values followed by different letters within each effect in each column are significantly different ($p < 0.05$).

3.7. Blood Biochemical Components

Cadmium levels at 100 and 150 mg/kg diet decreased ($p < 0.05$) blood total protein, total albumen and A/G ratio compared to the control and 50 mg cadmium/kg diet at 20 weeks of age. Meanwhile, cadmium did not affect total globulin. On the other hand, creatinine level decreased ($p < 0.05$) at various cadmium levels compared to the control (Tables 5–8). Concerning the effect of feed additives at 20 weeks of age, both 300 mg ascorbic acid and 1.50% Clay increased ($p < 0.05$) total protein and albumen compared to the non-supplemented group but did not affect total globulin and A/G ratio level at both 20 and 24 weeks of age. Increasing cadmium levels increased ($p < 0.05$) ALT, AST, ALP, urea-N, and creatinine as compared to the control at 20 and 24 weeks of age. At 20 weeks of age, both 300 mg ascorbic acid and 1.50% Clay decreased ($p < 0.05$) serum ALT, AST and urea-N compared to the non-supplemented group but did not affect ALP and creatinine level at both 20 and 24 weeks of age. The interaction between cadmium level and feed additives had no effect on various serum parameters.

Table 5. Some blood parameters of Japanese quail layers as affected by dietary cadmium levels, feed additives and their interactions at 20 weeks of age.

Items	ALT, U/L	AST, U/L	Uric Acid, Mg/dL	Urea-N, Mg/dL	Creatinine Mg/dL	Alkaline Phosphatase U/L
Cadmium level (mg/kg diet)						
Control	33.88 ^{c}	54.83 ^{c}	5.42 ^{c}	12.95 ^{c}	0.70 ^{b}	174.55 ^{c}
50 mg Cd/kg	34.22 ^{c}	54.31 ^{c}	5.32 ^{c}	13.72 ^{c}	0.79 ^{a}	176.12 ^{c}
100 mg Cd/kg	37.42 ^{b}	59.21 ^{b}	5.98 ^{b}	16.23 ^{b}	0.83 ^{a}	182.37 ^{b}
150 mg Cd/kg	41.95 ^{a}	61.90 ^{a}	6.63 ^{a}	20.47 ^{a}	0.87 ^{a}	199.11 ^{a}
Feed additives						
Control	37.64 ^{a}	58.56 ^{a}	5.99	16.97 ^{a}	0.82	185.16
300 mg.vit C	35.82 ^{b}	56.63 ^{b}	5.61	14.84 ^{b}	0.77	180.60
1.50% clay	36.41 ^{b}	56.25 ^{b}	5.68	14.84 ^{b}	0.79	180.01
Interaction						
Without cadmium						
Control	33.88	54.83	5.42	12.95	0.68	174.55
300 mg ascorbic acid	31.86	52.29	4.89	11.22	0.69	168.10
1.50% Clay	32.94	52.40	5.05	11.18	0.73	167.68
50 mg Cadmium						
Control	35.48	56.56	5.29	13.95	0.82	179.50
300 mg ascorbic acid	35.79	53.22	5.39	13.43	0.77	173.90
1.50% Clay	33.40	53.15	5.27	13.79	0.79	174.95
100 mg Cadmium						
Control	38.50	60.11	6.15	19.26	0.87	185.46
300 mg ascorbic acid	35.95	59.37	5.86	14.92	0.81	182.10
1.50% Clay	37.82	58.16	5.93	14.51	0.79	179.54
150 mg Cadmium						
Control	42.71	62.75	7.10	21.73	0.92	201.11
300 mg ascorbic acid	41.68	61.66	6.32	19.80	0.83	198.32
1.50% Clay	41.46	61.29	6.47	19.89	0.85	197.89
SEM	2.84	2.31	1.10	2.49	0.07	9.11
p-value						
Cadmium level	0.001	0.004	0.009	0.001	0.007	0.001
Feed additives	0.023	0.241	0.170	0.047	0.110	0.07
Interaction	0.082	0.173	0.354	0.477	0.209	0.119

$^{a–c}$ Values followed by different letters within each effect in each column are significantly different ($p < 0.05$).

Table 6. Some blood parameters of Japanese quail layers as affected by dietary cadmium levels, feed additives and their interactions at the end of (experiment 24 weeks of age).

Items	ALT, U/L	AST, U/L	Uric Acid, Mg/dL	Urea-N, Mg/dL	Creatinine Mg/dL	Alkaline Phosphatase U/L
Cadmium level (mg/kg diet)						
Control	34.65 [b]	55.18 [c]	5.43 [b]	13.03 [c]	0.71 [b]	174.92 [c]
50 mg Cd/kg	32.58 [c]	54.20 [c]	5.32 [c]	12.86 [c]	0.78 [b]	174.27 [c]
100 mg Cd/kg	34.90 [b]	57.75 [b]	5.46 [b]	14.66 [b]	0.76 [b]	178.57 [b]
150 mg Cd/kg	39.92 [a]	60.76 [a]	6.34 [a]	19.58 [a]	0.83 [a]	195.36 [a]
Feed additives						
Control	36.01 [a]	57.58 [a]	5.93 [a]	15.68 [a]	0.80	183.12 [a]
300 mg.vit C	34.97 [b]	56.39 [b]	5.44 [b]	14.56 [b]	0.74	179.11 [b]
1.50% clay	35.30 [b]	55.68 [b]	5.47 [b]	14.04 [b]	0.77	177.72 [b]
Interaction						
Without cadmium						
Control	34.65	55.18	5.43	13.03	0.71	174.92
300 mg ascorbic acid	34.23	52.54	5.39	11.64	0.70	172.15
1.50% Clay	34.01	52.79	5.18	11.13	0.75	168.15
50 mg Cadmium						
Control	33.11	55.36	5.68	12.68	0.80	175.34
300 mg ascorbic acid	32.41	54.16	5.09	12.74	0.79	173.97
1.50% Clay	32.24	53.07	5.20	13.17	0.78	173.51
100 mg Cadmium						
Control	35.25	58.50	5.71	16.14	0.83	183.61
300 mg ascorbic acid	33.82	58.12	5.33	14.66	0.71	176.72
1.50% Clay	35.62	56.64	5.33	13.19	0.75	175.38
150 mg Cadmium						
Control	41.04	61.31	6.89	20.87	0.88	198.63
300 mg ascorbic acid	39.41	60.74	5.96	19.22	0.78	193.61
1.50% Clay	39.32	60.24	6.16	18.67	0.81	193.84
SEM	3.71	3.27	0.93	2.08	0.07	8.75
p-value						
Cadmium level	0.001	0.001	0.007	0.001	0.042	0.001
Feed additives	0.015	0.049	0.038	0.020	0.715	0.039
Interaction	0.228	0.109	0.512	0.099	0.617	0.176

[a–c] Values followed by different letters within each effect in each column are significantly different ($p < 0.05$).

Table 7. Some blood parameters as affected by dietary cadmium levels, feed additives and their interactions at 20 weeks of age.

Items	Total Protein, G/dl	Total Albumin, G/dl	Total Globulin, G/dl	A/G ratio
Cadmium level				
Control	5.41 [a]	3.20 [a]	2.21	1.46 [a]
50 mg Cd/kg	5.28 [a]	3.06 [a]	2.22	1.39 [a]
100 mg Cd/kg	5.08 [b]	2.86 [b]	2.22	1.30 [b]
150 mg Cd/kg	4.58 [c]	2.37 [c]	2.21	1.08 [c]
Feed additives				
Control	4.81 [b]	2.63 [c]	2.18	1.22
300 mg.vit C	5.32 [a]	3.11 [a]	2.21	1.42
1.50% clay	5.12 [a]	2.87 [b]	2.87	1.28
Interaction				
Without cadmium				
Control	5.19	2.99	2.20	1.37
300 mg ascorbic acid	5.65	3.54	2.11	1.69
1.50% Clay	5.39	3.05	2.34	1.31

Table 7. *Cont.*

Items	Total Protein, G/dl	Total Albumin, G/dl	Total Globulin, G/dl	A/G ratio
50 mg Cadmium				
Control	5.07	3.05	2.02	1.53
300 mg ascorbic acid	5.55	3.23	2.32	1.41
1.50% Clay	5.22	2.90	2.33	1.24
100 mg Cadmium				
Control	4.72	2.39	2.33	1.02
300 mg ascorbic acid	5.23	3.04	2.19	1.39
1.50% Clay	5.28	3.15	2.13	1.48
150 mg Cadmium				
Control	4.27	2.09	2.18	0.97
300 mg ascorbic acid	4.86	2.63	2.23	1.18
1.50% Clay	4.60	2.38	2.21	1.08
SEM	0.45	0.39	0.15	0.22
p-value				
Cadmium level	0.001	0.005	0.211	0.001
Feed additives	0.001	0.006	0.167	0.092
Interaction	0.210	0.090	0.223	0.170

[a–c] Values followed by different letters in each column are significantly different ($p < 0.01$).

Table 8. Some blood parameters as affected by dietary cadmium levels, feed additives, and their interaction at 24 weeks of age.

Items	Total Protein G/dL	Total Albumin G/dL	Total Globulin G/dL	A/G Ratio
Cadmium level				
Control	6.09 [a]	3.52 [a]	2.56 [a]	1.38 [c]
50 mg Cd/kg	5.98 [a]	3.60 [a]	2.37 [b]	1.54 [b]
100 mg Cd/kg	5.89 [b]	3.48 [b]	2.11 [d]	1.66 [a]
150 mg Cd/kg	4.73 [c]	2.68 [c]	2.05 [d]	1.30 [d]
Feed additives				
Control	5.43 [c]	3.22	2.21 [b]	1.47 [b]
300 mg.vit C	5.81 [a]	3.41	2.39 [a]	1.44 [b]
1.50% clay	5.68 [b]	3.46	2.22 [b]	1.57 [a]
Interaction				
Without cadmium				
Control	6.09	3.52	2.56	1.38
300 mg ascorbic acid	6.60	3.91	2.69	1.46
1.50% Clay	6.11	3.70	2.41	1.56
50 mg Cadmium				
Control	5.79	3.59	2.20	1.68
300 mg ascorbic acid	6.24	3.74	2.51	1.50
1.50% Clay	5.90	3.48	2.41	1.44
100 mg Cadmium				
Control	5.28	3.17	2.11	1.50
300 mg ascorbic acid	5.60	3.48	2.12	1.64
1.50% Clay	5.89	3.80	2.09	1.84
150 mg Cadmium				
Control	4.56	2.59	1.96	1.32
300 mg ascorbic acid	4.80	2.57	2.23	1.15
1.50% Clay	4.83	2.86	1.97	1.45
SEM	0.67	0.81	0.21	0.33
p-value				
Cadmium level	0.001	0.001	0.006	0.034
Feed additives	0.005	0.208	0.017	0.009
Interaction	0.254	0.310	0.278	0.189

[a–d] Values followed by different letters within each effect in each column are significantly different ($p < 0.05$).

3.8. Cadmium Residues in Egg Components

Pollution of quail diets with Cd had a highly significant ($p < 0.01$) effect on cadmium residues in egg components (white, yolk and shell) at 20 and 24 weeks of age as shown in Table 9. Data showed that ascorbic acid or natural clay had a significant ($p < 0.01$) effect on cadmium residues in egg components. Results showed that Cd residues in egg components at 20 and 24 weeks of age were significantly ($p < 0.01$) affected by the interaction between Cd level and the dietary supplementation of ascorbic acid or natural clay. The highest value recorded (37.59 ± 2.03 mg) was found in yolk with high dietary Cd level (150 ppm) and without additives at 20 weeks of age.

Table 9. Cadmium residual in tissues of Japanese quail layers as affected by dietary cadmium levels, feed additives and their interaction at 20 and 24 weeks of age.

Items	Cadmium Residual (mg/kg DM)					
	20 w			24 w		
	White	Yolk	Shell	White	Yolk	Shell
Cadmium level (mg/kg diet)						
Control	0.13 ^{d}	0.36 ^{d}	0.18 ^{d}	0.13 ^{c}	0.25 ^{d}	0.15 ^{c}
50 mg Cd/kg	6.41 ^{c}	12.43 ^{c}	9.57 ^{c}	6.21 ^{b}	5.32 ^{c}	3.52 ^{b}
100 mg Cd/kg	14.31 ^{b}	17.31 ^{b}	16.71 ^{b}	7.72 ^{b}	11.03 ^{b}	7.98 ^{b}
150 mg Cd/kg	26.73 ^{a}	37.15 ^{a}	33.65 ^{a}	22.09 ^{a}	23.74 ^{a}	25.13 ^{a}
Feed additives						
Control	14.11 ^{a}	19.29 ^{a}	17.12 ^{a}	10.63 ^{a}	12.03 ^{a}	10.92 ^{a}
300 mg vit C	10.05 ^{b}	16.61 ^{b}	12.87 ^{b}	7.19 ^{b}	9.45 ^{b}	8.22 ^{b}
1.50% clay	9.35 ^{c}	15.15 ^{c}	13.68 ^{c}	6.52 ^{b}	8.56 ^{c}	6.98 ^{c}
Interaction						
Without cadmium						
Control	0.13 ^{a}	0.36 ^{a}	0.18 ^{a}	0.13 ^{a}	0.25 ^{a}	0.15 ^{a}
300 mg ascorbic acid	0.24 ^{a}	0.32 ^{a}	0.29 ^{a}	0.24 ^{a}	0.32 ^{a}	0.21 ^{a}
1.50% Clay	0.21 ^{a}	0.28 ^{a}	0.25 ^{a}	0.22 ^{a}	0.21 ^{a}	0.22 ^{a}
50 mg Cadmium						
Control	8.08 ^{bc}	11.74 ^{c}	9.43 ^{bc}	5.15 ^{c}	5.21 ^{bc}	3.49 ^{bc}
300 mg ascorbic acid	5.70 ^{ab}	9.35 ^{b}	7.82 ^{b}	3.44 ^{b}	3.46 ^{b}	3.14 ^{b}
1.50% Clay	6.68 ^{b}	10.61 ^{bc}	8.89 ^{bc}	3.95 ^{bc}	5.76 ^{c}	3.17 ^{b}
100 mg Cadmium						
Control	16.31 ^{d}	23.20 ^{e}	20.09 ^{d}	13.17 ^{d}	16.24 ^{de}	13.61 ^{d}
300 mg ascorbic acid	9.92 ^{c}	14.88 ^{d}	12.70 ^{cd}	6.46 ^{cd}	9.93 ^{d}	5.26 ^{cd}
1.50% Clay	9.19 ^{c}	14.34 ^{d}	11.62 ^{c}	4.29 ^{bc}	6.47 ^{cd}	4.51 ^{c}
150 mg Cadmium						
Control	29.43 ^{f}	37.99 ^{g}	34.94 ^{f}	23.08 ^{g}	25.89 ^{g}	28.17 ^{g}
300 mg ascorbic acid	25.06 ^{de}	33.75 ^{fg}	30.83 ^{e}	20.72 ^{f}	23.80 ^{f}	24.03 ^{f}
1.50% Clay	23.07 ^{de}	31.64 ^{f}	30.74 ^{e}	19.03 ^{e}	21.02 ^{e}	23.11 ^{e}
SEM	1.86	2.94	2.33	2.59	1.40	1.66
p-value						
Cadmium level	0.001	0.001	0.001	0.003	0.001	0.002
Feed additives	0.008	0.001	0.001	0.011	0.01	0.008
Interaction	0.001	0.001	0.001	0.011	0.008	0.001

$^{a–g}$ Values followed by different letters within each effect in each column are significantly different ($p < 0.05$).

4. Discussion

The body weight loss produced by Cd polluted diets (Table 2) might be due to the wide toxic effect of Cd on the whole-body processes in the bird. It has been reported that long term Cd exposure causes depletion of liver and muscular glycogen due to its action on the enzymes involved with glycogenesis and energy metabolism and inducing the oxidative stress in liver and kidney [26]. Also, decreasing body weight might be mediated by the Cd effect on the small intestine, where the nutrients are absorbed or through a dysfunction in the renal tubules resulted from the destructive effect of Cd on kidney. It would lead to an enhanced urinary excretion of some food nutrients which causes a decrease in the utilization of these nutrients in the body [26,27]. Decreasing body weight was also recorded in Japanese quail layers exposed to graded dietary Cd concentration of 40, 80, and 120 ppm [28]. Author attributed this result to the different doses of administration. Some studies on poultry have shown that supplemental ascorbic acid given in feed or by injection improved performance of chickens. Dietary supplementation with ascorbic acid increased the growth rate by about 4.5% [29]. These results agree with Shit et al. [30] who found that dietary L-ascorbic acid supplementation significantly ($p < 0.05$) increased body weight as of laying Japanese quail compared to the control group. On the other hand, these results disagree with those of Attia et al. [31] who reported that the addition of ascorbic acid had no effect on the body weight of broiler chicks until 42 d of age. The improvement in live body weight by clay may be due to delaying the transit time of digesta through the digestive tract by 2 to 2.5 h and promoting absorption of nutrients.

Digestibility of organic matter, fat, and nitrogen free extract and the nitrogen utilization were increased by supplementary Zeolite (aluminosilicate mineral) [32]. The present results agree with those obtained by Ayyat et al. [33] who found that supplementing clay (1%) in silver Montazah layers diet improved body weight. At any Cd level, ascorbic acid or clay supplementation in quail layer diets recorded higher body weight than the un-supplemented one. The results showed that Cd poisoning can be partially reduced by providing supplementary ascorbic acid or natural clay.

According to our results, Cd polluted diets caused 12.50% mortality rate. This result is in disagreement with Bokori et al. [7] who stated that dietary Cd pollution of 25 or 75 ppm did not cause indicative signs of mortality rate in chickens as compared to the control during the experimental period for nine months ago. Due to Klassen and Liu [34], the lethality associated with exposure to cadmium is related to cardiotoxicity and hepatotoxicity. Contrarily, Erdogan et al. [15] clarified that mortalities were not related to toxicity of Cd because no abnormal signs were observed during clinical observation or at autopsy examination in broiler chicks. Ascorbic acid has been reported to protect cells involved in the immune response such as lymphocytes, macrophages and plasma cells against oxidative damage of quail layer cells. Some studies on poultry have shown that supplemental ascorbic acid given in feed or drinking water or by injection of chickens reduced the mortality percentage by about 5% [29].

The significant decrease in egg number in the Cd polluted diets Japanese quail found in the present study (Table 2) could have resulted from alterations in the egg formation pathway or may be through the suppression of calcium metabolism [35]. Similarly, Rahman et al. [35] showed reduced egg production in quails. Decreased egg production was also observed in laying hens with single dietary exposures of 60 ppm radioactive Cd [36]. The dose and period of Cd exposure may be an important factor altering egg production potentialities which could be confirmed by the findings that showed no effect of Cd contamination on the egg production of pheasants given drinking water containing 1.5 mg cadmium/L for only 12 weeks [37]. Ascorbic acid or clay supplementation significantly increased egg number (Table 2). These results agree with Njoku and Nwazota [38] who clarified that ascorbic acid supplementation to laying hens' diets caused a proportional increase in the number of eggs laid. The presence of antioxidant (ascorbic acid) could adversely inhibit the oxidative protein denaturation and improve nutrients digestibility and feed efficiency. It could be also a reason for rising layers performance [39]. Present results agree with Moghaddam et al. [40] who found that dietary Zeolite addition at levels of 1.5%, 3%, and 4.5% caused a significant ($p < 0.01$) increase in egg production of Hy-Line hens. Therefore, supplementation of Zeolite can be used to extract ammonia by ion exchange

which helps to remove stress-forming ammonia from the hen intestine and thereby increasing egg production [41]. On the other hand, Lemser et al. [42] indicated that supplementation of rye-based broiler diets with natural clay minerals showed a negative effect on egg production.

Generally, the results indicated that the use of the cation exchange is capable to reduce the uptake and influence the distribution of heavy metals in poultry tissues. Evans et al. [43] concluded that the use of synthetic and natural Zeolite improved the performance of poultry. In line with our results, Rahman et al. [35] showed that egg weight of Japanese quail layers was markedly affected by Cd administration with injection levels of 0.1, 0.3, 1, 3, and 10 mg/kg body weight/day up to the end of the experimental period (14 days). Authors attributed this action to alterations in the egg formation pathway. This is may also returned to that quails exposed to Cd pollution produced fewer eggs and had lower egg weights than controls. Ascorbic acid or clay supplementation significantly ($p < 0.01$) increased egg weights (Table 2). This is because quails fed diets supplemented with ascorbic acid or clay produced more eggs and had higher egg weights than those fed diet without feed additives. These results are confirmed by Njoku and Nwazota [38] who clarified that ascorbic acid supplementation to laying hens' diets (250 ppm) showed highest mean egg weight but statistically no variation was drawn among the groups. According to Elliot and Edwards [44], natural Zeolite as a Clinoptolite bearing rock material was found to increase egg weight by incorporation in the hens' diet at an inclusion rate of less than 10%. Moghaddam et al. [40] found that dietary added Zeolite in levels of 1.5, 3 and 4.5% caused a significant ($p < 0.05$) increase in egg weight of Hy-Line hens. In contrast, Ozturk et al. [45] found that dietary Zeolite (aluminosilicate mineral) supplementation of 0%, 2%, 4%, 6%, and 8% as Clinoptilolite had no significant effect on egg weight of Babcock laying hens. The obtained results reported decreased egg mass due to Cd pollution (Table 4). It is worth noting that egg mass significantly decreased ($p < 0.01$) with increasing dietary Cd level which agree with results reported by Baykov et al. [46]. In a converse trend, results showed increased egg mass due to ascorbic acid or clay supplementation. These results agree with Moghaddam et al. [41] who demonstrated that Zeolite at levels of 1.5, 3 and 4.5% caused significant ($p < 0.01$) increases in egg mass of Hy-Line hens.

The poor feed conversion due to Cd pollution could be due to the highly significant decrease in egg mass as exposed to Cd pollution. Long-term cadmium exposure causes depletion of liver and muscular glycogen. This effect might reduce nutrient metabolism and feed utilization [35]. Feed conversion value depends mainly on the amount of feed consumed and the egg mass. In this context, it is worth noting that egg mass in ascorbic acid or clay groups were significantly ($p < 0.05$) higher than un-supplemented group (Table 4). These results are in harmony with Denli et al. [47] who found that dietary ascorbic acid improved feed conversion ratio in laying hens under stress. On the contrary, Soltani et al. [48] postulated that values of feed conversion rate of laying hens were not affected by dietary ascorbic acid ($p > 0.05$) with level of 250 mg/kg as compared to the control group.

For blood parameters, our results in Tables 7 and 8 fully agree with those obtained by Abou-Kassem et al. [19] who found that the increased levels of cadmium in quail diets significantly decreased ($p < 0.05$) total protein, total albumen and A/G ratio compared to the control group with no effects of various levels of clay or vitamin E supplementations. Also, Hashem et al. [49] found that cadmium levels at 100 mg/kg diet statistically decreased serum total proteins, albumin and globulins values. It was clear that cadmium levels at 100 and 150 mg/kg diet significantly increased ($p < 0.05$) ALT, AST, uric acid, urea-N, creatinine, and alkaline phosphatase levels compared to control and 50 mg cadmium/kg diet at 20 and 24 weeks of age of Japanese quails. Urea-N appears to be the most useful variable for the detection of pre-renal causes of renal failure with Cd toxicity. But the effects of feed additive and the interaction between cadmium level and feed additive at 20 and 24 weeks of age were not affecting ($p > 0.05$) on these traits (Tables 5 and 6). In a partially disagreement, Rambeck and Kollmer [50] showed that the addition of dietary ascorbic acid had a great protective effect on kidney damage from cadmium intake. Hashem et al. [49] reported that group received cadmium at level of 100 mg /kg diet significantly increased ($p < 0.05$) blood ALT, AST, uric acid and creatinine level compared to control group. The increase in ALT and AST enzyme levels and the outflow of

these enzymes to the blood from the liver due to Cd hepatotoxic effect is considered as an indicator of hepatocellular damage. ALT activity was significantly increased, while ALP activity was significantly decreased in plasma of rats fed 5 mg Cd /kg [51]. Cadmium revealed a significant increase in ALP activities when compared to the control group (Table 6). Our results assure the prophylactic potential of ascorbic acid or clay to prevent or decrease cadmium-induced toxic manifestations. ALP in blood is considered as an indicator of mineral status and bone mineralization especially Ca and P which plays an important role in the homeostasis of the body and ensures appropriate conditions for biological activities such as energy utilization, nucleic acid synthesis and bone mineralization [52].

The hepatocellular injury due to cadmium could be attributed to the cadmium-induced generation of free radicals [53]. The activity of AST and ALT enzymes used as stress indicators of evaluation for impairment and damage of tissue liver which produced from Cd toxicity and attributed the elevated activities of ALT and AST enzymes to the outflow from the liver cytosol to the blood. Also, increasing AST and ALT attributed to the hepatotoxic effect, hepatocellular damage or cellular degradation by Cd in quail liver. Toxicity with cadmium may be due to changes in liver carbohydrate metabolism especially activation of liver glycogenolysis and glycolysis as well as increased levels of plasma glucose [54]. From these explanations, the linkage between liver damage, energy metabolism, productive traits, and the residues of cadmium in quail egg components is clarified. Conversely, Erdogan et al. [15] reported that different Cd levels had no effects on AST and ALT activities.

Long-term exposure to cadmium leads to pathological changes in the quail kidneys which contains morphological changes are initially limited to proximal tubular epithelial cell degeneration followed by cellular atrophy, interstitial fibrosis, and glomerular sclerosis and finally associated with biochemical evidence of renal tubular dysfunction. These explanations ensured by Abdo and Abdalla [55] and Abou-Kassem et al. [19] who reported that kidney function tests elevated in Cd treated groups indicating the toxic effect of cadmium which was presumably due to nephrotoxic effect of cadmium on renal tubules and glomeruli.

Sato et al. [56] postulated that Cd transfer from laying hen to eggs was restricted after the maternal bird was exposed to Cd. furthermore; Cd accumulates in the follicle walls of ovary. These results suggest that the follicle walls might play a role in protecting the follicle yolks against Cd toxicity. These findings agree with Herzig et al. [57] who found that Cd level of all investigated tissues (liver and leg muscle) were significantly ($p < 0.05$) increased when chicken received 1.47 mg Cd within 10 days. Results of this study are in disagreement with Toman et al. [37] who found that the hepatic Cd concentration of pheasants exposed to Cd (1.5 mg/ L water) were under the limit of detection (0.01 mg/kg) in all sample collections (after 4, 8, and 12 w of the experiment).

Our results showed that ascorbic acid or natural clay had a significant ($p < 0.01$) effect on cadmium residues in egg components. On the contrary, Erdogan et al. [15] showed that ascorbic acid supplementation to the diet did not prevent Cd accumulation in several organs of chicks. These results agree with Attia et al. [31] who reported that natural clay addition to lead contaminated diets clearly reduced the level of lead residues in the viscera and muscles. Natural clay prevents the lead toxicity by reducing lead absorption in the intestinal tract and increasing fecal excretion [31].

5. Conclusions

It can be concluded that the consumption of polluted diets with heavy metals such as Cd causes deleterious effects on the productive performance of laying Japanese quails. The addition of ascorbic acid or natural clay to the diet of laying Japanese quails caused beneficial effects on productive performance and diminished the toxic effect of Cd on productive results during the treatment period.

Compliance with Ethical Standards: All procedures by this study were in accordance with international ethical standards. The research involved no human participants.

Author Contributions: D.E.A.-K. designed the study plan. M.E.A.E.-H. helped in conducting the experiment and collected literature. D.E.A.-K, A.E.T. and M.E.A.E.-H. analyzed the data and drafted the manuscript. J.S.A., S.N.M. and A.A.A. provided a technical help in writing the manuscript. All authors have read and agreed to the published version of the manuscript.

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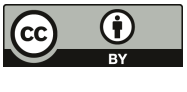

animals

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Article

Impact of Multienzymes Dose Supplemented Continuously or Intermittently in Drinking Water on Growth Performance, Nutrient Digestibility, and Blood Constituents of Broiler Chickens

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Simple Summary: Supplementing enzymes in diet can improve animal performance, carcass traits, physiological status, and reduce cost of feeding due to improving feed utilization. Enzyme supplementation in water is simple to apply, disseminates, and the contact with the substrates is faster. In addition, water supplementation may lessen the negative effects of aggressive heat exposure on enzyme activities when the pelleting temperature exceeds 85 °C and can replace expensive post-pelleting spraying systems. We investigated the effect of different doses of enzymes supplemented in water either continuously or intermittently on growth performance, digestibility of nutrients, and blood profiles. Results indicated that intermittent supplementation of enzymes at 1 mL/L drinking water and continuous supplementation at 0.5 mL/L drinking water can be investigated in further experiments as a tool to improve broiler performance and European production index.

Abstract: The aim of this work was to study the continuous or intermittent impact of a multienzyme supplement on growth performance, nutrient digestibility, and blood metabolites of broilers, and to evaluate production index of dietary supplementation. A total of 315 unsexed day-old Arbor Acres broiler chicks were randomly distributed to seven treatments groups, keeping initial body weights similar, in 35-floor pens (replicates) of nine chicks per replicate (pen) and five experimental units per treatment. All experimental groups were fed the same basal diet and administered seven multienzyme treatments: the 1st group (control) did not receive any enzyme supplementation; the 2nd, 3rd, and 4th groups were administered multienzymes at 0.5, 1.0, and 1.5 mL/L drinking water, respectively. Each enzyme supplemented-group was divided into two subgroups, with additives being applied either continuously (24 h/day) or intermittently (12 h/day) from 1 to 35 days old. Regardless of administration method, multienzyme supplements at 1.0 mL/L water along with a corn-soybean meal diet increased the body weight gain (BWG) by 7.8% compared to 0.5 mL/L water during days 1–21 of age. In addition, 1.5 mL/L water significantly improved BWG by 5.1% of broilers compared to 0.5 mL/L water during days 1–35 of age. Enzyme supplementation at 1.5 mL/L water significantly enhanced feed conversion ratio (FCR) by 4.3% during days 1–21 of age, and FCR by 5.2% and European production index (EPI) by 10.4% during days 1–35 of age compared to the group on 0.5 mL/L water. For the whole period, there were improvements of beneficial consideration in BWG (4.0%), FCR (4.0%), and European production index (8.2%) due to continuous multienzyme supplementation at 0.5 mL/L water compared to the same dose added intermittently. A similar trend was observed due to intermittent multienzymes at 1 mL/L drinking water that resulted in increased BWG by (6.4%) and improved FCR by (6.7%) and EPI by (12.7%). Intermittent administration significantly

increased feed intake of broilers during 22–35 days of age compared to continuous supplementation. Multienzymes at different doses did not significantly affect the digestibility of nutrients, blood serum biochemical constituent, inner body organs, and markers of functions of liver and renal organs. In conclusion, the highest BWG and the best FCR and EPI for the whole period were from broilers given continuous 1 and 1.5 mL/L drinking water or intermittent multienzyme supplementation at 1.5 mL/L drinking water. Furthermore, intermittent supplementation of enzymes at 1 mL/L drinking water and continuous supplementation at 0.5 mL/L drinking water can be investigated in further experiments as a tool to improve broiler growth performance and economic traits and to decrease the cost of enzyme application.

Keywords: broilers; multienzymes; administration method; enzyme dose; performance; blood constituents

1. Introduction

Diet composition is a key factor affecting the response to enzyme supplementation in poultry [1–3]. Vegetable based-diets contain anti-nutritive substances such non-starch polysaccharides (NSP), tannins, trypsin inhibitor, and phytic acid that negatively influence animal performance, digestibility of nutrients, environment, and decrease gut health and feed utilization [4–7].

Enzymes are commonly employed to decrease anti-nutritional substances and to improve animal performance [8–11]. Multienzymes containing carbohydrases and phytase were found to enhance the utilization of energy, protein, and minerals by chickens [12–14], suggesting that higher amounts of alternative feedstuffs could be used in the presence of enzymes [15]. Rye, wheat, and barley grains, which contain high levels of soluble-NSP, particularly arabinoxylans (pentosans) pectin, and β-glucans may reduce the rate of gut emptying and affect small intestinal transit time, block fats from digestion and thus absorption, therefore, cannot be incorporated into chickens' diets at high concentrations unless exogenous enzymes are adequately applied. It has been well documented that the high level of soluble NSP in rye increases digesta viscosity and the stickiness of droppings, which results in poor poultry performance [16,17].

Evidently, enzyme additions to corn, wheat, barley, and rye diets have been shown to significantly improve body weight gain (BWG) and feed conversion ratio (FCR) in broilers [11,16,18]. Generally, these enzymes are hypothesized to work in two steps, described as an ileal phase and a cecal phase [19,20]. During the ileal phase, enzymes remove fermentable substrates. During the cecal phase, degradation products of sugars, such as xylose and xylo-oligomers, are fermented by cecal bacteria, stimulating the production of volatile fatty acids (VFA) and the growth of specific beneficial bacteria [6]. In the process of depolymerization various polysaccharides in the diet, enzymes may produce manno-, galacto-, gluco-, or xylo-oligomers, which are similar to prebiotics and which may facilitate the proliferation of health-promoting bacteria such as *Lactobacillus* and Bifidobacterium [21]. Cellulase is a viscosity-reducing enzyme and is a group of enzymes that hydrolyze cellulose or β-(1,4)-glucan [2,14]. Protease enhanced degradation of soybean meal protein in the gut notably, and the mode of action of protease are wholly allied with the digestibility [14]. This observation has been evidenced by a significant increase in growth and an improvement in gut health and FCR when broilers were fed corn-based diets supplemented with enzymes [20,22,23].

In the available literature, there is a large body of results on feed supplementation with enzymes on broiler performance in contrast to the use of enzymes in drinking water. However, enzyme supplementation in water is more simple to apply, disseminate, and contact with the substrates is faster [24]. In addition, water supplementation of enzymes may lessen the harmful effects of aggressive heat exposure on enzyme activities when the pelleting temperature exceeds 85 °C and can replace expensive post-pelleting sparing systems [25]. Enzyme supplementation in drinking water significantly

increased the body weight of broilers during different periods of growth, from 14 to 35 days of age, from 20 to 41 days, and from 20 to 40 days of age [26–29]. In addition, broilers that received multienzymes through drinking water recorded the highest weekly weight gain when compared to those given enzymes through the feed, and both groups had higher growth than unsupplemented controls [24]. Thus multienzymes through drinking water at 0.5 g/L had a positive and growth-boosting effect in broiler chickens. Recently, feed additives may be used intermittently and resulted in similar growth performance and more economic benefits than continuous supplementation [30].

The enzymes' influence on blood constituents may shed light on the impact of enzymes on metabolic processes. However, contradictory results were found in the literature. Multienzyme supplementations at 0.5–1 mL/L water significantly decreased alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST), showing an improvement in liver leakage markers [26–29]. On the other hand, plasma total protein was higher in enzyme supplemented-groups than of controls, but albumin, globulin and albumin/globulin ratio, ALT, and AST were not affected [13]. Enzymes also increased total protein, albumin, globulin and albumin/globulin ratio compared to controls, but liver leakage markers (AST and ALT) were not affected by enzymes [3,31]. However, an enzyme cocktail did not influence the plasma biochemical constituents or the indices of the liver function of broilers [23,32].

Hence, this study aimed to evaluate the effects of multienzymes given in water, either continuously or intermittently, on productive performance, nutrient digestibility, carcass characteristics, and indices of the liver and renal functions of broiler chicks from 1 to 35 days of age.

2. Materials and Methods

All procedures were approved by the Deanship of Scientific Research (DSR), King Abdulaziz University under proposal number D-182-155-1440 H, that recommends animal rights, welfare and minimal stress, and did not cause any harm or suffering to animals according to the Royal Decree number M59 in 14/9/1431H.

2.1. Chick, Supplement, Design, and Husbandry

A total of 315 unsexed day-old Arbor Acres broiler chicks were obtained from the Cairo hatchery, wing banded, and randomly distributed to seven treatment groups, keeping initial body weight similar, in 35-floor pens (experimental unit) of nine chickens per pen and five replicates per treatment. All experimental groups were fed the same basal diet (Table 1) and were given seven multienzymes (Caplix® is a product of Vetoquinol India; Caplix® contains multienzymes 100,000,000 U/L cellulase, 1,500,000 U/L xylanase, 250,000 U/L amylase, and 400,000 U/L protease) treatments as follows: the 1st group (control) did not receive enzyme supplementations; the 2nd, 3rd, and 4th groups were given multienzymes (Caplix®) at 0.5, 1.0, and 1.5 mL/L drinking water, respectively. Each supplemented group was divided into two subgroups, in which the additives were administrated continuously (24 h/day) or intermittently (12 h/day), respectively, during the 1st through to the 35th day of age. The enzyme was added to water tank daily for those given continuous access, while it was added from 8 am to 8 pm for those intermittently supplemented. The water system (tank, tube, and cups) was flushed with clean water in-between enzyme applications to avoid the residual of enzymes in the water system. The water used was tap water recommended for human consumption in Egypt. Water intake was not determined herein due to limited research facility.

Table 1. The ingredients and determined and calculated composition of the experimental diets.

Ingredients	Diets, % as Fed Basis	
	Starter (1–21 d of Age)	Grower (22–35 d of Age)
Maize	51.3	51.9
Rye	0.0	5.0
Soybean meal (44% Crude protein)	32.8	24.4
Vegetable oil	2.25	2.0
Full fat soybean meal	10.0	13.0
Dicalcium phosphate	1.8	1.6
Limestone	1.0	1.0
L-Lysine	0.10	0.15
DL-Methionine	0.15	0.2
Vitamins and minerals premix *	0.3	0.3
NaCl	0.3	0.45
Calculated [1] and determined [2] composition (% as fed basis)		
Dry matter [2]	89.15	88.85
Metabolizable energy (MJ/kg) [1]	12.73	12.98
Crude protein [2]	22.8	21.2
Total lysine [1]	1.33	1.23
Total methionine [1]	0.50	0.52
Total methionine+cysteine [1]	0.87	0.87
Ash [2]	5.41	5.76
Calcium [1]	0.91	0.85
Available phosphorus [1]	0.46	0.41
Ether extract [2]	6.42	6.68
Crude fiber [2]	3.55	4.48

* Vitamins and minerals mix. provides per kg diet: Vit. A, 12,000 IU, vit. E (dl-α-tocopheryl acetate) 20 mg, menadione 2.3 mg, Vit. D3, 2200 ICU, riboflavin 5.5 mg, calcium pantothenate 12 mg, nicotinic acid 50 mg, Choline 250 mg, vit. B12 10 µg, vit. B6 3 mg, thiamine 3 mg, folic acid 1 mg, d-biotin 0.05 mg. Trace mineral (mg/kg of diet): Mn 80 Zn 60, Fe 35, Cu 8, Selenium 0.1 mg. [1] Calculated,[2] Determined.

2.2. Housing and Husbandry

Chicks were housed in floor brooders in a semi-opened house. Their light schedule was 23 h light up to 21 days of age, followed by 20 h of light until slaughter. The average outdoor minimum and maximum temperature and relative humidity during the experimental period were 22.3 and 25.6 °C and 55.4% and 58.3%, respectively. The housing temperature was 32 °C during the 1st week and declined gradually by 2 °C each week and was then stabilized at 28 °C until slaughter. Chicks were vaccinated against the most common diseases, such as Newcastle disease (ND), infectious bursal disease (IBD), and infectious bronchitis (IB), under veterinary care. The experimental diets were formulated to meet the nutrient requirements of broiler chickens [33]. The ingredients and the calculated [33] and determined [34] composition of the experimental basal diets fed during the two phases of broiler production (starter and growing periods) are shown in Table 1. Crumble feed and water were given ad libitum.

2.3. Response Traits

Broilers in each replicate were weighed (g) at 1, 21, and 35 d of age, and the BWG (g/chick) was calculated. Feed intake was recorded for each replicate (g/chick) and thereby FCR (g feed/g gain), and survival rate (SR, 100 - mortality rate) during 1–21, 22–35, and 1–35 d of age were calculated. The European production efficiency index was calculated [8].

The apparent digestibility of crude protein (CP), ether extract (EE), crude fibre (CF), and ash was performed using five replicates of three males housed in metabolic metal cages per treatment in a separate trial [8]. The excreta of the total tract were collected using the total tract apparent digestibility method during 36–40 days of age [8]. To test the apparent nutrient digestibility, the experimental

period was 5 days: 2 days of adaptation and 3 days of the collection period in which non-marked excreta during days 38 and 40 of age was discarded. During the collection period, feed intake and voided excreta were recorded daily. To identify the excreta derived from the tested diets, 1% of ferric oxide was supplemented to the tested diet at the 1st and last day of collection, 38 and 40 days of age, respectively [35]. Hence, non-marked excreta of the 1st and 3rd days of collection period were discarded. The excreta was collected from each replicate, cleaned from feed, feather, and scales and pooled for the three days, dried in a force ventilated oven, stored in glass jars, and kept for further analyses. Nitrogen as a faecal nitrogen was determined after separation of urinary nitrogen according to [36] while EE, CF, and the ash content of the excreta as well as that of the feed were determined according to [34]. The digestibility of any nutrient was calculated using (input-output/input) × 100.

Five animals were taken randomly from each treatment group to represent all treatment replications and slaughtered according to the Islamic method after being fasted overnight. The carcass and inner organs were separated, weighed, and expressed as relative to live BWG as cited by [9]. The empty gizzard and intestinal were used to estimate gizzard and intestinal percentage.

At 35 days of age, five blood samples (5 mL per sample) of each treatment were collected in clean non-heparinized tubes. The serum was separated by centrifugation at 1500× *g* for 10 min at 4 °C, and stored at −18 °C until analysis. The serum profiles were determined using commercial diagnostic kits (Diamond Diagnostics Company, Cairo, Egypt). The serum total protein and albumin concentrations (g/dL) was established, according to Henry et al. [37] and Doumas et al. [38], respectively. The globulin concentrations (g/dL) were calculated as the difference between total protein and albumin. The activities (μ/L) of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes were determined according to the method described by Reitman and Frankel [39]. Serum creatinine and urea were determined by [40]. Total cholesterol was determined, as defined by Watson [41].

2.4. Statistical Analysis

Statistical analyses were performed using the GLM procedure of the statistical analysis software of the SAS Institute [42] using one-way ANOVA to test for the effects of seven treatments. In addition, a contrast analysis was used after exclusion of the control group to compare intermittent vs. continuous treatment. The linear and non-linear effects of enzymes dose were tested. In addition, a factorial analysis was run after excluding the control-unsupplemented treatment to check the result of enzyme level, administration method, and the interaction. Before analysis, all percentages data of digestibility measurements, carcass traits, and inner organs were transformed to arcsines to normalize data distribution. The pens were the experimental units. The mean differences at $p \leq 0.05$ were tested using the Student–Newman–Keuls test. The p value between 0.05 and 0.10 was considered a trend.

3. Results

It should be noted that results are presented according to the effect of contrasting intermittent vs. continuous methods of application, and then to the effect of treatments effects.

3.1. Growth, Feed Intake, and Survival Rate

The impact of administering multienzymes in drinking water on the growth and survival of broiler chickens are summarized in Table 2. Differences between (intermittent vs. continuous) were not significant for growth, FCR, and EPI during the experimental periods.

Treatments had no significant effects on BWG during 1–21 days ($p < 0.143$) days of age but the effect approached meaningful ($p < 0.099$) during 22–35, and ($p < 0.06$) during the whole period showing the highest growth of groups on 1.5 mL/L drinking water administrated either intermittently or continuously.

Regardless of administration method, enzyme level showed a significant effect on the growth of broilers during days 1–21 of age showing that groups supplemented with 1 mL/L enzymes increased

growth compared to the group on 0.5 mL/L water. During days 22–35 of age, chicken that received the level of enzymes at 1.5 mL/L exhibited numerically ($p < 0.061$) higher BWG than those only given levels of 0.5 and 1 mL/L. For the whole period, 1.5 mL level of enzymes resulted in the growth of broilers more than 0.5 mL/L water.

Table 2. Effects of multienzymes supplemented continuously or intermittently on body weight gain and feed intake of Arbor Acres broiler chicks during 1–35 days of age.

Treatments	Body Weight Gain, g/Chick/Period/Days			Feed Intake, g/Chick/Period/Days		
	1–21	**22–35**	**1–35**	**1–21**	**22–35**	**1–35**
Administration Method						
Int	609	1051	1660	957	1839 ^{a}	2795
Con	615	1067	1682	954	1832 ^{b}	2787
Enzyme level						
0.5	590 ^{b}	1038	1627 ^{b}	957	1835	2792
1.0	636 ^{a}	1041	1677 ^{ab}	955	1827	2792
1.5	611 ^{ab}	1099	1710 ^{a}	955	1833	2788
Treatments						
Control	577	991	1568	958	1839	2797
0.5 × Int	568	1027	1595	956	1837	2793
0.5 × Con	611	1048	1659	957	1835	2792
1.0 × Int	637	1032	1669	954	1837	2791
1.0 × Con	634	1051	1685	956	1837	2793
1.5 × Int	621	1096	1717	960	1838	2798
1.5 × Con	600	1103	1703	949	1830	2779
SEM	20.2	27.7	36.4	4.29	2.22	4.79
p value						
Contrast						
Int vs. Con	0.147	0.589	0.225	0.896	0.048	0.770
Treatments	0.143	0.099	0.060	0.717	0134	0.174
Enzyme level	0.047	0.061	0.046	0.919	0.392	0.720
Administration method	0.669	0.489	0.402	0.542	0.038	0.141
Interaction	0.197	0.963	0.473	0.351	0.352	0.129

a,b: Differences among means within a column within each factor not sharing similar superscripts are significant at $p < 0.05$; Int: intermittent; Con: continuous; SEM: standard error of the mean.

There no significant effect of administration method and the interaction between enzyme level and administration method on the growth of broilers during different periods.

Feed intake of broiler chickens during most of the experimental periods was significantly affected by the administration method (intermittent vs. continuous), except for days 22–35 of age in which broilers in the intermittent group consumed significantly more feed than the continuous one. Treatments also had no significant impact on feed intake during the different experimental period, nor there were significant effects due to the enzyme level and the interaction between enzyme level and administration method. There were no deaths in this experiment; the survival rate was 100% during the experimental period, and thus death-rate data are not presented.

3.2. Feed Conversion Ratio and European Production Index

The effects of administering multienzymes in drinking water on the FCR and EPI of broiler chickens are summarized in Table 3. The FCR and EPI were not significantly affected by the administration method (intermittent vs. continuous) during the experimental period. In addition, treatments did not affect FCR during most of the trial period except that FCR during days 1–35. In addition, the difference within 0.5, 1, and 1.5 mL levels was not significant between the two application methods. The results revealed that the group supplemented continuously with 1.5 mL/L water either continuously or

intermittently show the best FCR, while the worst FCR was from a group that was supplemented with 0.5 mL/L intermittently.

Table 3. Effects of multienzyme supplemented continuously or intermittently on feed conversion ratio and European production index of Arbor Acres broiler chicks during 1–35 days of age.

Treatments	Feed Conversion kg/kg/Period/Days			EPI
	1–21	22–35	1–35	
Administration Method				
Int	1.58	1.75	1.69	290
Con	1.56	1.72	1.66	299
Enzyme level				
0.5	1.64 ^{a}	1.77	1.72 ^{a}	279 ^{b}
1.0	1.51 ^{ab}	1.77	1.67 ^{ab}	296 ^{ab}
1.5	1.57 ^{b}	1.67	1.63 ^{b}	308 ^{a}
Treatments				
Control	1.68	1.86	1.79 ^{a}	260
0.5 × Int	1.70	1.79	1.76 ^{a}	268
0.5 × Con	1.57	1.75	1.69 ^{ab}	290
1.0 × Int	1.50	1.79	1.67 ^{ab}	293
1.0 × Con	1.51	1.76	1.66 ^{b}	299
1.5 × Int	1.55	1.68	1.63 ^{b}	309
1.5 × Con	1.58	1.66	1.63 ^{b}	307
SEM	0.056	0.046	0.039	12.5
p Value				
Contrast				
Int vs. Con	0.112	0.570	0.228	0.229
Treatments	0.115	0.075	0.047	0.065
Enzyme level	0.039	0.053	0.036	0.049
Administration method	0.474	0.438	0.361	0.359
Interaction	0.188	0.975	0.515	0.558

a,b: Differences among means within a column within each factor not sharing similar superscripts are significant at $p < 0.05$; EPI: European production index; Int: intermittent; Con: continuous; SEM: standard error of the mean.

Irrespective of administration method, enzyme level significantly affect FCR during days 1–21 and FCR and EPI during days 1–35 of age, showing 1.5 mL/L water enhanced FCR and EPI compared to 0.5 mL/L water. The EPI was not significantly affected by administration method (intermittent vs. continuous) as well as treatments.

3.3. Apparent Digestibility of Nutrients

Data concerning the effects of the administration method and different enzyme treatments on the apparent digestibility of the nutrients of broiler chicks are shown in Table 4. There were no significant effects for the administration method (intermittent vs. continuous) and for the treatments on the apparent digestibility of nutrients. The results showed that there was a trend ($p \leq 0.11$) for higher crude fiber digestibility of the group supplemented with enzymes continuously compared to that on intermittent supplementation. A trend towards increasing digestibility of crude protein ($p \leq 0.10$), ether extracts ($p \leq 0.11$), and ash ($p \leq 0.07$) was shown because of different enzyme treatments compared to the control.

3.4. Carcass Traits and Inner Body Organs

The carcass characteristics and body organs of broilers as affected by the method of administration and different enzyme treatments are shown in Table 5. The percentages of the liver, heart, pancreas, abdominal fat, spleen, bursa, and thymus were not significantly affected by method of administration (intermittent vs. continuous). However, there was a trend ($p < 0.09$) of increased dressing (%) because

of constant supplementation compared to the intermittent one, but reduced gizzard ($p < 0.089$) and pancreas ($p < 0.059$) percentage. There were no significant differences in the carcass traits and body organs due to different enzyme treatments.

Table 4. Effects of multienzymes supplemented continuously or intermittently on apparent nutrient digestibility (%) during 36–40 days of age of Arbor Acres broiler chicks.

Treatments	Apparent Digestibility, %				Ash%
	Dry Matter	Crude Protein	Ether Extract	Crude Fiber	
Administration Method					
Int	75.1	80.1	85.2	21.8	37.8
Con	75.8	80.9	86.1	23.8	39.0
Treatments					
Control	72.3	73.8	77.9	13.2	33.5
0.5 × Int	74.7	76.3	81.2	20.0	40.1
0.5 × Con	77.4	79.4	84.5	20.0	36.1
1.0 × Int	75.2	79.8	84.9	21.0	33.8
1.0 × Con	74.6	82.5	87.8	25.5	40.9
1.5 × Int	75.5	84.1	89.4	24.3	39.5
1.5 × Con	75.3	80.9	86.1	26.0	39.9
SEM	1.19	1.53	1.63	1.46	2.18
p value					
Contrast					
Int vs. Con	0.510	0.510	0.500	0.110	0.510
Treatments	0.330	0.100	0.110	0.320	0.070

Int: intermittent; Con: continuous; SEM: standard error of the mean.

Table 5. Effects of multienzymes supplemented continuously or intermittently on carcass and organs characteristics of Arbor Acres broiler chicks during 1–35 days of age.

Treatments	Relative Weight (%)						
	Dressing	Gizzard	Liver	Heart	Intestinal	Pancreas	Abdominal Fat
Administration method							
Int	72.9	1.77	2.71	0.672	3.90	0.374	0.994
Con	74.1	1.61	2.84	0.693	4.60	0.331	1.18
Treatments							
Control	69.6	1.90	3.21	0.735	5.98	0.390	1.48
0.5 × Int	72.1	1.86	2.75	0.678	4.46	0.398	1.18
0.5 × Con	73.3	1.69	3.03	0.680	5.54	0.312	1.36
1.0 × Int	72.8	1.82	2.86	0.646	3.66	0.376	0.877
1.0 × Con	74.5	1.63	2.99	0.707	4.09	0.352	1.07
1.5 × Int	73.9	1.63	2.52	0.692	3.58	0.347	0.921
1.5 × Con	74.5	1.52	2.50	0.691	4.16	0.329	1.11
SEM	0.82	0.113	0.200	0.052	0.421	0.027	0.170
p value							
Contrast							
Int vs. Con	0.090	0.089	0.445	0.617	0.471	0.059	0.185
Treatments	0.822	0.950	0.757	0.795	0.714	0.396	0.999

Int: intermittent; Con: continuous; SEM: standard error of the mean.

3.5. Lymphoid Organs

The lymphoid organs of broilers as affected the method of administration (intermittent vs. continuous) and different enzyme treatments are shown in Table 6. The percentages of the spleen, bursa,

and thymus were not significantly affected by method of administration (intermittent vs. continuous). There were no significant differences in the lymphoid organs due to different enzyme treatments.

Table 6. Effects of multienzymes supplemented continuously or intermittently on lymphoid organs of Arbor Acres broiler chicks during 1–35 days of age.

Treatments	Relative Weight, %		
	Spleen	Bursa	Thymus
	Administration method		
Int	0.157	0.295	0.551
Con	0.153	0.274	0.517
	Treatments		
Control	0.172	0.288	0.709
0.5 × Int	0.149	0.283	0.572
0.5 × Con	0.145	0.289	0.560
1.0 × Int	0.161	0.279	0.518
1.0 × Con	0.163	0.273	0.398
1.5 × Int	0.162	0.321	0.562
1.5 × Con	0.152	0.260	0.591
SEM	0.003	0.006	0.034
	p value		
ContrastInt vs. Con	0.777	0.341	0.496
Treatments	0.939	0.397	0.460

Int: intermittent; Con: continuous; SEM: standard error of the mean.

3.6. Blood Serum Biochemical Constituents

There was no significant effect of the administration method (intermittent vs. continuous) on albumin, globulin, albumin/globulin ratio, total cholesterol, but a trend towards ($p < 0.075$) increasing serum protein was shown in the continuous group compared to intermittent group Table 7.

Table 7. Effects of multienzymes supplemented continuously or intermittently on biochemical constituents of blood serum of Arbor Acres broiler chicks during 1–35 days of age.

Treatments	Biochemical Constituents of Blood Serum				
	Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin/Globulin Ratio	Total Cholesterol (mg/dL)
			Administration method		
Int	5.44	2.87	2.57	1.29	187
Con	6.28	3.46	2.82	1.61	195
			Treatments		
Control	4.36	2.54	1.82	1.60	192
0.5 × Int	5.11	2.70	2.41	1.34	174
0.5 × Con	5.74	3.44	2.30	1.64	189
1.0 × Int	5.94	3.04	2.90	1.16	199
1.0 × Con	6.71	3.44	3.27	1.20	191
1.5 × Int	5.26	2.86	2.40	1.36	188
1.5 × Con	6.41	3.50	2.91	1.98	205
SEM	0.562	0.275	0.511	0.456	10.49
			p value		
			Contrast		
Int vs. Con	0.075	0.131	0.546	0.397	0.355
Treatments	0.894	0.819	0.821	0.817	0.426

Int: intermittent; Con: continuous; SEM: standard error of the mean.

3.7. Markers of the Liver and Renal Functions

Results for liver and renal functions of the broiler chicks as they were influenced by the administration method (intermittent vs. continuous) and different enzyme treatments are shown in Table 8. There was no significant effect of the administration mehtod (intermittent vs. continuous) on most of the indices of the liver and renal functions except for serum AST ($p < 0.079$) and Urea ($p < 0.044$). Both were higher in the group given enzymes continuously than those of the group supplemented with enzymes intermittently. There was no significant effect of different enzyme treatments on the markers of the liver and renal functions of the broiler chicks.

Table 8. Effects of multienzymes supplemented continuously or intermittently on liver function and renal function of Arbor Acres broiler chicks during 1–35 days of age.

Treatments	Liver Function			Renal Function		
	ALT, u/L	AST, u/L	ALT/AST	Urea, g/dL	Creatinine, g/dL	Urea/Creatinine Ratio
Administration Method						
Int	48.1	59.5	1.23	23.6	1.21	19.9
Con	50.0	62.3	1.26	26.6	1.26	21.5
Treatments						
Control	52.0	60.0	1.16	25.5	1.28	20.2
0.5 × Int	47.6	58.4	1.23	22.2	1.27	18.4
0.5 × Con	52.0	62.4	1.21	29.0	1.24	23.8
1.0 × Int	49.2	64.0	1.30	25.0	1.16	21.6
1.0 × Con	48.0	64.8	1.36	26.6	1.25	21.5
1.5 × Int	47.6	56.0	1.18	23.5	1.21	19.6
1.5 × Con	50.0	59.6	1.2	24.2	1.28	19.3
SEM	1.56	1.88	0.047	1.80	0.094	1.75
p value						
Contrast						
Int vs. Cont	0.153	0.079	0.603	0.044	0.570	0.278
Treatments	0.208	0.654	0.717	0.195	0.816	0.219

Int: intermittent; Con: continuous; ALT: alanine aminotransferase; AST: aspartate aminotransferase; SEM: standard error of the mean.

4. Discussion

The use of enzymes in feed to overcome the anti-nutritional factors, to increase feed utilization, and to improve performance as well as to decrease stress has received considerable attention [3,4,8,11,16,43]; so, also is the use of enzymes in water [26–29], with water application inducing superior performance [24]. This advantage may be due to faster distribution, application, and availability [24,25] and greater use of water as well [27]. However, the effect of the enzymes depends on diet composition (target substrate), the dose of the enzyme, and the age of chickens [5,9]. However, the activity of enzymes was not determined herein by direct enzymatic method, but by growth performance and digestibility of diets instead, showing some positive effects depend on the age of broilers, and dose of enzymes.

The use of enzymes is also restricted by its cost-benefit ratio [44]; hence, we investigated the effect of different doses of multienzymes continuously (24 h/d) or intermittently (12 h/d) administered with the aim of improving the performance of the animals and reducing the costs of supplementation on broiler performance and the EPI. The results indicate that at 1 and 1.5 mL of enzyme supplementation either continuously or intermittently yields superior effects on biological and economic value than the low dose of enzymes with a low dosage and intermittent administration could yield better economic benefits under similar production performance due to low cost of supplementation.

The results indicate that the effect of the method of administration (intermittent vs. continuous) and different enzyme treatments are dependent on the age of the chickens ($p < 0.034$). It was observed

that the level of enzyme supplemented at 1 mL/L water improved growth (7.8%) of chickens during only days 1–21 of age compared to 0.5 mL/L supplementation. During days 22–35 and 1–35 of age, enzyme level at 1.5 mL resulted in higher growth of broilers by 5.9% and 5.1%, respectively, compared to 0.5 mL. This indicates that enzymes supplemented continuously at 1.5 mL/L water of a corn-soybean diet at 22–35 days of age was adequate to improve growth and FCR. On the other hand, the dose of multienzymes given intermittently to induce similar improvements in FCR was 1 mL/L. Either continuous or intermittent supplementation with 1.5 mL/L water was more efficient for increasing growth (8.6%–9.9%) and improving FCR (7.7%) and EPI (19.3%) for the whole experimental period compared to unsupplemented control even not significantly different from the 1 mL multienzyme treatments, but obviously economically beneficial. Similar results were reported previously [17,45]. The linear and non-linear components of enzymes concentration showed a weak linear effect on BWG during 1–21 days of age ($p < 0.089$) and FCR during 1–21 days of ($p = 0.076$). In the literature, various cereals responded differently to enzyme supplementation with a greater effect on viscous grains such as rye [9,18,23]. These results indicate that the enhancing effect of increasing dose of enzymes on growth and FCR of broilers occurs during the 1–21 days of age period.

Multienzymes increased the digestibility of CP, EE, and CF and ash numerically of the corn-soybean diet containing 5% rye, and the saturation response was attained in the group supplemented with 1 mL/L water due to lack of response to further increase in enzyme concentration. This could explain the positive effect of enzymes on the performance of broilers found herein. The positive response of the corn-soybean meal diet to multienzymes indicates the presence of insoluble components, such as NSP in rye, which are not digested by broiler chickens and may limit the utilization of some nutrients or negatively influence gut health, nutrient digestibility, and dietary metabolizable energy [4,7,43,46]. The results cited herein are similar to those reported by [8,20,43] and can be explained by the improvement in gut architecture and health [4,6,47].

Dressing percentage increased (1.6%) due to enzyme supplementation continuously in comparison to the intermittent method, showing the positive effect of continuous enzyme supply on the availability of nutrients. It seems that increasing nutrient availability due to continuous supplementation will be used for tissue growth.

The results of this study indicate that enzyme supplementation through different methods at different doses did not affect lymphoid organs (spleen, bursa, and thymus) of broilers. There was an increase in serum protein and albumin (no specific immune protein) due to enzyme supplementation when continuously administered; this may be a reflection of the increase in digestibility of CP observed herein. This positive relationship between enzymes and immunity was recently reported [48,49]. Further evidence indicates a lack of adverse effects of enzymes on the markers for liver leakage and renal function, as enzyme supplementation in water significantly decreased AST and ALT, showing a decrease in liver leakage enzymes, showing an improvement in liver function [26–29].

5. Conclusions

The highest BWG and the best FCR and PI for the whole experimental period were from broilers given continuous or intermittent multienzyme supplementation at 1.5 mL/L drinking water suggesting further experiments should be undertaken to test the possibility of constant vs. intermittent application of multienzymes due to 50% saving in the cost of enzyme supplementation. In addition, intermittent supplementation of enzymes at 1 mL/L drinking water and continuous supplementation at 0.5 mL/L drinking water can be investigated in further experiments as a tool to improve broiler performance and European production index.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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A Mixture of Exogenous Emulsifiers Increased the Acceptance of Broilers to Low Energy Diets: Growth Performance, Blood Chemistry, and Fatty Acids Traits

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Simple Summary: Since fat energy is cheaper than carbohydrate energy, it is profitable to increase fat content in broiler diets. One of the factors that limits using high levels of fat in broiler diets is the indigestion of fat, because bile secretion in broilers is not efficient in the first days of age. In this sense, using exogenous emulsifiers in the high-fat diet enhanced fat utilization and digestive metabolism. In the current study, birds fed the basal diet and another two low-energy diets (−50 kcal/kg than control) with or without emulsifiers (500 g/ton). The obtained results revealed that the emulsifier's supplementation to low-energy diets enhanced fat utilization and resulted in positive effects on growth performance, nutrients utilization, lipid peroxidation, and modified plasma lipid profiles in broilers.

Abstract: To investigate the influence of emulsifiers on broilers fed low-energy diets, the birds were distributed into three sets—the control was fed the basal diet, the second group was fed diets 50 kcal/kg less than control, and the third group was fed diets 50 kcal/kg less than control and supplemented with 500 g/ton of emulsifiers. The used mixture of exogenous emulsifiers contains phosphatidyl choline, lysophosphatidyl choline, and polyethylene glycol ricinoleate. Although the feed intake was not meaningfully affected by dietary low-energy level with emulsifier inclusion ($P = 0.42$), the weight gain and FCR were clearly enhanced ($P = 0.005$ and $P = 0.044$, respectively). Protein and lipids utilization were decreased by reducing energy level, but they were increased by emulsifier supplementation ($P = 0.022$ and $P = 0.011$, respectively). Liver thiobarbituric acid-reactive substances (TBARs) and muscle palmitic acid concentrations were decreased by reducing the energy level and emulsifier's supplementation ($P = 0.014$ and $P = 0.042$, respectively). However, muscle total lipids and α-tocopherol, oleic acid, linoleic acid, and α-linolenic acid were not affected by dietary treatments ($P > 0.05$). Interestingly, the plasma total cholesterol, HDL-cholesterol, total protein, and globulin were decreased in the low-energy group without emulsifier but they were increased by emulsifier supplementation ($P = 0.008$, $P = 0.005$, $P = 0.037$, and $P = 0.005$, respectively). It could be concluded that the mixture of emulsifier supplementation to low-energy diets enhanced fat utilization

and resulted in positive effects on the growth performance, nutrient utilization, lipid peroxidation, and modified plasma lipid profiles in broilers. Getting such benefits in broilers is a necessity to reduce the feed cost and consequently the price of the product, which will lead to improved welfare of mankind.

Keywords: broilers; blood chemistry; emulsifiers; growth; nutrient utilization; TBARs

1. Introduction

Fats and oils are the most essential energy sources in broilers' diets as a worthy way for gathering the high energy demands for the highest growth rates of broiler chickens [1,2]. In addition, the evident merits of high-caloric-density lipids result in an excellent caloric impact [3,4]. The failure of the broilers to gain lipids is assigned to bad emulsification rather than the shortage in lipase secretion, which led to great interest in the possibility of using exogenous emulsifiers to improve the utilization of lipids in broiler chickens [5]. Several previous studies indicated that supplementation with bile acids or bile salts improve the utilization of dietary fat by chicks because of limited endogenous secretion [6,7].

Big amounts of fat are used in broilers feed, especially strains that require high-energy diets [8]. In various situations, the actual utilization of lipids is specified by the cost linkage between energy content and yellow corn energy content [9]. Since fat energy is cheaper than carbohydrate energy, it is profitable to increase fat content in broiler diets. One of the factors that limits using high levels of fat in broiler diets is the indigestion of fat, because bile secretion in broilers is not efficient in the first days of age [10]. In this time, using exogenous emulsifiers in the high-fat diet enhances fat utilization and digestive metabolism [3,5]. Moreover, San Tan et al. [11] recommended that supplementation of emulsifiers in the early stages of age improved digestion and absorption of the fats and enhanced growth performance in broilers. Although, supplemented bile acids (including cholic acid and chenodeoxycholic acid) and bile salts (taurocholate) improved the absorption of fat in broilers [10]. However, supplemented bile acid in the diets was not economically applicable due to the high cost. Thus, emulsifiers might be included in broilers' diets to reduce the surface tension of water [5]. Bontempo et al. [12] illustrated that emulsifier supplementation in broiler diets consisting of a vegetal bidistilled oleic acid and glycerol polyethylene glycol ricinoleate had a positive effect on growth performance, feed efficiency, carcass dressing, and plasma lipid metabolism. Furthermore, Siyal et al. [5] showed that the supplementation of emulsifiers improved the growth performance of broiler chickens by increasing fatty acid digestibility. However, the effects of emulsifiers (in association with low-energy diets) on growth performance, blood chemistry, and fatty acids traits have been rarely thoroughly investigated, even though the interest in using exogenous emulsifiers has increased in the last several decades. Thus, in this study, it could be hypothesized that the utilization of fats can be increased by emulsifiers, which in turn could enhance the growth performance of broilers. In this sense, the current investigation evaluated the influence of emulsifier supplementation into low-energy diets by the attenuation of growth, feed efficiency, and muscle fatty acid profiles in broilers.

2. Material and Methods

2.1. Experimental Design and Diet Preparation

The study was approved by the Ethics Committee of Local Experimental Animals Care Committee and conducted in accordance with the guidelines of Kafrelsheikh University, Egypt (Number 4/2016 EC). Three hundred one-day-old male birds (Ross 308) were kept in bens and divided randomly into 3 treatments, and each treatment divided into 4 replicates (25 birds/rep). The first treatment was served as control and fed on control diets containing the optimized energy requirements (3000, 3100, and 3180 kcal/kg) for starter, grower, and finisher diets, respectively. The second treatment was fed

diets 50 kcal/kg less than control and the third treatment was fed diets 50 kcal/kg less than control and supplemented with 500 g/ton of emulsifiers (Table 1). The emulsifiers used in this study were called Liposorb® and provided from CEVA POLCHEM PVT. LTD., India. Liposorb® contains three types of emulsifiers (Phosphatidyl Choline (PC), Lysophosphatidyl Choline (LPC), PolyEthylene Glycol Ricinoleate (PEGR)), and the optimum dose is 500 g Liposorb®/ton feed. The dose used in the current study was selected based on the study of Bontempo et al. [12] and Zhao et al. [13].

The birds were placed inside a room equipped with 12 pens (3 treatments/4 replicates each) with a chain feeder system and automatic nipple cup drinker in a completely randomized design. Feed and water were provided for ad libitum consumption for 35 days. The light cycle and temperature were the same in the experimental groups. The photoperiod was 24 h of light from day 0 to day 7 and 23 h of light from day 7 to the end of the trial. Room temperature was 25–29 °C with proportional moisture between 50% and 70% during the trial. Birds' body weighing was on record every three days, and feed consumption was on file every day over the empirical period.

2.2. Final Sampling

At the end of the trial period, 36 chicks (12 birds/treatment) were randomly chosen, weighed, and gently slaughtered to collect the breast muscle, liver, abdominal fat, and heart organs for offal weight. The blood samples were collected into heparinized test tubes and centrifuged at 5900 × g for 10 min at 4 °C to collect the plasma and finally kept at −20 °C for further analysis. Three days before the end of the trial, 20 birds per group were housed in batteries for digestibility tests where the excreta and feed were collected. Subsequently, the samples were desiccated by the drying kiln at 60 °C for 24 hrs. Then, the dried samples were grinded and kept for protein, lipid, and fiber analysis by following the standard procedure [14].

2.3. Blood Biochemical Analysis

Plasma triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-cholesterol), low-density lipoprotein cholesterol (LDL-cholesterol), glucose, glutamic oxalacetic transaminase (GOT), total protein, albumin, and globulin were tested calorimetrically by using trade kits (Diamond Diagnostics, Egypt) according to the steps outlined by the manufacturer.

Muscle total lipid content and fatty acid profile analysis were measured using gas-liquid chromatography (GLC) according to the method of Saleh [15].

Liver thiobarbituric acid retroactive substances (TBARs) concentration was tested by the process of Goodla et al. [16]. The α-tocopherol concentration in muscle was tested by the HPLC according to the method of Faustman et al. [17].

2.4. Statistical Analysis

Shapiro–Wilk and Levene's tests confirmed normal distribution and variance homogeneity. All statistical differences were assessed by one-way analysis of variance tests (SPSS version 17, SPSS Inc., Ill., USA) with Tukey's multiple test where differences in experimental groups occurred. The level of significance was accepted at $P < 0.05$. All data are presented as means ± standard error (SE).

Table 1. Composition of the experimental diets.

Ingredient	Basal Diet			Low Energy Diet (−50 kcal)			Low Energy Diet (−50 kcal) + Emulsifier		
	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher
Yellow Corn	559.5	601.5	659.5	570.5	612.5	670.5	570	612	670
Soybean meal (44%)	325	276	215	324	275	214	324	275	214
Soy oil	15	22	25	5	12	15	5	12	15
Toxin binders	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Broiler concentrate (45%) [1] + Premix [2]	100	100	100	100	100	100	100	100	100
Emulsifiers	0	0	0	0	0	0	0.5	0.5	0.5
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000
Calculated analysis									
CP (%)	23.09	21.26	19.02	23.09	21.26	19.0.	23.085	21.26	19.02
ME (kcal/Kg)	3003	3093	3177	2955	3045	3128	2954	3043	3127
Ca (%)	1.01	0.98	0.88	1.01	0.99	0.88	1.01	0.98	0.88
Lysine (%)	1.352	1.217	1.152	1.349	1.215	1.149	1.35	1.216	1.15
Methionine (%)	0.57	0.55	0.52	0.57	0.55	0.52	0.57	0.55	0.52

[1] Broiler Concentrate—crude protein, 45%; ME, 2800 kacl/kg; calcium, 9%; phosphorus, 2.74%; Na, 1.9%; CL, 1.5%; lysine, 2.8%; methionine, 2.5%. [2] Premix (g/kg feed)—calcium hydrogen phosphate, 20; calcium carbonate, 8.15; sodium chloride, 5.1; (µg/kg feed): iron sulfate, 400; copper sulfate, 31.5; zinc sulfate, 176; manganese sulfate, 152; sodium iodate, 0.55; sodium selenite, 0.27. Retinol, 1.4; DL-α-tocopherol acetate, 6.5; thiamine hydrochloride, 2.6; riboflavin, 6.5; pyridoxine hydrochloride, 1.30; calcium D-pantothenate, 10.4; nicotinic acid, 26; (µg/kg feed): menadione sodium bisulfite, 650; D-biotin, 70; choline chloride, 780; pteroylglutamic acid, 520; cyanocobalamin, 26; cholecalciferol, 13.

3. Results

3.1. Growth Performance and Carcass Composition

Feed intake was not influenced by a low-energy diet supplemented with an emulsifier ($P = 0.42$), however, body weight gain and FCR were enhanced ($P = 0.005$ and $P = 0.044$, respectively) (Table 2). Crude protein utilization and ether extract utilization were decreased by reducing the energy level, but they were increased by emulsifier supplementation ($P = 0.022$ and $P = 0.011$, respectively) (Table 2). However, crude fiber utilization and mortality rate were not influenced by dietary treatments ($P = 0.32$) (Table 2). Carcass % and abdominal fat % were decreased by reduced energy level, but they were increased by adding emulsifier ($P = 0.045$ and $P = 0.018$, respectively). On the other hand, breast and thigh muscles, heart, and liver percentages were not affected by the reduction of energy or addition of emulsifier ($P > 0.05$) (Table 2).

Table 2. Effect of test diets on growth performance, nutrient utilization, and organ weights in broilers.

Item	Treatment			*P*-Value
	Basal Diet	Low Energy Diet (−50 kcal)	Low Energy Diet (−50 kcal) + Emulsifier	
Initial body weight (g)	42.23 ± 0.14	42.10 ± 0.23	42.13 ± 0.24	0.900
Body weight gain (g/34 day)	1769 ± 10.00 [a]	1624 ± 41.00 [b]	1761 ± 12.00 [a]	0.005
Feed intake (g/34 day)	3043 ± 80.00	2883 ± 85.00	2994 ± 86.00	0.420
Feed conversion ratio (g gain/g feed)	1.68 ± 0.05[b]	1.73 ± 0.06 [a]	1.66 ± 0.06 [b]	0.044
Crude protein utilization (%)	68.00 ± 0.50 [a]	64.30 ± 1.10 [b]	67.00 ± 0.70 [a]	0.022
Crude fiber utilization (%)	34.50 ± 0.70	33.00 ± 0.60	34.30 ± 0.90	0.320
Ether extract utilization (%)	46.75 ± 1.50 [a]	42.00 ± 0.50 [b]	45.75 ± 0.60 [a]	0.011
Mortality (%)	2.00 ± 1.15	3.00 ± 1.00	2.00 ± 1.15	0.767
Organ weight (% body weight)				
Carcass	68.86 ± 0.80 [a]	66.11 ± 1.30 [b]	68.39 ± 0.60 [a]	0.045
Breast muscle	23.46 ± 0.38	22.23 ± 0.93	23 ± 0.22	0.360
Thigh muscle	19.65 ± 0.50	18.78 ± 0.90	19.4 ± 0.50	0.630
Abdominal fat	1.88 ± 0.15 [a]	1.47 ± 0.05 [b]	1.77 ± 0.05 [a]	0.018
Liver	2.47 ± 0.08	2.44 ± 0.07	2.48 ± 0.06	0.901
Heart	0.81 ± 0.02	0.85 ± 0.02	0.8 ± 0.02	0.122

[a,b] Values expressed as means ± SE. Means within the same row with different superscripts differ ($P < 0.05$).

3.2. Blood Biochemical Parameters

Plasma total cholesterol, HDL-cholesterol, total protein, and globulin were decreased in the low-energy diet group without emulsifier but they were increased by emulsifier supplementation ($P = 0.008$, $P = 0.005$, $P = 0.037$, and $P = 0.005$, respectively) (Table 3). However, plasma LDL-cholesterol, triglycerides, glucose, GOT, albumin, creatinine, and uric acid were not influenced by dietary treatments ($P > 0.05$) (Table 3).

Table 3. Effect of test diets on blood plasma parameters.

Item	Treatment			*P*-Value
	Basal Diet	Low-Energy Diet (−50 kcal)	Low-Energy Diet (−50 kcal) + Emulsifier	
Triglycerides (mg/ml)	102.00 ± 4.20	97.80 ± 4.60	99.13 ± 3.30	0.752
Total cholesterol (mg/ml)	146.00 ± 2.50 [a]	131.00 ± 3.40[b]	134.00 ± 3.80 [b]	0.008
HDL- cholesterol (mg/ml)[1]	27.25 ± 0.31 [a]	24.00 ± 0.87 [b]	25.75 ± 0.56 [ab]	0.005
LDL- cholesterol (mg/ml)[2]	109.00 ± 3.70	103.00 ± 2.40	101.00 ± 2.10	0.137
Glucose (mg/ml)	180.40 ± 8.50	177.00 ± 9.30	175.00 ± 7.40	0.891
GOT (U/l)[3]	253.00 ± 9.90	241.00 ± 8.30	245.00 ± 10.00	0.667
Total protein (mg/dL)	4.20 ± 0.10 [a]	3.75 ± 0.08 [b]	4.06 ± 0.16 [ab]	0.037
Albumin (mg/dL)	2.23 ± 0.04	2.22 ± 0.04	2.20 ± 0.07	0.933
Globulin (mg/dL)	1.91 ± 0.08 [a]	1.48 ± 0.06 [b]	1.81 ± 0.11 [a]	0.005
Creatinine (mg/ml)	0.625 ± 0.05	0.55 ± 0.03	0.59 ± 0.03	0.365
Uric acid (mg/ml)	5.40 ± 0.30	5.70 ± 0.40	5.75 ± 0.31	0.672

[a,b] Values expressed as means ± SE. Means within the same row with different superscripts differ ($P < 0.05$). [1] HDL, high-density lipoprotein; [2] LDL, low-density lipoprotein; [3] GOT, glutamic oxaloacetic transaminase.

3.3. Fatty Acid Profiles

Liver TBARs and muscle palmitic acid concentrations were decreased by reducing the energy level and supplementation with emulsifier ($P = 0.014$ and $P = 0.042$, respectively). However, muscle total lipids and α-tocopherol, oleic acid, linoleic acid, and α-linolenic acid were not affected by the tested diets ($P > 0.05$) (Table 4).

Table 4. Effect of test diets on liver thiobarbituric acid retroactive substances (TBARs), muscle total lipid, α-tocopherol, and fatty acid profile contents in muscle.

Item	Treatment			*P*-Value
	Basal Diet	Low Energy Diet (−50 kcal)	Low Energy Diet (−50 kcal) + Emulsifier	
Liver				
TBARs (nmol MDA/g)	3.60 ± 0.08 [a]	2.85 ± 0.22 [b]	2.73 ± 0.2 [b]	0.014
Muscle				
Total lipid (%)	3.03 ± 0.11	2.98 ± 0.08	2.93 ± 0.13	0.812
α-Tocopherol (mg/100 g)	0.21 ± 0.01	0.20 ± 0.01	0.22 ± 0.02	0.690
Fatty acid (mg/100 mg fat)				
Palmitic acid (16:00)	1.36 ± 0.04 [a]	1.23 ± 0.03 [b]	1.19 ± 0.04 [b]	0.042
Oleic acid [18:1 (n-9)]	1.07 ± 0.06	1.07 ± 0.02	1.13 ± 0.01	0.520
Linoleic acid [18:2 (n-6)]	1.88 ± 0.23	1.88 ± 0.21	1.92 ± 0.23	0.986
Omega-3 [18:3 (n-3)]	0.06 ± 0.02	0.05 ± 0.02	0.07 ± 0.01	0.731

[a,b] Values expressed as means ± SE. Means within the same row with different superscripts differ ($P < 0.05$).

4. Discussion

The growth performance was influenced by the reduction of the energy rate in this study. Normally, birds utilize the energy for life maintenance and body building. Subsequently, when birds were fed low-energy diets, the priority of energy utilization can be used for life preservation and in turn, the growth performance might be negatively affected. Following this hypothesis, the results of the present study displayed reduced body weight gain and increased FCR following feeding with a low-energy diet. However, dietary emulsifier presented a practical strategy to increase the growth performance and feed utilization in broilers fed low-energy diets in the present study (high body weight with low FCR). These results are in line with previous studies [11,12,18]. The obtained results revealed that feed intake was not affected by the experimental diets in the present study. Similarly,

Kaczmarek et al. [19] reported that dietary emulsifier did not influence the feed intake in broilers. The increased growth performance in the present study might be related to the improvement in crude protein and fat utilization. San Tan et al. [11] and Zhang et al. [18] also illustrated that the inclusion of emulsifiers improved fat digestion and absorption as well as the nutrient digestibility and consequently resulted in enhancing the growth performance in broilers. The emulsifiers are reported to increase the integration of micelles in the gut lumen, which in turn increases the fat digestibility [13]. Thus, it might be hypothesized that the improved digestibility of the fat is an effect of emulsifiers on proteolysis [5,7].

The abdominal fat % was significantly decreased by reduced energy level, but it was increased by emulsifier inclusion in the present study. Indeed, a higher rate of dietary metabolizable energy can increase abdominal fat [20,21]. Similarly, Zaman et al. [22] reported that abdominal fat was increased by using diets with high metabolizable energy content. High-energy diets could increase the bulk of fat in broilers' bodies, and in turn, raises the level of abdominal fat when compared with low-energy diets [23].

By including an emulsifier in low-energy diets, plasma HDL-cholesterol and globulin concentrations were increased, while, plasma LDL-cholesterol, triglycerides, and glucose concentrations were decreased in the current study. Plasma GOT, albumin, creatinine, and uric acid concentrations were not influenced by the tested diets. To the knowledge of the authors, the impact of emulsifier on HDL:LDL levels in the blood plasma of broilers has not been documented yet. Emulsification could reduce the level of free fatty acids and total cholesterol in plasma by lowering the secretion of lipoprotein molecules in the blood [24]. It has been reported that total cholesterol, triglycerides, and HDL-cholesterol of broilers fed feed including plant oil or animal fat were not influenced by dietary emulsifiers [25,26]. On the contrary, broilers fed dietary emulsifier (sodium stearoyl-2-lactylate) displayed low blood triglycerides in comparison with those fed high-energy diets without the addition of emulsifier [27].

The discrepancy of the production and removal of oxidants from the organism cells is called oxidative stress [28]. Poor nutritional value of the poultry feeds is among the main reasons for the oxidative stress [29]. However, including feed additives such as antioxidants and emulsifiers in poultry diets has been recognized as an effective strategy to alleviate the impaired effects induced by oxidants on broiler performance [5,30]. TBARs are an indirect marker of oxidative stress, but they are a direct marker of lipid damage caused by increased oxygen under stressful conditions, and α-tocopherol has a crucial role as an antioxidant protecting the lipids from peroxidation [31,32]. In the present study, dietary emulsifiers lowered the TBAR levels in the liver of birds, which confirms that the lipid peroxidation was decreased by feeding emulsifiers [33]. In addition, the level of α-tocopherol is relatively increased by feeding emulsifier, which might be involved in reducing the lipid peroxidation in the liver [34,35]. These results mention that the inclusion of emulsifier in the feed of broilers could enhance the meat quality variables, such as drip loss and tenderness. Outcomes of the current experiment are in accordance with Attia and Kamel [36], who documented that TBARs were reduced by increasing soy lecithin rate in rabbit diets. Such refinements in this study agreed with Al-Daraji et al. [37]. Similarly, emulsifiers have neuroprotective and antioxidative properties, and they diminishes liver damage and enhances oxidative strength [27]. King et al. [38] stated that oxidative stabilization might be related to the ability of phospholipids to pool a hydrogen atom from the amino group that moves the oxidized phenolic molecule of the real antioxidant. Moreover, Judde et al. [39] elucidated that the antioxidative properties of emulsifiers depend on fatty acid structure and tocopherol concentration.

The muscle palmitic acid content as saturated fatty acids was decreased by reduced energy and emulsifier, however, muscle oleic acid, linoleic acid, and α-linolenic acid as unsaturated fatty acids were not significantly affected in this study. The emulsifiers can aid to fix the free fatty acids that are seldom soluble by themselves in bile salt micelles, and in this way they raise the digestibility of saturated fatty acids [25].

In the present study, we found that the inclusion of exogenous emulsifiers in broilers' diets with low energy clearly enhanced the weight gain and reduced the feed conversion ratio. Further, the protein

and lipid utilization was increased by emulsifier supplementation. Interestingly, the liver TBARs and muscle palmitic acid concentrations were decreased by reducing energy level and supplementation with emulsifier, which confirms the protective role of emulsifiers against oxidative stress. The plasma total cholesterol, HDL-cholesterol, total protein, and globulin were also increased by emulsifier supplementation. The obtained results are in agreement with previous studies investigating the importance of using emulsifiers in poultry diets [12,40–43]. However, further experiments are needed by using different alternative fat sources with different emulsifier additions and doses to explore their effects on the fatty acid profiles and the performances of broilers in different periods of age.

5. Conclusions

It could be concluded that dietary supplementation of a mixture of emulsifiers in low-energy diets exhibited similar or more effective effects on growth performance, nutrient utilization, lipid peroxidation, and modified plasma lipids than the high-metabolizable energy diet in broiler chickens. Due to the continuous rise of ingredient price and energy cost, the obtained results confirm the concept of using emulsifiers in low-energy diets to reduce the cost of poultry feeding and in turn increase the profitability as well as reduce the price of the product, leading improved welfare of mankind. However, future studies are required to clarify the mechanistic role of emulsifiers in improving the performances of broiler feed diets with low energy using advanced molecular tools.

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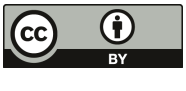

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Modelling Methionine Requirements of Fast- and Slow-Growing Chinese Yellow-Feathered Chickens during the Starter Phase

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Simple Summary: In poultry production, consuming diets with low or excessive methionine levels leads to negative effects on growth performance. The requirements of methionine may differ among the fast and slow-growing breeds; therefore, the optimal dietary methionine level should be estimated for each. In this study, six dietary methionine levels were evaluated to estimate the optimal level for fast and slow-growing yellow feathered chicken breeds. The quadratic polynomial and exponential asymptotic regression showed that the optimal methionine requirements for maximal growth performance were 0.50% and 0.53% in the fast-growing breed, and 0.48% and 0.52% in the slow growing breed.

Abstract: Two experiments were carried out to investigate the dietary methionine requirement for fast and slow-growing Chinese yellow-feathered breeds during the starter phase, based on growth variables and regression models. In Experiment 1, a total of 2880 one-day-old Lingnan chicks (fast growing breed) were used to test the methionine requirement from 1 to 21 days of age for males and females separately. Of each gender, 1440 birds were allocated into 6 dietary methionine levels (0.28%, 0.32%, 0.37%, 0.43%, 0.50% and 0.63%), each with 6 pen replicates of 40 chicks. Experiment 2 had the same design with Guangxi chicks (slow growing breed) from 1 to 30 d of age. Results indicated that significant nonlinear or quadratic responses to increasing dietary methionine levels were observed in body weight, daily gain, feed intake and feed conversion ratio of both breeds. In summary, the quadratic polynomial regression showed that the optimal methionine requirements for maximal growth performance of Lingnan chickens were 0.52–0.58% in males, 0.51% in females, and 0.53% in mixed genders. The corresponding values for Guangxi breed were 0.53% in males by quadratic polynomial regression and 0.43% in females, and 0.48% to 0.49% in mixed sexes by exponential asymptotic models.

Keywords: amino acids; nutrient requirements; growth indices; modeling; Chinese yellow-feathered chickens

1. Introduction

With the rapid development of economy in China, the second largest worldwide producer of chicken meat, market demands for nutrient-rich and tasty meat have been in continuous increase, which is boosting the industry of Chinese yellow-feathered chickens. The contribution of such chicken type in production has been growing; 3.7 billion birds annually with more than 30% of whole chicken meat shares in recent years [1]. The indigenous chickens have a recent increase in their commercial importance due to their favorable meat color and flavor, which highlights the need for comprehensive studies to enhance their feeding standards. However, only few researches on their nutrient requirements were conducted up till now [2–5].

Some essential amino acids are often added as pure to poultry diets to ensure the optimal balance required for poultry in order to maximize the production efficiency. Globally, methionine (Met) is considered to be the first limiting amino acid for poultry fed on typical corn–soybean meal-based diets [6]. Met is an essential amino acid doing numerous vital biological activities in animals' body [7,8]; it has an important role in protein synthesis in addition to enhancing the antioxidant capacity of the organism via participating as a precursor of glutathione that eliminates the reactive oxygen species in the body cells [9,10]. Additionally, the Met is required for the polyaminase synthesis that mediates cell and nucleus division process, and is considered as the most common donor of methyl group consumed in the DNA methylation process [11–14]. Met, therefore, is required for ensuring normal growth performance in poultry. The dietary supplementation of Met in poultry is commonly used to fulfil the bird requirement of Met and to achieve rapid correction of any deficiency of some nutrients; Met is a precursor of cysteine, succinyl-CoA, creatine, homocysteine and carnitine. Additionally, optimizing the Met level in poultry diets can contribute in reducing the nitrogen emission of nitrogen into the surrounding environment [13,14].

Chinese yellow-feathered chicken is a general name, which consists of quite a few breeds, mainly referring to Chinese local breeds and the improved breeds. Chinese yellow-feathered chickens grow slowly and are reared for a longer time compared with white-feathered broilers. The Lingnan yellow-feathered chicken breed is classified according to its growth rate to fast (1.47–2.30 kg, marketable at 8–10 weeks), medium (1.00–2.27 kg BW, marketable 9–14 weeks), and slow growing (1.06–1.88 kg BW, marketable 12–25 weeks) [3,15,16]. The Guangxi yellow-feathered chicken is a native, slow growing and light-body type breed with good meat quality in China (1.02–1.6kg BW, marketable 13–22 weeks). The nutrient requirements of broilers differ among the different growth stages, which are divided into three periods in Lingnan yellow-feathered broilers: starter (1–21 days old), grower (22–42 days old), and finisher (>42 days), and four phases in Guangxi yellow feathered broilers: starter (1–30 days), grower (31–60 days), early finisher (61–90 days), and later finisher (>90 days old) phases [15,16].

The Met requirement of feeding standard of chicken (CNY/T33-2004) is mainly for medium-growing yellow-feathered broilers [12]. As the dietary Met requirement for the yellow-feathered meat type chickens has not been fully determined or optimized, the present study aimed to estimate it for males and females of Lingnan (a typical fast-growing yellow feathered) and Guangxi (a slow-growing yellow-feathered) chicken breeds during the starter period.

2. Materials and Methods

2.1. Birds, Diet and Management

Two experiments were carried out to estimate the Met requirement for Lingnan (Exp1) and Guangxi (Exp2) yellow-feathered chicken breeds following the same experimental design. Before trial, the Met content of the basal diet ingredients used in both experiments was determined by ion-exchange chromatography on an automatic amino acid analyzer (L8800, Hitachi, Japan), according to the procedures described by Xi et al. [17]. In each experiment, a total of 2880 one-day-old chicks (50% males + 50% females) were randomly assigned to 6 dietary Met levels, each contained 12 identical floor pens of 40 birds, of which 6 pens were males and 6 were females (n = 240 birds from each

sex/treatment). The average initial body weights (g) were 33.88 ± 0.26, 38.75 ± 0.32, and 36.32 ± 0.28 for male, female and mixed sexes of Lingnan chicks, and 31.50 ± 0.23, 31.02 ± 0.25, and 31.20 ± 0.24 for male, female, and mixed sexes of Guangxi chicks. All birds were housed in one environmentally controlled room with dry wooden shaving flooring, continuous artificial lighting was provided from incandescent lamps, and room temperature was maintained at 30 °C in the 1st week and gradually reduced to 25 °C on the 4th week. All chicks were managed according to the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences Management Guide (GAASIAS-2016-017). The experimental diets were offered from d 1 to d 21 for the Lingnan breed (Exp1) and from d 1 to d 30 for the Guangxi breed (Exp2). Dietary treatments included a Met unsupplemented corn–soybean meal basal diet (Table 1) and the basal diets supplemented with 0.04%, 0.09%, 0.15%, 0.22%, and 0.35% Met in the form of DL-Met (Evonik Industries AG, Hanau-Wolfgang, Germany); the final dietary Met concentrations were 0.28%, 0.32%, 0.37%, 0.43%, 0.50%, and 0.63%.

Pelleted feed and drinking water were freely available to chicks. The different diets were prepared three weeks prior to the trial to allow time for checking content homogeneity, in terms of dry matter, ash, crude protein, ether extract, crude fiber, and amino acid concentrations.

Table 1. Composition of the basal diet (air-dry basis, %).

Ingredient (%)	Amount	Nutrients [2]	Value
Corn	63.62	AME (MJ/kg)	(12.55)
Soybean meal	20.01	Crude protein (%)	21
Pea protein concentrate	9.68	Lysine (%)	1.2
Soybean oil	2.56	Met [3] (%)	0.28
Calcium Carbonate	1.35	Cys [3] (%)	0.26
Dicalcium phosphate	1.9	Threonine (%)	0.84
Mineral premix [1]	0.20	Tryptophan (%)	0.21
Vitamin premix [1]	0.02	Isoleucine (%)	0.89
Salt	0.49	Valine (%)	0.99
Choline chloride (50%)	0.10	Calcium (%)	1.0
L-Threonine	0.07	Non-phytate phosphorus (%)	0.45
Total	100		

[1] Totally provided per kg of diet: 1500 IU vitamin A; 200 IU vitamin D3; 10 IU vitamin E; 0.5 mg vitamin K3; 1.8 mg thiamin; 3.6 mg riboflavin; 3.5 mg pyridoxine; 0.01 mg cyanocobalamin; 10 mg pantothenic acid; 30 mg niacin; 0.55 mg folic acid; 0.15 mg biotin; 500 mg choline; 80mg Fe; 8 mg Cu; 80 mg Mn; 60 mg Zn; 0.35 mg I; 0.3 mg Se. [2] Values were calculated based on the data provided by Feeding Standard of Chicken (Ministry of Agriculture, China, 2004). [3] Analyzed values.

2.2. Growth Performance

Birds were weighed at the beginning (day 1) and end of each experiment to record the initial and final body weights (FBW), which were used to calculate the average daily gain (ADG). Average daily feed intake (ADFI) was measured on a per pen basis for the entire experimental period, and the feed conversion ratio (FCR) was calculated. Mortality was checked daily and dead birds were weighed in order to adjust the feed intake calculations.

2.3. Statistical Analysis

Data were subjected to one-way ANOVA using the GLM procedure of SAS (version 9.3, SAS Inst., Cary, NC, USA, 2014). Tukey–Kramer test was used for means comparison, and pairwise comparisons among the means were assessed by Duncan's multiple-range tests at $p < 0.05$. All data were expressed as means and SEM, derived from ANOVA error mean square. When the main effect was significant ($p < 0.05$), linear and quadratic effects of Met content were determined. For key performance variables, the dietary methionine requirement of the birds was estimated using quadratic polynomial (QP) or exponential asymptotic (EA) models by the NLIN procedure of SAS (SAS Institute, 2014).

QP model:

$$Y = c + bX + aX^2 \quad (1)$$

where a = quadratic coefficient, b = linear coefficient, c = intercept. The requirement of Met was defined as Met = −b/(2 × a).

EA model:

$$Y = a + b \times (1 - EXP(-c \times (X - d))) \quad (2)$$

where a = relative response to the diet containing the lowest Met (deficient diet); b = difference between the minimum and the maximum response obtained with dietary Met; c = curve slope coefficient; d = Met level of the deficient diet). The optimal Met was defined as Met = (-ln (0.05)/c) + d, using 95% of the asymptotic response, since the exponential curve never reaches the asymptotic point [18–20]. The suitability of the different models was evaluated by the correlation coefficient (R^2), Akaike information criteria (AIC) and mean square error values (MSE).

3. Results

3.1. Growth Performance of Lingnan Broilers Aged 1 to 21 Days (Exp 1)

The growth performance traits of male, female and mixed male and female Lingnan yellow-feathered chickens fed different dietary Met levels between 1 and 21 days of age are shown in Tables 2–4, respectively. The increase in the dietary Met level showed linear and quadratic effects on the final BW, ADG, ADFI and FCR (linear, $p < 0.01$; quadratic, $p < 0.01$) of males; and final BW, ADG and FCR (linear, $p < 0.01$; quadratic, $p < 0.01$) of females; and final BW, ADG and ADFI (linear, $p < 0.01$; quadratic, $p < 0.01$) of mixed genders. According to the EA and QP regression, the optimal dietary Met level for the highest body weight were 0.54% and 0.55% in males, 0.47% and 0.51% in females, and 0.50% and 0.53% in mixed genders. The corresponding EA and QP values for the highest ADG were 0.54% and 0.55% in males, 0.47% and 0.51% in females, 0.50% and 0.53% in mixed genders. With regard to these results, it is worth mentioning that obtaining the same optimum Met requirement value for maximal final BW and ADG is logically expected; certainly, because these two variables are linearly correlated, where the ADG is calculated as the (final BW—initial BW) /days. This, therefore, led to the same values of R2 and the same calculated Met requirements for these two variables (Tables 2–4, Figures 1–3). Additionally, the EA and QA models showed that the optimal Met level for ADFI were 0.58% and 0.50% in males, and 0.51% and 0.47% in females, and those of daily feed intake estimated 0.50% and 0.52% in males, and 0.52% and 0.53% in mixed genders. Goodness-of-fit results for the growth performance functions are shown in Figures 1–3. The high R^2, and lowest AIC and MSE values of QP model are shown in Figure 1A–C, Figure 2A,B, Figure 3A–C. The results indicated that the QP model is more adequate for predicting the optimal Met requirements for maximal growth performance in Lingnan broilers, which showed that the optimal Met requirements for Lingnan male, female and mixed sexes were 0.52% to 0.58%, 0.51%, and 0.53%, respectively.

Table 2. Effects of total dietary methionine level on growth performance of male Lingnan yellow-feathered chickens aged 1–21 days.

Variables	Total Dietary Methionine Levels (%)						SEM [1]	*p*-Value		
	0.28	0.32	0.37	0.43	0.50	0.63		Met Level	Linear	Quadratic
Final body weight (g)	291.67 ^{d}	322.44 ^{c}	349.26 ^{b}	359.79 ^{ab}	380.44 ^{a}	374.56 ^{a}	8.62	<0.0001	<0.0001	<0.0001
Average daily gain (g)	12.28 ^{d}	13.74 ^{c}	15.02 ^{b}	15.52 ^{ab}	16.50 ^{a}	16.22 ^{a}	0.41	<0.0001	<0.0001	<0.0001
Average daily feed intake (g)	24.13 ^{d}	25.77 ^{c}	27.19 ^{b}	28.70 ^{a}	28.89 ^{a}	28.09 ^{ab}	0.47	<0.0001	<0.0001	<0.0001
Feed conversion ratio (g feed: g weight)	1.97 ^{a}	1.88 ^{b}	1.81 ^{bc}	1.82 ^{bc}	1.75 ^{c}	1.73 ^{c}	0.003	<0.0001	<0.0001	<0.0001

Variables	Model	Regression Equation [2]	TotalDietary Met Level (%)	TotalDaily Met Allowance (g/day)	*SSR*[3]	*p*-Value
Final body weight (g)	Quadratic Polynomial	$Y = 15.81 + 1340.52X - 1225.08X^2$	0.55	0.149	12162.24	<0.0001
	Exponential Asymptotic	$Y = 291 + 88.99 \times (1 - EXP(-11.42 \times (X - 0.28)))$	0.54	0.146	12218.97	<0.0001
Average daily gain (g)	Quadratic Polynomial	$Y = -0.86 + 63.83X - 58.34X^2$	0.55	0.149	27.58	<0.0001
	Exponential Asymptotic	$Y = 12.25 + 4.24 \times (1 - EXP(-11.42 \times (X - 0.28)))$	0.54	0.146	27.71	<0.0001
Average daily feed intake (g)	Quadratic Polynomial	$Y = 7.04 + 83.86X - 80.13X^2$	0.52	0.141	28.29	<0.0001
	Exponential Asymptotic	$Y = 24.03 + 4.58 \times (1 - EXP(-13.85 \times (X - 0.28)))$	0.50	0.135	31.50	<0.0001
Feed conversion ratio		$Y = 2.54–2.74X + 2.34X^2$	0.58	0.157	0.212	<0.0001

In the same row, means not sharing a similar superscript (a, b, c, d) differ significantly ($p < 0.05$), the number of replicates was used as the experimental unit (n = 6). [1] Standard error of the mean from ANOVA (n = 6). [2] Where Y is final body weight, average daily gain, average daily feed intake or feed conversion ratio and X is total dietary content of methionine. [3] SSR = sum of squared residuals.

Table 3. Effects of total dietary methionine level on growth performance of female Lingnan yellow-feathered chickens aged 1–21 days.

Variables	Total Dietary Methionine Levels (%)						SEM [1]	*p*-Value		
	0.28	0.32	0.37	0.43	0.50	0.63		Met Level	Linear	Quadratic
Final body weight (g)	315.34 ^{c}	332.84 ^{b}	368.9 ^{a}	377.26 ^{a}	380.81 ^{a}	370.91 ^{a}	4.73	<0.0001	<0.0001	<0.0001
Average daily gain (g)	13.17 ^{c}	14.00 ^{b}	15.72 ^{a}	16.12 ^{a}	16.29 ^{a}	15.82 ^{a}	0.23	<0.0001	<0.0001	<0.0001
Average daily feed intake (g)	28.53 ^{b}	27.46 ^{c}	29.13 ^{ab}	29.48 ^{a}	27.69 ^{c}	29.19 ^{ab}	0.15	<0.0001	0.147	0.342
Feed conversion ratio (g:g)	2.17 ^{a}	1.96 ^{b}	1.852 ^{bc}	1.83 ^{c}	1.70 ^{d}	1.85 ^{bc}	0.029	<0.0001	<0.0001	<0.0001

Table 3. *Cont.*

Variables	Model	Regression Equation [2]	Total Dietary Met Level, %	TotalDaily Met Fed Allowance, g/day	*SSR* [3]	*p*-Value
Final body weight (g)	Quadratic Polynomial	$Y = 46.14 + 1323.09X - 1285.88X^2$	0.51	0.145	7316.66	<0.0001
	Exponential Asymptotic	$Y = 311.9 + 67.2 \times (1 - EXP(-15.64 \times (X - 0.28)))$	0.47	0.134	7992.86	<0.0001
Average daily gain (g)	Quadratic Polynomial	$Y = 0.35 + 63.00X - 61.23X^2$	0.51	0.145	16.59	<0.0001
Feed conversion ratio	Exponential Asymptotic	$Y = 13.01 + 3.20 \times (1 - EXP(-15.64 \times (X - 0.28)))$	0.47	0.134	18.12	<0.0001
	Quadratic Polynomial	$Y = 3.86 - 8.47X + 8.38X^2$	0.51	0.145	0.281	<0.0001

In the same row, means not sharing a similar superscript (a, b, c, d) differ significantly ($p < 0.05$), the number of replicates was used as the experimental unit (n = 6). [1] Standard error of the mean from ANOVA (n = 6). [2] Where Y is final body weight, average daily gain, or feed conversion ratio and X is total dietary content of methionine. [3] SSR = sum of squared residuals.

Table 4. Effects of total dietary methionine level on growth performance of mixed male and female Lingnan yellow-feathered chickens aged 1–21 days.

Variables	Total Dietary Methionine Levels (%)						SEM [1]	*p*-Value		
	0.28	0.32	0.37	0.43	0.50	0.63		Met level	Linear	Quadratic
Final body weight (g)	303.50 ^{d}	327.65 ^{c}	359.10 ^{b}	368.53 ^{ab}	380.62 ^{a}	372.73 ^{ab}	3.87	<0.0001	<0.0001	<0.0001
Average daily gain (g)	12.72 ^{d}	13.87 ^{c}	15.37 ^{b}	15.82 ^{ab}	16.40 ^{a}	16.02 ^{ab}	0.18	<0.0001	<0.0001	<0.0001
Average daily feed intake (g)	26.33 ^{b}	26.62 ^{b}	28.16 ^{a}	28.72 ^{a}	28.29 ^{a}	28.64 ^{a}	0.19	<0.0001	<0.0001	<0.0001
Feed conversion ratio (g:g)	2.07 ^{a}	1.92 ^{b}	1.83 ^{c}	1.82 ^{c}	1.73 ^{d}	1.79 ^{cd}	0.017	<0.0001	0.123	0.108

Variables	Model	Regression Equation [2]	Total Dietary Met Level, %	Total Daily Met Fed Allowance, g/day	*SSR* [3]	*p*-Value
Final body weight (g)	Quadratic Polynomial	$Y = 30.97 + 1331.80X - 1255.48X^2$	0.53	0.147	4164.46	<0.0001
	Exponential Asymptotic	$Y = 301.3 + 77.56 \times (1 - EXP(-13.46 \times (X - 0.28)))$	0.50	0.139	4413.55	<0.0001
Average daily gain (g)	Quadratic Polynomial	$Y = -0.25 + 63.42X - 59.79X^2$	0.53	0.147	9.443	<0.0001
	Exponential Asymptotic	$Y = 12.62 + 3.69 \times (1 - EXP(-13.46 \times (X - 0.28)))$	0.50	0.139	10.008	<0.0001

Table 4. *Cont.*

Average daily feed intake (g)	Quadratic Polynomial	$Y = 17.71 + 41.35X - 38.39X^2$	0.53	0.147	14.597	<0.0001
	Exponential Asymptotic	$Y = 26.14 + 2.59 \times (1 - EXP(-12.47 \times (X - 0.28)))$	0.52	0.145	14.958	<0.0001

In the same row, means not sharing a similar superscript (a, b, c, d) differ significantly ($p < 0.05$), the number of replicates was used as the experimental unit (n = 6). [1] Standard error of the mean from ANOVA (n = 6). [2] Where Y is final body weight, average daily gain or average daily feed intake and X is total dietary content of methionine. [3] SSR = sum of squared residuals.

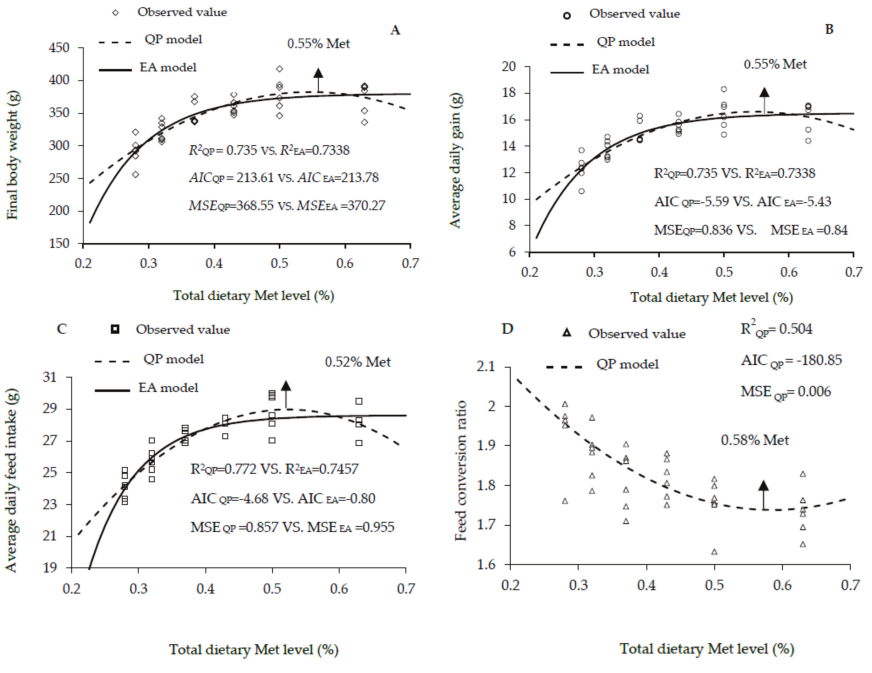

Figure 1. Regression models plot of growth performance as a function of total dietary Met level (0.28, 0.32%, 0.37%, 0.43%, 0.50%, and 0.63% Met) of male rapidly growing yellow-feathered chickens between 0 and 21 days of age. Correlation coefficient (R^2), Akaike information criteria (AIC), and mean squares error (MSE) are indicators for evaluating model fitness. (**A**) The optimum response arrow pointing at 0.55% Met by quadratic polynomial (QP) model. (**B**) The optimum response arrow pointing at 0.55% Met by QP model. (**C**) The optimum response arrow pointing at 0.52% Met by QP model. (**D**) The optimum response arrow pointing at 0.58% Met according to the QP model.

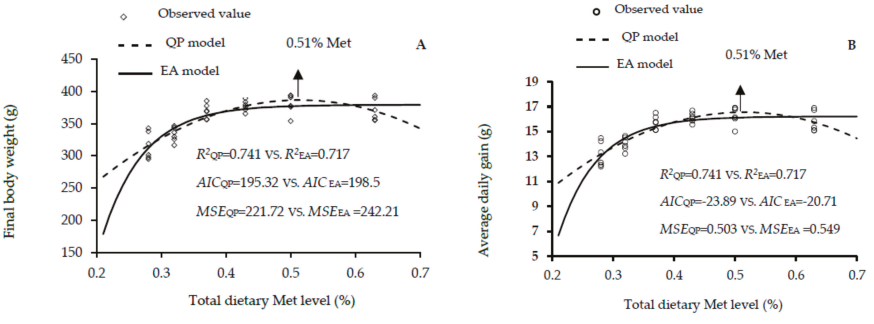

Figure 2. *Cont.*

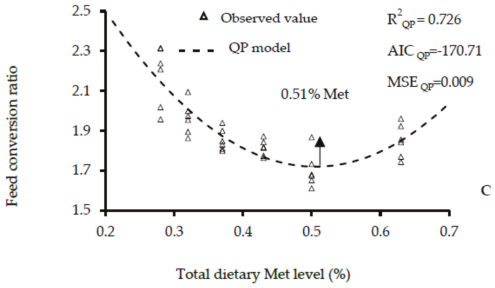

Figure 2. Regression models plot of growth performance as a function of total dietary Met level (0.28%, 0.32%, 0.37%, 0.43%, 0.50%, and 0.63% Met) of female rapidly growing yellow-feathered chickens between 0 and 21 days of age. Correlation coefficient (R^2), Akaike information criteria (AIC), and mean squares error (MSE) are indicators used in evaluating fitness of models. (**A**) The optimum response arrow pointing at 0.51% Met by the best fitting regression model. (**B**) The optimum response arrow pointing at 0.51% Met identified by the best fitting model. (**C**) The optimum response arrow pointing at 0.51% Met.

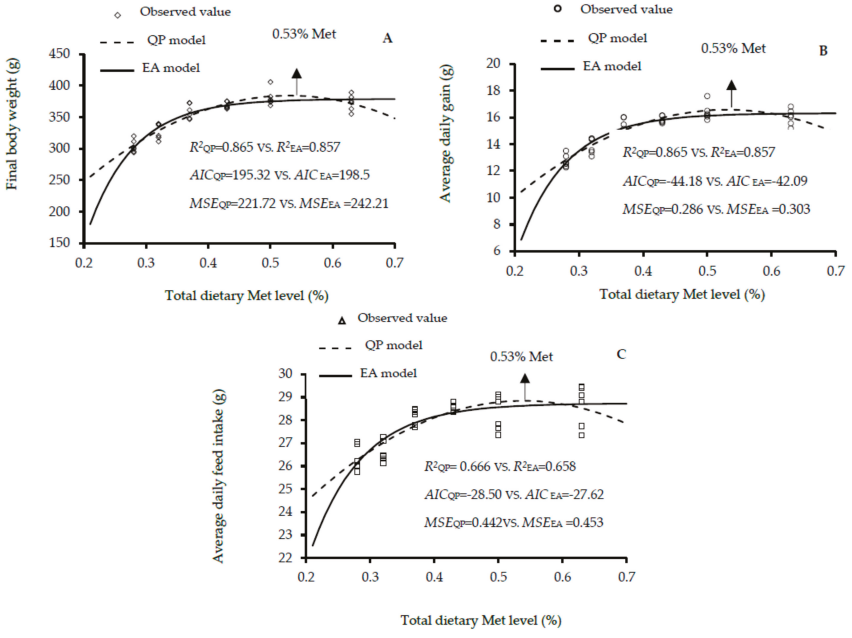

Figure 3. Regression models plot of growth performance as a function of total dietary Met level (0.28%, 0.32%, 0.37%, 0.43%, 0.50%, and 0.63% Met) of mixed sex rapidly growing yellow-feathered chickens aged 0 and 21 days of age. Correlation coefficient (R^2), Akaike information criteria (AIC) and mean squares error (MSE) are used as indicators for evaluation model fitness. (**A**) The optimum response arrow pointing at 0.53% Met according to QP model. (**B**) The optimum response arrow pointing at 0.53% Met by QP model. (**C**) The optimum response arrow pointing at 0.53% Met.

3.2. Growth Performance of Guangxi Broilers Aged from 1 to 30 Days (Exp 2)

The growth performance results of male, female and mixed genders of Guangxi yellow-feathered chickens as affected by dietary Met levels between 1 and 30 days of age are shown in Tables 5–7. The increase in dietary Met level showed linear and quadratic effects on the final BW (linear and quadratic,

$p < 0.01$), ADG (linear and quadratic, $p < 0.01$) and ADFI (linear, $p < 0.01$; quadratic, $p < 0.05$) of males; final BW, ADG, and ADG (linear, $p < 0.01$; quadratic, $p < 0.01$) of females; and final BW (linear, $p < 0.01$; quadratic, $p < 0.01$), ADG (linear, $p < 0.01$; quadratic, $p < 0.01$), and ADFI (linear and quadratic, $p < 0.05$) of mixed genders. According to the EA and QP regression models, the optimal Met for the highest final BW were 0.51% and 0.53 in males, 0.43% and 0.51% in females, and 0.48% and 0.52% in the mixed males and females. The EA and QP indicated that the optimal Met for the maximal ADG were 0.51 and 0.53% in males, 0.43% and 0.51% in females, and 0.48% and 0.52% in mixed genders. Additionally, the QP model showed that 0.49% was optimal for the best FCR in mixed genders. Fitness of the two growth performance models are shown in Figures 4–6. QP models showed higher R^2, lower AIC and MSE values, in estimating the optimal Met requirement (Figure 4a,b and Figure 6a,b) for growth performance of males, whereas the EA model in females and mixed sexes, had higher R^2, and the lower AIC and MSE values. These results reveal that the QP model are more fitting to predict Met requirement for growing Guangxi males (0–30 days), but the EA function was better fitting for females and mixed sexes. The QP models predicted the requirement of Met for males as 0.53%, and the EA models predicted the requirements of Met for females and mixed genders as 0.43% and 0.48% to 0.49%, respectively.

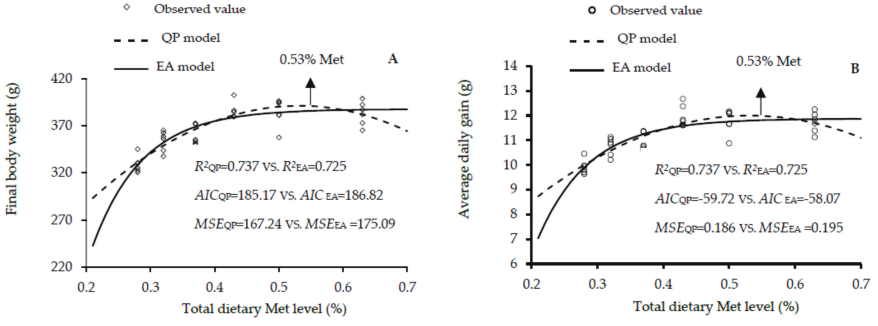

Figure 4. Regression models plot of growth performance as a function of total dietary Met level (0.28%, 0.32%, 0.37%, 0.43%, 0.50%, and 0.63% Met) of male slowly growing yellow-feathered chickens between 0 and 30 days of age. Correlation coefficient (R^2), Akaike information criteria (AIC) and mean squares error (MSE) are indicators used to evaluate fitness of models. (**A**) The optimum response arrow pointing at 0.53% Met by the best fitting model. (**B**) The optimum response arrow pointing at 0.53% Met by the best fitting regression model.

Table 5. Effects of total dietary methionine level on growth performance of male Guangxi yellow-feathered chickens aged 1 to 30 days.

Variables	Total Dietary Methionine Levels (%)						SEM [1]	*p*-Value		
	0.28	0.32	0.37	0.43	0.50	0.63		Met Level	Linear	Quadratic
Final body weight (g)	328.96 ^{c}	353.96 ^{b}	359.07 ^{b}	390.74 ^{a}	384.15 ^{a}	383.04 ^{a}	6.18	<0.0001	<0.0001	<0.0001
Average daily gain (g)	9.92 ^{c}	10.75 ^{b}	10.92 ^{b}	11.97 ^{a}	11.75 ^{a}	11.70 ^{a}	0.21	<0.0001	<0.0001	<0.0001
Average daily feed intake (g)	25.76 ^{b}	25.82 ^{b}	26.37 ^{ab}	28.21 ^{ab}	27.29 ^{ab}	28.84 ^{a}	0.82	0.0035	0.003	0.114
Feed conversion ratio (g:g)	2.6	2.41	2.41	2.35	2.32	2.46	0.08	0.1508		

Variables	Model	Regression Equation [2]	Total Dietary Met Level (%)	Total Daily Met fed Allowance (g/day)	*SSR*[3]	*p*-Value
Final body weight (g)	Quadratic Polynomial	$Y = 123.45 + 1007.30X - 947.59X^2$	0.53	0.143	5518.908	<0.0001
	Exponential Asymptotic	$Y = 328.50 + 59.13 \times (1 - EXP(-12.83 \times (X - 0.28)))$	0.51	0.138	5777.969	<0.0001
Average daily gain (g)	Quadratic Polynomial	$Y = 3.07 + 33.58X - 31.59X^2$	0.53	0.143	6.132	<0.0001
	Exponential Asymptotic	$Y = 9.90 + 1.97 \times (1 - EXP(-12.83 \times (X - 0.28)))$	0.51	0.138	6.42	<0.0001

In the same row, means not sharing a similar superscript (a, b, c) differ significantly ($p < 0.05$), the number of replicates was used as the experimental unit (n = 6). [1] Standard error of the mean from ANOVA (n = 6). [2] Where Y is final body weight or average daily gain and X is total dietary content of methionine. [3] SSR = sum of squared residuals.

Table 6. Effects of total dietary methionine level on growth performance of female Guangxi yellow-feathered chickens aged 1 to 30 days.

Variables	Total Dietary Methionine levels (%)						SEM [1]	*p*-Value		
	0.28	0.32	0.37	0.43	0.50	0.63		Met Level	Linear	Quadratic
Final body weight (g)	308.50 ^{c}	318.82 ^{bc}	329.90 ^{ab}	340.57 ^{a}	327.14 ^{ab}	332.34 ^{ab}	5.61	0.0023	0.011	0.001
Average daily gain (g)	9.25 ^{c}	9.59 ^{bc}	9.96 ^{ab}	10.31 ^{a}	9.87 ^{ab}	10.04 ^{ab}	0.19	0.0023	0.011	0.001
Average daily feed intake (g)	25.57	26.82	24.96	26.31	25.58	24.62	1.11	0.45		
Feed conversion ratio (g:g)	2.77	2.79	2.51	2.55	2.59	2.45	0.11	0.21		

Table 6. *Cont.*

Variables	Model	Regression Equation [2]	Total Dietary Met Level, %	Total Daily Met Fed Allowance, g/day	*SSR* [3]	*p*-Value
Final body weight (g)	Quadratic Polynomial	$Y = 210.40 + 493.91X - 482.58X^2$	0.51	0.131	5682.55	0.001
	Exponential Asymptotic	$Y = 307.6 + 25.8 \times (1 - EXP(-20.29 \times (X - 0.28)))$	0.43	0.110	5432.792	0.0006
Average daily gain (g)	Quadratic Polynomial	$Y = 5.98 + 16.46X - 16.09X^2$	0.51	0.131	6.314	0.001
	Exponential Asymptotic	$Y = 9.22 + 0.86 \times (1 - EXP(-20.29 \times (X - 0.28)))$	0.43	0.110	6.036	0.0006

In the same row, means not sharing a similar superscript (a, b, c) differ significantly ($p < 0.05$), the number of replicates was used as the experimental unit (n = 6). [1] Standard error of the mean from ANOVA (n = 6). [2] Where Y is final body weight or average daily gain and X is total dietary content of methionine. [3] SSR = sum of squared residuals.

Table 7. Effects of total dietary methionine level on growth performance of mixed male and female Guangxi yellow-feathered chickens aged 1 to 30 days.

Variables	Total Dietary Methionine Levels (%)						SEM [1]	*p*-Value		
	0.28	0.32	0.37	0.43	0.50	0.63		Met Level	Linear	Quadratic
Final body weight (g)	318.73 ^{c}	336.39 ^{bc}	344.49 ^{ab}	365.66 ^{a}	355.64 ^{ab}	357.69 ^{ab}	4.28	<0.0001	<0.0001	<0.0001
Average daily gain (g)	9.58 ^{c}	10.17 ^{bc}	10.44 ^{ab}	11.15 ^{a}	10.81 ^{ab}	10.88 ^{ab}	0.14	<0.0001	<0.0001	<0.0001
Average daily feed intake (g)	25.67	26.32	25.67	27.26	26.44	26.73	0.43	0.2473		
Feed conversion ratio (g:g)	2.68 ^{a}	2.60 ^{ab}	2.46 ^{b}	2.45 ^{b}	2.45 ^{b}	2.46 ^{b}	0.041	0.0105	0.027	0.016
Variables	**Model**		**Regression Equation [2]**			**Total Dietary Met Level, %**	**Total Daily Met Fed Allowance, g/day**		***SSR*[3]**	***p*-Value**
Final body weight (g)	Quadratic Polynomial		$Y = 166.93 + 750.60X - 715.09X^2$			0.52	0.137		3466.707	<0.0001
	Exponential Asymptotic		$Y = 318.0 + 42.06 \times (1 - EXP(-15.14 \times (X - 0.28)))$			0.48	0.126		3396.603	<0.0001
Average daily gain (g)	Quadratic Polynomial		$Y = 4.52 + 25.02X - 23.84X^2$			0.52	0.137		3.852	<0.0001
	Exponential Asymptotic		$Y = 9.56 + 1.40 \times (1 - EXP(-15.14 \times (X - 0.28)))$			0.48	0.126		3.774	<0.0001
Feed conversion ratio	Quadratic Polynomial		$Y = 3.60 - 4.58X + 4.41X^2$			0.49	0.129		0.874	0.016

In the same row, means not sharing a similar superscript (a, b, c) differ significantly ($p < 0.05$), the number of replicates was used as the experimental unit (n = 6). [1] Standard error of the mean from ANOVA (n = 6). [2] Where Y is final body weight, average daily gain or feed conversion ratio and X is total dietary content of methionine. [3] SSR = sum of squared residuals.

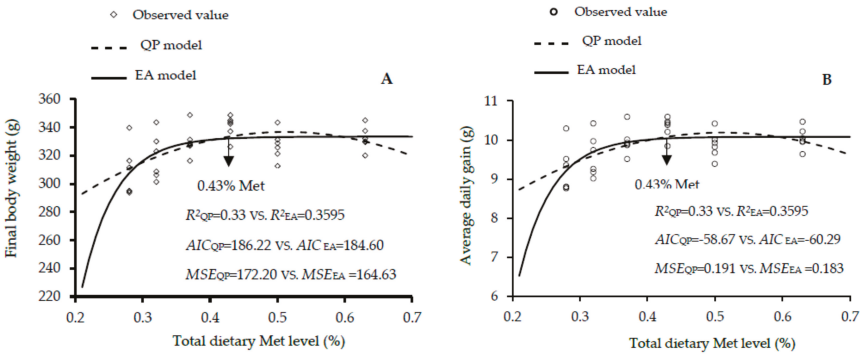

Figure 5. Regression models plot of growth performance as a function of total dietary Met level (0.28%, 0.32%, 0.37%, 0.43%, 0.50%, and 0.63% Met) of female slowly growing yellow-feathered chickens between 0 and 30 days of age. Correlation coefficient (R^2), Akaike information criteria (AIC) and mean squares error (MSE) are indicators used in evaluating fitness of models. (**A**) The optimum response arrow pointing at 0.43% Met by the best fitting model. (**B**) The optimum response arrow pointing at 0.43% Met identified by the best fitting regression model.

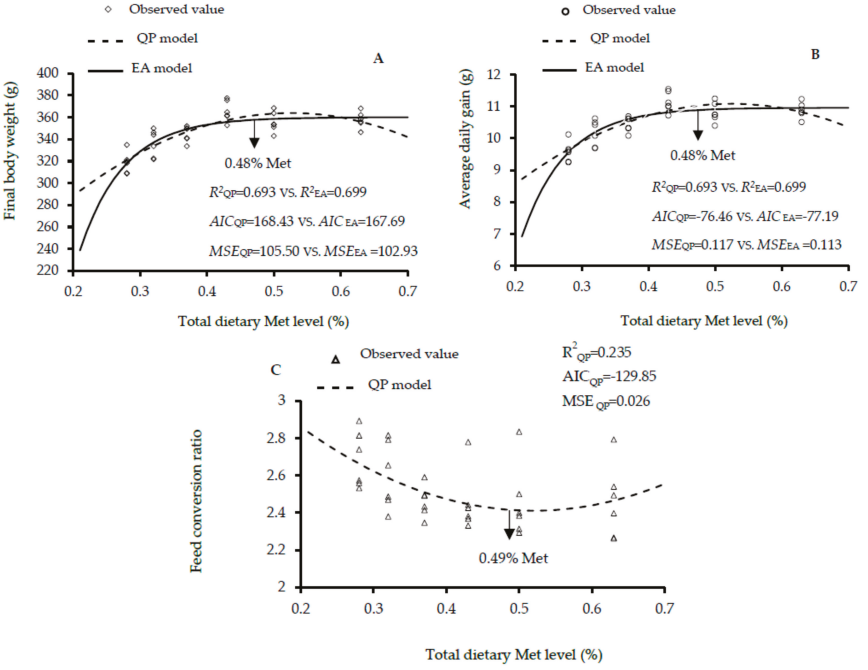

Figure 6. Regression models plot of growth performance as a function of dietary total Met level (0.28%, 0.32%, 0.37%, 0.43%, 0.50%, and 0.63% Met) of mixed sexes of slowly growing yellow-feathered chickens between 0 and 30 days of age. Correlation coefficient (R^2), Akaike information criteria (AIC) and mean squares error (MSE) are indicators of models fitness. (**A**) The optimum response arrow pointing at 0.48% Met by the best fitting model. (**B**) The optimum response arrow pointing at 0.48% Met identified by the best fitting model. (**C**) The optimum response arrow pointing at 0.49% Met.

4. Discussion

Diets having low or excessive Met levels could engender important influences on poultry performance [21]. An optimal dietary Met concentration could significantly improve growth performance of broiler chickens when the Met level was lower in the diet [4,5,22–24]. The results of the present study confirm that increasing the dietary Met level improved growth performance of Liangnan and Guangxi chicks. This improvement in growth performance is attributable to the important roles of Met in animal's body, as mentioned in the introduction. Tsiagbe et al. [25] demonstrated that the Met has direct influences on growth performance and immunity in meat-type chickens. In a like manner, Carew et al. [26] reported that Met deficiency reduces the growth and development of lymphoid organs, which have negative effects on growth. The excess of Met, higher than the requirement, is used to cover requirement of some important amino acids such as cystine; two molecules of Met are used in synthesizing one molecule of cystine [27], whereas Met requirement can be covered only by dietary methionine. Additionally, the results here indicated that an excess of dietary Met level did not synchronously improve growth performance of birds and even showed a tendency of negative effects, which was consistent with the findings of Jamroz et al. [28]. Excessive levels of dietary Met can have negative effects on growth. The results of D'Mello and D'Mello [29] indicated that the dietary addition of 20 or 40 g/kg of excess methionine decreased feed intake and reduced body weight gain. Edmonds and Baker [30] found that using an excess of Met at 4% of a corn-soybean meal diet containing 23% protein reduced body weight gain by 92%, whereas similar excesses of lysine, tryptophan, and threonine were far less toxic.

The Met requirement of Chinese yellowed-feathered chicks (unsexed) was 0.46% at the starter phase, according to the old estimations of [16]. According to the results here, however, the Met requirement of Chickens differed between males and females. These results agree with previous findings [4,5,17], which indicated that chicken males and females have different Met requirements. In the present study, the estimated Met requirements of male and female Lingnan, and male Guangxi chickens, determined according to EA and QP models, were higher than the old recommended level (0.46%) of FSC [16]; but the estimated Met requirement of female Guangxi chicken was lower than the recommended value [16]. According to NRC [27], the requirements of Met and Met + Cystine for commercial broiler chickens during the starter period (0–3 weeks) are 0.5% and 0.9% of the diet. The obtained Met requirements here were obtained in the presence of 0.26% cystine (Table 1) in the basal diet. The different Met requirements between breeds (fast and slow growing) or between both sexes is logically attributed to differences in growth rates and genetic potential [31]. Kalinowski et al. [24], reported that Ross 308 broilers optimized final BW (794 g) with dietary Met of 0.50% during 0–21 days; whereas Xi et al. [5], found that the yellow-feathered chicken only needed 0.433–0.435% Met (male) and 0.445–0.454% Met (female) for optimal 21 day BW (male: 351.12 g; female:314.37 g) in the starter phase. According to the available information on the FSC [16] and feeding management regulations of the yellow feathered-chicken [15], the Lingnan chicken breed is classified as a fast-growing breed, and the Guanxi chicken is a slow-growing breed; this can explain the differences in Met requirements obtained here between the two breeds. The different starter phase durations of the Lingnan (0–21 days), and Guangxi (0–30 days) are originally dependent on their growth rate and genetic potential, which have caused the differences in the optimal Met requirement of the two breeds. Similar results were reported by Kalinowski et al. [24] and Dozier et al. [32]. Additionally, several studies indicated that evaluating the response of more than one variable to a dietary nutrient makes it difficult to determine a unique value of the nutrient requirement, i.e., the optimal level of any nutrient required for obtaining the best result of growth rate, body weight, feed intake, feed conversion ratio, meat quality, or immunity indices can differ [2,3,33]; this can explain the different values of optimal Met level for the different growth variables here.

5. Conclusions

Supplementation of graded Met levels in basal diets of yellow feathered broilers during the starter period showed beneficial influences on their growth performance indices. The results indicated that the optimal Met requirement differed between the fast (up to 21 d of age) and slow-growing (up to 30 days of age) yellow-feathered chicken breeds as well as between males and females of each breed. The QP regression model was more appropriate for estimating the optimal Met requirements for the best body weight, average daily gain, feed intake and FCR indices of Lingnan (fast-growing) males (0.52% to 0.58%), females (0.51%), and mixed genders (0.53%), as well as for Guangxi (slow-growing) males (0.53%), whereas the EA model was found to be better for estimating the optimal Met requirement of Guangxi females (0.43%) and mixed genders (0.48% to 0.49%).

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Ameliorative Effects of Antibiotic-, Probiotic- and Phytobiotic-Supplemented Diets on the Performance, Intestinal Health, Carcass Traits, and Meat Quality of *Clostridium perfringens*-Infected Broilers

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Simple Summary: Necrotic enteritis is considered the most important economic problem for the poultry industry due to the sudden death rates of up to 50%. However, there is limited information concerning the ameliorative role of probiotic and/or phytobiotic compounds in the prevention of *Clostridium perfringens* infections in broilers. Hence, this trial is conducted to evaluate the influence of some antibiotic, probiotic and phytobiotic compounds (Maxus, CloStat, Sangrovit Extra, CloStat + Sangrovit Extra, and Gallipro Tect) on the growth performance, carcass traits, intestinal health, and meat quality of broiler chicks. The obtained in vivo results highlight that a probiotic- and/or phytobiotic-supplemented diet has many positive effects on the performance, organ weight, and meat quality of broilers. Besides, a notable reduction in the lesion score is observed with a combined probiotic and phytobiotic diet.

Abstract: The poultry industry needs efficient antibiotic alternatives to prevent necrotic enteritis (NE) infections. Here, we evaluate the effects of probiotic and/or prebiotic dietary supplementation on performance, meat quality and carcass traits, using only an NE coinfection model, in broiler chickens. Three hundred and twenty-four healthy Ross 308 broiler chicks are allocated into six groups. Taking a 35 d feeding trial, the chicks are fed a basal diet with 0.0, 0.1, 0.5, 0.12, 0.5 + 0.12, and 0.2 g Kg^{-1} for the control (T_1), Avilamycin (Maxus; T_2), live probiotic (CloStat (*Bacillus subtilis*);T_3), natural phytobiotic compounds (Sangrovit Extra (sanguinarine and protopine); T_4), CloStat + Sangrovit Extra (T_5), and spore probiotic strain (Gallipro Tect (*Bacillus subtilis* spores); T_6) treatments, respectively. Occurring at 15 days-old, chicks are inoculated with *Clostridium perfringens*. The obtained results reveal that all feed additives improve the performance, feed efficiency, and survival rate, and reduces the intestinal lesions score compared with the control group. The T_6 followed by T_3 groups show a significant ($p < 0.05$) increase in some carcass traits, such as dressing, spleen, and thymus percentages compared with other treatments. Also, T_5 and T_6 have significantly recorded the lowest temperature and pHu values and the highest hardness and chewiness texture values compared to the other treated groups. To conclude, probiotics combined with prebiotic supplementation improves the growth, meat quality, carcass characterization and survival rate of NE-infected broiler chickens by modulating gut health conditions and decreasing lesion scores. Moreover, it could be useful as an ameliorated NE disease alternative to antibiotics in *C. perfringens* coinfected poultry.

Keywords: probiotic; *Clostridium perfringens*; phytobiotic; broiler

1. Introduction

Clostridium perfringens is a Gram-positive bacterium which is common within ecosystems and healthy intestinal microflora [1]. *C. perfringens* is responsible for several diseases in humans, wildlife, and farm animals [2], and is the leading cause of necrotic enteric (NE) disease in farm animals, especially in poultry [3]. Regarding birds, NE disease is caused by specific strains of *C. perfringens*. It costs the global poultry industry over two billion dollars annually, mainly due to the high costs of antibiotics and inactive feed conversion [4,5]. Several studies have reported that *C. perfringens* bacteria could produce more than 15 different toxins [6,7]. Although all *C. perfringens* types can induce α-toxin [3], this kind of toxin causes serious enteric and intestinal diseases in animals and humans [8]. Infected birds show severe lesions of the jejunum and ileum, with the small intestine presenting a degenerated mucosa and is distended by gases produced by *C. perfringens* [9]. Signs of infection include depression, reduced mobility, and diarrhoea, which is the most visibly obvious symptom [10].

Several strategies are commonly used to alleviate the symptoms of enteritis in broilers, including the use of probiotics [11]. It has been recognized that administering antibiotics as feed additives can avoid mortalities induced by NE [12]. Avilamycin is an antibiotic of the oligosaccharides family and is widely active against Gram-positive bacteria [13]. Moreover, Avilamycin has been shown to have potent bactericidal effects on *C. perfringens* in vitro [11]. Similar results were found by Paradis et al. [11] and Mwangi et al. [14], who reported linear relationships between the level of Avilamycin in the feed and a reduction in NE mortalities, NE lesion scores, and intestinal *C. perfringens* count.

A probiotic is a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance [15–17]. Probiotics can interact with the host to improve immunity and intestinal morphology or stimulate the metabolism, thus reducing the risk of infection by opportunistic pathogens [18]. Probiotic bacteria also have been shown to produce molecules with antimicrobial activities, such as bacteriocins, which target specific pathogens or inhibit the adhesion of pathogens or the production of pathogenic toxins [19,20]. Moreover, beneficial bacteria can act as competition against pathogenic strains within the host [21]. A large number of studies have described the isolation of some strains belonging to the genera *Bacillus* and *Lactobacillus*, which exhibit anti-*C. perfringens* activity in vitro [22,23]. The supplementation of animal feed with *Bacillus* spores (*B. licheniformis*) also was tested and proven to be an efficient alternative to antibiotics when used in larger amounts and for longer periods [24]. When 20 day-old chicks, inoculated with low amounts of *C. perfringens*, were given a single dose of 10^9 *B. subtilis* spores, the colonization and persistence of *C. perfringens* were abolished. However, the *B. subtilis* strain alone was unable to affect *C. perfringens* in vitro [25]. Also, Sokale et al. [26] reported that using *Bacillus subtilis* as a feed supplement in broiler chicks increased the performance and reduced mortality in the chicks treated with *C. perfringens*. The supplementation of *B. subtilis* not only controlled *C. perfringens*-induced NE, but also improved the intestinal health of the broilers [27].

Several natural products such as herbs, spices and essential oils are categorized under the term botanic, phytobiotic or phytogenic compounds [28–30]. Phytobiotics are well identified for their antibacterial and pharmacological effects and, thus, are commonly used in broiler feed as growth promoters and alternative medicines [17]. A huge number of in vitro and in vivo studies have approved a varied range of activities for phytobiotics in poultry nutrition, like stimulation of feed intake, or antimicrobial, coccidiostatic, anthelmintic and immunostimulating actions [31]. Abudabos et al. [32] reported that using Sanguinarine as a feed supplement in broiler chicks (Ross 308) challenged with *Clostridium perfringens* enhanced performance, carcass traits and some blood biochemical parameters. Also, El–Sheikh et al. [33] informed that the prebiotic supplemented (Sanguinarine) diet could be an effective treatment alternative to antibiotics for controlling necrotic enteritis diseases in broilers. Diverse types of additives include phytobiotics (primary or secondary components of plants that contain bioactive compounds that exert a

positive effect on the growth and health of animals) which could be a beneficial strategy that regulates the gastro-intestinal microbial community and improve broiler health [34].

Found in the literature, the positive effects of both *B. subtilis* and phytobiotic compounds have been reported. However, their synergistic effects have not been described yet. The present study aims to evaluate the ameliorative effects of probiotic and phytobiotic compounds alone or in a combined form of two different types of *B. subtilis* on the performance, carcass traits, meat measurements, and intestinal health of *C. perfringens*-infected broilers during the starter and finisher phases.

2. Materials and Methods

The experiment was performed in cage pens under similar managerial and hygienic conditions in an environmentally controlled poultry unit at the Animal Production Department, College of Food Science and Agriculture Science, King Saud University. All protocols were chosen according to the experimentation guidelines of the Animal Use Ethics in Research Committee of King Saud University (approval number: SE-19-150).

2.1. Experimental Design and Feeding Regime

Altogether, 324 day-old broiler chicks (Ross 308 strain) were randomly distributed into six groups. Each group contained nine replicates, with six birds per replicate, and were used for a 35 d feeding trial period. Each group was assigned to one of the following dietary treatments: T_1; basal diet (control), T_2; diet supplemented with 0.1 g kg^{-1} of Maxus (antibiotic), T_3; diet supplemented with 0.5 g kg^{-1} of Clostat (natural probiotic strain), T_4; diet supplemented with 0.12 g kg^{-1} of Sangrovit (phytobiotic), T_5; diet supplemented with 0.5 g kg^{-1} of Clostat combined with 0.12 g kg^{-1} of Sangrovit, and T_6; diet supplemented with 0.2 g kg^{-1} of Gallipro Tect (spore probiotic). The chicks were fed a starter diet between days 0–14, which was then switched to a grower diet between days 15–35 (Table 1). After 14 d, all treated groups were inoculated with *C. perfringens* bacteria. Birds were fed ad libitum, and water was available at all times during the experimental period.

Maxus was manufactured by BIOFERM CZ, spol. Sro. Banskobystrická 461, 621 00 Brno-Řečkovice a Mokrá Hora, Czechia, as a source of Avilamycin antibiotics (each 1000 g, containing 100 g of Avilamycin) in feed diets, while, CloStat products were manufactured by KERMIN Ind., Inc., 2100 Maury Street Des Moines, IA 50317 USA (each 1 g, containing 2×10^7 CFU/g *Bacillus subtilis*). The Sangrovit Extra used phytobiotic compounds (extracts of Benzophenanthridine alkaloids (sanguinarine) and protopine) produced by Albitalia s.r.L., Co., Milano, Italy. Gallipro Tect were used as a source of a highly-selected strain (DSM17299) of *Bacillus subtilis* (*B. subtilis* 4×10^9 CFU/g DSM 17299), and was provided by Boege Alle Co., Hoersholm, Denmark.

2.2. Challenge with Clostridium perfringens Bacteria

The *C. perfringens* challenge model was performed as described by Prescott [35]. All treated groups received a *C. perfringens* challenge at a rate of 4×10^8 CFU g^{-1} via oral gavages on day 14, as recommended by Olkowski et al. [36], using the defined B positive bacteria *C. perfringens* isolated from a local farm. The identified bacterium was previously confirmed to be sensitive to antibiotics (Avilamycin) using the broth dilution method (MIC testing) [37]. An inoculum was equipped to contain nearly 10^8 cells of *C. perfringens* per ml and was processed at a ratio of 1:1.5 ration-to-broth [11]. The challenge feed was mixed with the treatment diet. After the administration of the challenged material, dead birds were counted as study mortalities. These birds underwent a necropsy evaluation to estimate the cause of death/disease.

Table 1. Composition of starter and finisher diets.

Ingredient	Treatment Period (0-35) Days	
	Starter (0–15)	Finisher (15–35)
Yellow corn	57.39	61.33
Soybean meal	27.00	22.80
Palm oil	2.20	2.80
Corn gluten meal	8.80	6.0
Wheat bran	0.00	3.0
Dicalcium phosphate (DCP)	2.30	2.09
Ground limestone	0.70	0.62
Choline chloride	0.05	0.05
DL-methionine	0.105	0.075
L-lysine	0.39	0.36
Salt	0.40	0.20
Threonine	0.17	0.17
V–M premix [1]	0.50	0.50
Total	100	100
Analysis		
Metabolizable energy (ME) (kcal/kg)	3000	3050
Crude protein (%)	23.0	20.5
Non-phytate P (%)	0.48	0.44
Calcium (%)	0.96	0.88
Digestible lysine (%)	1.28	1.15
Digestible methionine (%)	0.60	0.54
Digestible sulfur amino acids (%)	0.95	0.86
Digestible threonine (%)	0.86	0.77

[1] V–M premix; vitamin–mineral premix contains the following per kg: vitamin A, 2,400,000 IU; vitamin D, 1,000,000 IU; vitamin E, 16,000 IU; vitamin K, 800 mg; vitamin B1, 600 mg; vitamin B_2, 1600 mg; vitamin B_6, 1000 mg; vitamin B_{12}, 6 mg; niacin, 8000 mg; folic acid, 400 mg; pantothenic acid, 3000 mg; biotin 40 mg; antioxidant, 3000 mg; cobalt, 80 mg; copper, 2000 mg; iodine, 400; iron, 1200 mg; manganese, 18,000 mg; selenium, 60 mg; zinc, 14,000 mg.

2.3. Growth Performance Parameters

During the starter (0–14-days-old) and finisher (15–35-days-old) periods, the growth and feed efficiency parameters of the broiler chicks were estimated. The daily feed intake was calculated by subtracting the quantity of feed rejected from the feed offered. Additionally, live body weight was estimated at biweekly intervals, while the final body weight and total feed consumption were determined at the end of each trial period. The body weight gain (BWG) was measured by calculating the difference between the live body weight and final body weight for each trial period. The feed conversion ratio (FCR) was computed for each group, as mentioned by Abudabos et al. [32], using the following formula:

$$\text{FCR} = \text{Feed intake/Weight gain} \quad (1)$$

Meanwhile, the production efficiency factor (PEF) was calculated as suggested by Griffin [38], using the following formula:

$$\text{PEF} = (\text{Livability} \times \text{Live weight (kg)})/(\text{Age in days} \times \text{FCR}) \times 100 \quad (2)$$

During the feeding trial, the number of deaths was counted to calculate the survival rate, as mentioned by Hussein et al. [39], using the following formula:

$$\text{Survival rate (SR)\%} = (\text{the number of the surviving broilers / the initial number of broilers}) \times 100 \quad (3)$$

2.4. Carcass Measurements and Lesion Scores

Ten broiler chicks were randomly collected from each group to estimate the organ weights (heart, gizzard, liver, bursa, spleen, thymus, small and large intestine, and ceca). Chicks were weighed before sacrifice. All internal organs were weighed immediately after slaughter. The gizzard was weighed after its content was removed. The small intestine was measured by determining the distance between

the site of the duodenum emergence from the gizzard and the beginning of the ceca. The organ weight was calculated relative to the live body weight.

According to the Hofacre et al. [40] procedure, two birds per pen were examined for gross intestinal lesions. The characteristic of necrotic enteritis was defined using the description of the Long et al. [41] study. The lesion scores were outlined as follows: 0 = none, 1 = mild, 2 = moderate and 3 = marked (severe) [42].

2.5. Meat Characteristics

At the end of the finisher period (35-days-old chicks), three birds were randomly selected per pen to estimate the meat characteristics followed by the Chen et al. [43] method. After euthanasia, the jugular vein was cut, the feathers, heads, and shanks were removed, and the remaining carcasses were dissected. The left and right breast from each bird were used for the quality measurements. The breast samples were stored at −80 °C until further analysis. At the time of the analysis, frozen muscles were thawed overnight in a chiller at 4 °C.

The pH of the breast muscle was measured twice (after slaughtering and 24 h postmortem), using a microprocessor pH-Meter (Model PH 211, Hanna Instruments, Padova, Italy). The core temperature in the breast muscle was measured after slaughtering with a portable digital thermocouple (Eco Scan Series, Temp JKT, Eu tech Instruments, 7 Gul Circle, level 2M, Keppel Logistic Building, Singapore 629563). The colour values of the CIELAB Color System (L*(lightness), a* (redness), and b* (yellowness)) were determined for the breast muscles 15 min and 24 h after slaughtering, using a Chroma meter (Konica Minolta, CR-400-Japan) following the method used by Castellini et al. [44].

The myofibril fragmentation index (MFI) of the breast muscle was determined by multiplying the absorbance value at 540 nm, as described by Culler et al. [45]. The water-holding capacity (WHC) was determined based on the technique described by Hamm [46] and following the modification performed by Wilhelm et al. [47], using the following equation:

$$\mathrm{WHC} = 100 - [(\mathrm{Wi} - \mathrm{Wf}/\mathrm{Wi}) \times 100] \quad (4)$$

where, Wi and Wf are the initial and final sample weights, respectively.

The drip loss (DL) was determined as a percentage based on the initial sample weight. The cooking loss (CL) was determined as the difference between the initial and final weights. Then, cooked samples were used to evaluate the shear force according to the procedure described by Wheeler et al. [48], under a 200 mm/min crosshead speed. The texture profile analysis (TPA) values were estimated using a Texture Analyzer (TA–HD–Stable MicroSystems, Golborne, Warrington WA3 3GR, England) equipped with a compression-platen attachment. The variables determined included the hardness (maximum force needed to compress the sample), cohesiveness (ratio between the total energy required for the first and second compression), springiness (the ability of a sample to recover to its original form after the removal of the compressing force), and chewiness (a resultant of springiness × hardness × cohesiveness).

2.6. Statistical Analysis

The data underwent a one-way ANOVA using a completely randomized design. Before analysis, the data were examined for normality of distributions and homogeneity of variance. Percentage data were subjected to arcsine transformation before analysis. SPSS 22 analysis software was used for all statistical analyses. Data are expressed as the mean ± SEM, with a statistical significance level of $p \leq 0.05$. Significant differences between means were determined using Duncan's Multiple Range [49].

3. Results

3.1. Growth Performance and Intestinal Lesions Score

The effects of some feed additives on the total feed intake (TFI), body weight gain (BWG), feed conversion ratio (FCR), and protein efficiency ratio (PEF) of broilers in the starter and finisher periods are shown in Table 2. During the starter period, our results revealed no significant differences between all treatment and non-treatment groups. T_4 produced the lowest TFI values, while T_3 and T_1 exhibited the highest. Regarding the FCR values, both the T_4 and T_5 treatments had lower values than those of the other treatments. The dietary supplementation of probiotic bacteria increased the PEF (except for T_3) compared with the control group.

Table 2. Effects of some feed additives on the growth performance, survival rate, and intestinal lesion score of broilers challenged with *C. perfringens* during the starter and finisher feeding periods.

Measurements	Treatment						*p*-Value	
	T_1	T_2	T_3	T_4	T_5	T_6	SEM	Sig.
Starter Period (0–14 days-old)								
IBW (g)	36.7	36.9	36.8	36.8	36.9	36.8	0.04	NS
BW 14 d (g)	449	443.1	448	445.5	446.6	448.1	1.089	NS
TFI (g)	561.1	549.9	558.6	531.1	546.8	555.0	2.049	NS
BWG (g)	412.3	406.2	411.2	408.7	409.7	411.3	0.831	NS
FCR (g:g)	1.36	1.35	1.36	1.30	1.33	1.35	0.05	NS
PEF	189.0	206.4	189.2	217.0	213.8	209.0	1.049	NS
Finisher Period (15–35 days-old)								
FBW (g)	1593.3 ^{b}	1807.4 ^{a}	1827.0 ^{a}	1824.3 ^{a}	1823.1 ^{a}	1798.9 ^{a}	1.121	***
TFI (g)	2325.6	2366.7	2353.2	2382.6	2379.9	2404.4	2.865	NS
BWG (g)	1144.0 ^{c}	1364.3 ^{b}	1379.0 ^{a}	1378.8 ^{a}	1376.5 ^{a}	1350.8 ^{b}	2.961	***
FCR (g:g)	2.03 ^{a}	1.73 ^{bc}	1.71 ^{c}	1.73 ^{bc}	1.73 ^{bc}	1.78 ^{b}	0.03	***
PEF	215.6 ^{c}	301.5 ^{b}	305.0 ^{ab}	310.0 ^{a}	307.6 ^{ab}	288.5 ^{b}	3.76	***
SR (%)	97.2 ^{b}	99.8 ^{a}	99.6 ^{a}	99.8 ^{a}	99.8 ^{a}	99.6 ^{a}	0.30	***
Lesions score	2.50 ^{a}	0.67 ^{b}	0.66 ^{b}	0.67 ^{b}	0.33 ^{c}	0.50 ^{b}	0.26	***

IBW, initial body weight; BW 14 d, body weight at 14 days of age; FBW, final body weight; TFI, total feed intake; BWG, bodyweight gain; FCR, feed conversion ratio; PEF, protein efficiency ratio; and SR, survival rate. SEM, mean values of the standard error. Mean values of three replicates with deferent letters (^{a,b,c}) in the same row are significantly different ($p < 0.05$). Sig, significance; NS, non-significance; *** significance at $p < 0.001$.

During the finisher period, all experimental additives significantly increased ($p < 0.01$) the FBW, BWG, FCR, PEF, survival rate (SR), and decreased the intestinal lesions score compared with those of the control group (Table 2). However, the TFI was not significantly affected by the supplemented diets. The T_3, T_4 and T_5 groups had higher BWG values compared with those of the other groups. Additionally, the T_3-treated group exhibited the best FCR values, while the different treatment groups produced intermediate FCR values. Regarding the PEF values, compared with the control, all experimental additives were significantly enhanced ($p < 0.05$). The SR (%) and lesions score were enhanced considerably by the dietary treatments at 15–35 days of age (Table 2).

3.2. Carcass Measurements

The effects of supplemented diets on carcass traits after a 35-day feeding trial are presented in Table 3. Regarding the T_6 treatment group, the dressing percentage showed significant ($p < 0.05$) increases. Also, the T_1 and T_3 treatment groups recorded the highest significant thymus values, while the lowest values were recorded for T_4. Meanwhile, the highest significant ($p < 0.05$) spleen weight percentage value was recorded in the T_5 group.

Table 3. Effects of some feed additives on the carcass traits of broilers challenged with *C. perfringens* during the starter and finisher feeding periods.

Measurements	Treatment						*p*-Value	
	T_1	T_2	T_3	T_4	T_5	T_6	SEM	Sig.
DP (%)[10]	65.7 ^{b}	67.4 ^{b}	67.3 ^{b}	67.5 ^{b}	67.4 ^{b}	68.3 ^{a}	0.65	**
Breast (%)[10]	24.8	25.6	24.9	26.2	25.6	25.8	0.94	NS
Leg (%)[10]	19.4	19.5	19.5	18.6	19.5	19.4	0.67	NS
Fat (%)[10]	1.35	1.25	1.37	1.08	1.30	1.45	0.18	NS
Liver (%)[10]	2.58	2.48	2.47	2.82	2.43	2.23	0.14	NS
Bursa (%)[10]	0.17	0.19	0.16	0.15	0.20	0.19	0.02	NS
Spleen (%)[10]	0.11 ^{ab}	0.11 ^{ab}	0.09 ^{b}	0.12 ^{ab}	0.13 ^{a}	0.10 ^{b}	0.09	**
Thymus (%)[10]	0.52 ^{a}	0.49 ^{ab}	0.57 ^{a}	0.42 ^{c}	0.49 ^{ab}	0.45 ^{b}	0.04	*
Gizzard (%)[10]	3.52	2.85	2.90	3.08	3.38	3.10	0.21	NS
Heart (%)[10]	0.53	0.45	0.48	0.60	0.57	0.50	0.04	NS

DP, dressing percentage. SEM, mean values of the standard error. Mean values of three replicates with different letters (^{a,b,c}) in the same row are significantly different ($p < 0.05$). Sig, significance; NS, non-significance; *, significance at $p < 0.05$; **, significance at $p < 0.01$.

3.3. Meat Characteristics

The effects of feed additives on broiler meat quality parameters are presented in Table 4. The T_3 and T_5 treatments resulted in the lowest pHi values, with the highest values observed in T_2. Conversely, the pHu was significantly reduced by the treatments, with the lowest values recorded in T_5 and T_6. Furthermore, the Temperature was significantly reduced in T_6 followed by T_5-treated groups compared with other treated groups. However, no significant differences were demonstrated among the other treatment groups concerning the colour component after slaughter (L15 and b15) and 24 h (L24, a24, and b24) at 35 days of age.

Table 4. Effects of some feed additives on the meat quality of broilers challenged with *C. perfringens* during a feeding trial period.

Measurements	Treatment						*p*-Value	
	T_1	T_2	T_3	T_4	T_5	T_6	SEM	Sig.
pHi	6.58 ^{ab}	6.63 ^{a}	6.52 ^{b}	6.59 ^{ab}	6.55 ^{b}	6.60 ^{ab}	0.03	**
pHu	6.00 ^{b}	6.07 ^{a}	6.05 ^{ab}	6.05 ^{ab}	5.93 ^{c}	5.94 ^{c}	0.02	***
Temperature (°C)	27.82 ^{ab}	28.43 ^{a}	28.98 ^{a}	27.03 ^{ab}	26.10 ^{b}	24.84 ^{c}	0.32	***
L^*_{15}	42.29	45.22	45.56	44.11	44.40	44.38	0.93	NS
a^*_{15}	6.32	4.78	6.09	7.26	5.37	4.97	0.64	NS
b^*_{15}	2.34	2.68	2.68	1.91	2.23	1.73	0.55	NS
L^*_{24}	52.69	52.70	52.63	53.35	53.84	53.60	0.81	NS
a^*_{24}	5.11	5.85	5.57	6.75	5.51	5.76	0.60	NS
b^*_{24}	6.57	5.77	5.58	5.52	7.50	6.50	0.71	NS

pHi, meat pH after slaughter; pHu, meat pH after 24 h; Temp., carcass temperature after slaughter; L15, a15, and b15, colour components after slaughter; and L*24, a*24, and b*24, colour components after 24 h. SEM, mean values of the standard error. Mean values of three replicates with different letters (^{a,b,c}) in the same row are significantly different ($p < 0.05$). Sig, significance; NS, non-significance; **, significance at $p < 0.01$; ***, significance at $p < 0.001$.

The results presented in Table 5 reveal the effects of feed additives on meat characteristics, such as the Cooking Loss (CL), Water Holding Capacity (WHC), Myofiber Fragmentation Index (MFI), Shear Force (SF) and texture profile analysis (TPA) at 35 days of age under a *C. perfringens* challenge test. All treatment groups showed no significant differences concerning the CL, WHC, MFI, and SF. The T_6 and T_5 treatments exhibited significantly increased ($p < 0.001$) hardness and chewiness values compared with those of other treatment groups, respectively. Although the dietary supplementation showed no significant differences in Springiness and Cohesiveness values between all treated groups.

Table 5. Effects of some feed additives on the meat characteristics of broilers challenged with *C. perfringens* during a feeding trial period.

Measurements	Treatment						*p*-Value	
	T_1	T_2	T_3	T_4	T_5	T_6	SEM	Sig.
CL%	25.98	26.85	30.56	29.65	29.38	28.87	1.61	NS
WHC	0.25	0.31	0.28	0.29	0.28	0.28	0.02	NS
MFI	108.43	90.70	91.27	110.55	109.12	110.67	5.14	NS
SF (kg)	1.26	1.54	1.44	1.44	1.40	1.40	0.18	NS
Texture Profile Analysis (TPA)								
Hardness (kg)	1.09 ^{b}	0.69 ^{c}	0.71 ^{bc}	1.21 ^{ab}	1.49 ^{a}	1.51 ^{a}	0.12	***
Springiness	0.58	0.61	0.62	0.59	0.58	0.60	0.02	NS
Cohesiveness	0.53	0.51	0.53	0.52	0.50	0.52	0.01	NS
Chewiness	0.34 ^{b}	0.22 ^{c}	0.24 ^{c}	0.37 ^{ab}	0.43 ^{a}	0.47 ^{a}	0.04	***

CL, cooking loss; WHC, water holding capacity; MFI, Myofiber Fragmentation Index; SF, shear force; and TPA, texture profile analysis. SEM, mean values of the standard error. Mean values of three replicates with different letters (^{a,b,c}) in the same row are significantly different ($p < 0.05$). Sig, significance; NS, non-significance; ***, significance at $p < 0.001$.

4. Discussion

Necrotic enteritis (NE) has become one of the most critical problems in the poultry industry [50]. Feed additives, including antibiotics, prebiotics, and probiotics, have frequently been used for improving the health, growth, and feed efficiency parameters of animals [51]. Recently, interest in incorporating probiotics and antibiotics into broiler treatments has been rapidly increasing [52]. Several studies have shown that the addition of probiotics has positive effects on the growth rate, feed utilization, feed efficiency, and mortality rate [53,54]. However, the efficacy of probiotics depends upon the selection of more efficient strains, manipulation of genes, a combination of several strains, and the combination of probiotics and synergistically=acting components [55]. The use of multi-strain probiotics seems to be the best way of potentiating the efficacy of probiotics, as it beneficially affects the host by improving growth-promoting bacteria with competitive antagonism against pathogenic bacteria in the gastrointestinal tract [56].

4.1. Growth Performance

The performance results obtained in the starter period and the finisher period are presented in Table 2. The IBW, TFI, BWG, and FCR of all groups receiving feed-supplemented diets and basal diets had no significant differences among them. However, the BWG, FBW, PEF, FCR, and SR during the finisher phase, at 15–35 days-old, were significantly ($p < 0.05$) improved in all supplemented diets compared to the control group. These results are similar to those of Khaksefidi and Ghoorchi [57], who found that the BWG of birds fed a diet supplemented with 50 mg/kg of *Bacillus subtilis* was significantly higher during the finisher period (22–42 d) than birds fed the control diets. Additionally, the feed conversion ratio of birds fed a diet supplemented with probiotics significantly reduced from 22 to 42 d compared with birds fed the control diets. Consequently, Patel et al. [55] indicated that the dietary supplementation of combined probiotics and prebiotics (Protexin) at 100 g/ton of feed significantly enhanced the BWG, along with an improved FCR, and benefitted without any adverse effects on the feed intake, mortality, or carcass characteristics. Also, Anjum et al. [58] and Singh et al. [59] observed similar results. The improvement of all performance parameters may be due to the biological role of probiotics in altering the intestinal pH, which modifies both the microbial population and nutrient absorption, ultimately improving the efficiency of feed utilization [60]. Moreover, increased feed intake and digestive enzyme secretions also are detected in animals' fortified phytobiotic-supplemented feed [61]. Growth enhancement through the use of phytobiotics probably depends on the synergistic effects among complex active molecules present in phytobiotics [39,62].

The mortality rate in this study was low (finisher period), representing the positive effect of feed additives on the mortality rate (Table 2). These findings were similar to those found by Abdel–Hafeez et al. [63] and Riad et al. [64], who indicated that the addition of probiotics as feed additives decreased the mortality rate. The reduction in mortality was attributed to the inhibitory effects of these additives toward enteric microorganisms via modifying the intestinal pH [63]. Compared to a wide range of antibiotics (including Avilamycin), a significant decrease in mortality was seen for treated broilers with 2 g/kg Bio–Mannan-oligosaccharides (Bio–MOS) as a source of prebiotic, depending on the age of birds [65,66]. Moreover, there are major modes of action by which broiler performance is improved by proposed oligosaccharide prebiotics, such as control of type-1fimbriae pathogenic bacteria (mannose-sensitive lectin), immune modulation effects, and modulation of intestinal morphology and expression of mucin and brush border enzymes [67].

4.2. Carcass Traits

The mean values for the carcass characteristics, dressing percentage, and organ weight (%) relative to the bodyweight of the broilers are presented in Table 3. The dressing percentage was significantly ($p < 0.05$) increased in the T_6 group compared with those of the other treatments. However, non-significant differences were observed in the other carcass measurements amongst the treatment groups. Some organ weight (spleen and thymus) significantly increased in probiotic supplemented diets alone or in combination with prebiotics compared with other treated groups. Generally, the inclusion of probiotics in broiler rations had no extra additional benefits for the organ weight or carcass yield [55]. Also, Salamkhan et al. [68], Panda et al. [69], and Patel et al. [55] reported that the dressing percentage did not differ significantly ($p \leq 0.05$) between the probiotics-fed broilers and control broilers. To contrast, Banday and Risam [70] and Kabir et al. [71] observed a significant ($p \leq 0.05$) improvement in the dressing percentage in groups supplemented with probiotics. Nevertheless, Sarangi et al. [72] reported a highly significant increment in breast yield in birds supplemented with prebiotics. Jin et al. [73] attributed the lower fat deposition in birds treated with probiotics to how the product could interfere in the availability of fat for lipogenesis in the birds.

4.3. Meat Quality Parameters

The effects of antibiotic-, probiotic-, and phytobiotic-supplemented diets on the measurements of meat characteristics under *C. perfringens* infection in broilers are presented in Table 4. The change in pH is one of the most significant changes that can occur during rigor mortis and can affect meat quality characteristics, such as texture, colour, and WHC [74]. Fletcher et al. [75] reported that post-mortem pH measurements are a good indicator of meat characteristics. Generally, a rapid post-mortem pH decline in breast meat can lead to protein denaturation, which may result in a pale colour and low WHC [76]. During this trial, the temperature and pH values obtained after slaughter and 24 h post-slaughter showed significant ($p \leq 0.05$) enhancements for all supplemented diets compared with the group fed the basal diet. These results are similar to those found by Battula et al. [77] and Corzo et al. [78] where the bacterial challenge had negative impacts on the temperature and pH of the breast muscle.

Additionally, the cooking loses (CL) is a measure of the percentage of water lost during cooking as a result of shrinkage. Consequently, the degree of shrinkage upon cooking is directly correlated with a loss of juiciness. During this trial, the CL, WHC, and SF showed non-significant differences between the control and treatment groups. During a subsequent study, Pelicano et al. [79] examined the effects of *Lactobacillus*- and *Bacillus subtilis*-based probiotics on SF and reported non-significant values compared with the control group. However, the MFI of the breast muscle was influenced by the treatments ($p \leq 0.05$), as shown in Table 4. The MFI decreased in T_2 and T_3 compared with the other treatment groups. Myofibrillar fragmentation is the extent of myofibril destruction caused by homogenization [80]. Olson and Stromer [81] reported that MFI values are correlated with other muscle indices, such as SF and tenderness. Therefore, it can be concluded that probiotic supplementation causes less damage to the myofibrils [76].

TPA is an instrumental measurement of the sensory attributes of chicken breasts which imitates the conditions to which food is subjected to in the mouth [76]. The TPA, which includes hardness, cohesiveness, springiness, and chewiness, is shown in Table 5. The treatments had no significant effects on any TPA variable ($p \leq 0.05$). All tested groups were very tender, except for the T_6 and T_5 supplemented groups, which had hardness values that were significantly ($p \leq 0.001$) higher than those of the other treatment groups. The data presented in this trial aligns with that of Angelovicova et al. [82] who noticed that probiotics (CloStat) moderately affect the hardness, cohesiveness, springiness, and chewiness of cooked breast meat. Also, Lisowski et al. [83] investigated a beneficial effect of the prebiotic on breast muscle weight and carcass percentage. It could be supposed on the one hand, that using prebiotics in broiler diets has a positive influence on muscle weight [84].

5. Conclusions

To conclude, fortified feed diets with 0.2, 0.5, 0.12 g kg^{-1} of *Bacillus subtilis* spores (Gallipro Tect), live *Bacillus* strain (CloStat) and phytobiotic natural compounds (Sangrovit Extra) respectively, alone or in combined form, could promote growth and reduce the NE mortality rates. Additionally, using these probiotic and phytobiotic compounds as an alternative for antibiotics in feed diets could be a useful strategy to ameliorate the harmful effects of *C. perfringens* bacterium on broilers, in terms of the performance, feed efficiency, meat quality and carcass characteristics regarding necrotic enteritis infections.

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Olive Cake Meal and *Bacillus licheniformis* Impacted the Growth Performance, Muscle Fatty Acid Content, and Health Status of Broiler Chickens

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Simple Summary: The extraction of oils from olives usually results in large quantities of olive cake meal (OCM), which has a high nutritional value. The OCM is used successfully in livestock and poultry feeding, but due to the high fiber content, alternative methods of treating OCM must be considered. To increase the efficiency of OCM in broiler chickens' diet, it can be mixed with suitable microorganisms with beneficial effects. Hence, the current study investigated the influence of OCM and *Bacillus licheniformis* (BL) on the growth, nutrient utilization, blood chemistry, and muscle fatty acid profile of broilers. Birds were divided into six experimental groups (control, OCM (2%), OCM (4%), BL, OCM (2%)/BL, and OCM (4%)/BL groups). The results revealed that the inclusion of BL with OCM diets improved the fat utilization and, accordingly, increased the growth, nutrient utilization, and antioxidative response in broilers.

Abstract: Olive cake meal (OCM) is characterized by its high nutritional value and is used as an alternative source of protein and fats in poultry diets. However, due to the high percentage of fiber in OCM, beneficial bacteria cells are used to improve the digestion rates. Therefore, the influence of OCM and *Bacillus licheniformis* (BL) on the growth, nutrient utilization, blood chemistry, and muscle fatty acid profile of broilers was exclusively examined in this study. Three hundred and sixty birds were randomly divided into six experimental groups (6 replicates/10 birds each): Control, OCM (2%), OCM (4%), BL, OCM (2%)/BL, and OCM (4%)/BL groups. Although feed intake was not meaningfully influenced by dietary treatments, weight gain was enhanced and feed conversion ratio was reduced ($p < 0.05$). The abdominal fat was lowered in broilers fed OCM (2%), OCM (4%), OCM (2%)/BL, and OCM (4%)/BL diets without a difference to those fed BL only ($p < 0.05$). Interestingly, blood total protein, albumin, Newcastle disease (ND) titer, and high-density lipoprotein (HDL) cholesterol were significantly increased, while total cholesterol was decreased by the mixture of OCM and BL ($p < 0.05$). Muscle oleic and linoleic acids, as well as vitamin E, increased significantly in broilers fed both OCM (4%) and BL, while linolenic acid increased in all groups except those fed BL and control diets ($p < 0.05$). Liver malondialdehyde (MDA) was decreased by feeding BL or both OCM at 2% or 4% and BL ($p < 0.05$). In conclusion, the inclusion of BL to OCM diets resulted in improved fat utilization and, accordingly, enhanced growth, nutrient utilization, and antioxidative response in broilers. Based on the obtained results, it is recommended to use BL to improve the nutritional value of OCM and to increase the feed utilization of OCM by broilers.

Keywords: broilers; alternative ingredients; probiotics; growth; lipid peroxidation

1. Introduction

As a result of increasing demand, limited supply, and a dramatic increase in the prices of feed ingredients, suitable alternative sources for poultry feed have recently been intensively studied [1,2]. Feed cost may account for more than 70% of the total production costs of broilers [3,4]. Any reduction in feed costs, which still preserves the health status of broilers, is bound to have a direct positive effect on the profitability of poultry production. A considerable effort has been applied to find alternative and sustainable protein sources to be included in broilers diets [5]. In this context, among the available plant protein alternatives, olive cake meal which has high nutritional value (lipids, 13–15%, and proteins, 9–10%), with a high level of non-starch polysaccharides (NSP) (xyloglucan and xylan-xyloglucan complexes) [3,6–8]. The extraction of oils from olives usually results in large quantities of olive cake meal. The olive cake meal is available in several countries around the world at reasonable prices and can be used as a plant ingredient in the feed of broilers [7]. Potential problems in olive cake meal feeding exist due to the existence of fiber and high levels of unsaturated fatty acids, which can cause high fatty acid pre-oxidation, malnutrition, and lower palatability [3]. Olive cake meal was used successfully in poultry diets of up to 10% of the total ration [3,9]. To increase the efficiency of olive cake meal in broilers' diet, it can be mixed with suitable microorganisms to obtain beneficial effects.

A probiotic is defined as "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [10]. Using probiotic-enriched diets is an inexpensive practice that can be adopted by both small- and large-scale farmers, and which can offer several benefits from increasing broilers' growth to increasing immune parameters and disease resistance [11,12]. The use of probiotics has increased due to its remarkable beneficial effects on microbiota and gut health in swine [13], poultry [14], and rabbits [15–17]. In parallel, the significant role of probiotic bacterium on growth, intestinal microbiota, and immunological responses in broilers has been demonstrated [14,18,19]. Indeed, lactic acid bacterial species are unique strains of probiotics authorized by the Food and Drug Administration for administration in animals [10,20]. Furthermore, dietary *Bacillus licheniformis* has been shown to increase growth performance and feed efficiency due to the secretion of digestive enzymes that can increase the digestibility of nutrients in the animal's gut [21–25].

This offers a new topic for researchers, where a combination of the plant by-products and the probiotics mixture is used in poultry feeds. Al-Harthi [3] concluded that there was no adverse effect on the performance of broilers when fed a dietary olive cake meal and *Saccharomyces cerevisiae* blend. Sateri et al. [9] were also able to include up to 8% olive cake meal with a digestive enzyme mixture in the diet of broilers. To date, no data are available about the use of olive cake meal mixed with *B. licheniformis* in the diet of broilers.

With the continued increase in broiler production, it is necessary to find non-traditional alternative ingredients for use in the preparation of feed [2]. Therefore, the objective of the current study was to evaluate the effects of olive cake meal mixed with *B. licheniformis* on the performance parameters, the muscle fatty acid content, and the blood parameters of broilers.

2. Materials and Methods

2.1. Birds and Experimental Design

All of the experimental procedures in this study followed the guidelines set by the Institutional Animal Care and Use Committee at Kafrelsheikh University (Number 4/2016 EC). A total of 360 one-day-old male broiler chickens (45.7 g) were placed inside a room equipped with 36 floor bins (10 birds each) (6 treatments/6 replicates each, stocking density was 10 birds/m^2) with a chain feeder system and automatic nipple cup drinker. The bins were arranged by placing the first replicate of each treatment then the second replicate of each treatment until the sixth replicate of each treatment to follow the completely randomized design (CRD). The first group served as control and was fed basal diets without any additives. The second and third groups were fed diets containing 2% and

4% olive cake meal (OCM; Al-Sabeel Al-Gadidah Company, Tanta, Al-Gharbia, Egypt); the fourth group was fed control diets with probiotic (*Bacillus licheniformis* (BL), Dutch State Mines Company, DSM 17236; the recommended inclusion level was 100 g/ton of feed to achieve a target inclusion of 8×10^{12} colony-forming units (CFU)/kg); the fifth and sixth groups were fed diets containing 2% and 4% OCM with BL. The compositions of the experimental diets are presented in Table 1. *B. licheniformis* is a commercial probiotic product called GalliPro® Tech DSM 17236, and this probiotic strain was isolated from soil and is a non-GMO; the recommended dose, as provided by Interpharma® Company, Egypt, was used. The diets were presented to the birds ad libitum. The photoperiod was maintained as a 21 h light/3 h dark cycle. After the brooding period, the room temperature was kept between 24 and 26 °C, with relative humidity from 50% to 60% throughout the experiment.

Table 1. Composition of the experimental diets *.

Ingredients (g/kg)	Control			OCM (2 %)			OCM (4 %)		
	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher
Yellow corn	532	583	641	512	563	621	492	543	601
Soybean meal (44%)	361	305	230	361	305	230	361	305	230
Gluten (62%)	45	46	59	45	46	59	45	46	59
Soybean oil	22	27	30	22	27	30	22	27	30
Dicalcium phosphate	16	15	15	16	15	15	16	15	15
DL-Methionine	2	1.8	1.2	2	1.8	1.2	2	1.8	1.2
L-Lysine	1.3	1.4	2.4	1.3	1.4	2.4	1.3	1.4	2.4
Threonine	0.5	0.3	0.1	0.5	0.3	0.1	0.5	0.3	0.1
$CaCo_3$	12	12	11	12	12	11	12	12	11
NaCl	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Premix **	3	3	3	3	3	3	3	3	3
$NaHCO_3$	1.5	1.5	1.6	1.5	1.5	1.6	1.5	1.5	1.6
K_2CO_3	0.2	0.5	2.2	0.2	0.5	2.2	0.2	0.5	2.2
Olive cake meal ***	0	0	0	20	20	20	40	40	40
Chemical analysis									
CP (%)	23.04	21.02	19.02	23.07	21.04	19.04	23.1	21.07	19.07
ME (Kcal/kg)	2951	3042	3143	2950	3041	3142	2950	3040	3141
Ca, %	0.93	0.892	0.834	0.93	0.892	0.834	0.93	0.892	0.834
Avp. P (%)	0.428	0.401	0.389	0.428	0.402	0.39	0.428	0.402	0.39
Fiber (%)	3.949	3.659	3.271	4.183	3.893	3.505	4.417	4.127	3.739
Na (%)	0.194	0.194	0.197	0.193	0.194	0.197	0.193	0.193	0.197
Cl (%)	0.252	0.251	0.25	0.251	0.25	0.249	0.25	0.249	0.249

* The basal diet fed to the chicks was formulated to meet the NRC [24] recommendations for broiler chickens; ** Premix (Hero mix® ,Hero pharm, Cairo, Egypt). Composition (per 3 kg): vitamin A 12,000,000 IU, vitamin D3 2,500,000 IU, vitamin E 10,000 mg, vitamin K3 2000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1500 mg, vitamin B12 10 mg, niacin 30,000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid 10,000 mg, manganese 60,000 mg, zinc 50,000 mg, iron 30,000 mg, copper 4000 mg, iodine 300 mg, selenium 100 mg, and cobalt 100 mg). *** Olive cake meal (OCM) analysis (crude protein (CP; 9%), metabolizable energy (ME; 3320 Kcal/kg), Ca; 0.021%, available phosphorus (Avp.; 0.29%), ether extract; 13.7%, fiber; 11.6%).

2.2. Growth Performance and Carcass Parts

Bird body weight was measured individually every week. However, feed intake was measured daily (on a group basis per pen) throughout the experimental period. The feed conversion ratio per bird was calculated. At 35 days, all birds were weighed individually and sorted from the smallest to the heaviest in weight. Then, 36 birds (1 bird per replicate; 6 birds per treatment) were slaughtered and then dissected to measure the weights of the breast muscle, the thigh muscle, the liver, and the abdominal fat.

2.3. Blood Samples and Plasma Biochemical Analysis

At 35 days, blood samples from 36 birds (1 bird per replicate; 6 birds per treatment) were collected from the wing vein immediately before slaughtering, gathered into heparinized test tubes, and then rapidly centrifuged (3000 rpm for 20 min at 5 °C) to separate the plasma. Plasma was stored at −20 °C pending analysis. Plasma total cholesterol, high-density lipoprotein (HDL), glutamic oxalacetic transaminase (GOT), total protein, albumin and globulin, and uric acid were measured calorimetrically by using a commercial chickens' kit (Diamond Diagnostics, Cairo, Egypt), according to the procedure outlined by the manufacturer, using spectrophotometric analysis. Serum antibody titers against Newcastle disease (ND) were determined using the hemagglutination inhibition (HI) test using standard methods qualified in the manual of the World Organisation for Animal Health (OIE) [26].

2.4. Muscle Biochemical Analysis

The analysis of muscle fatty acids was conducted in 36 birds (1 bird per replicate; 6 birds per treatment) from the breast muscle (pectoral superficial muscle) by gas-liquid chromatography (GLC) according to the procedure of Saleh [27]. The concentration of muscle vitamin E and liver malondialdehyde (MDA) was determined according to Ohkawa et al. [28].

2.5. Statistical Analysis

The differences between the treatment groups and the control group were analyzed with a General Linear model using SPSS (version 17.0: SPSS Inc., Chicago, USA). Two-way ANOVA was applied to determine the effects of *B. licheniformis* supplementation (BL), olive cake meal inclusion (OCM), and their interaction (BL × OCM) (2 × 3 factorial design). Duncan's new multiple range tests were used to identify which treatment conditions were significantly different from each other at a significance level of $p < 0.05$.

3. Results

3.1. Growth Performance and Organ Weight

Body weight gain (WG), feed conversion ratio (FCR), and abdominal fat were significantly influenced by OCM, BL, and their interaction ($p < 0.05$) (Table 2). Body WG showed higher ($p < 0.05$) levels in broilers fed OCM (4%), OCM (2%)/BL, and OCM (4%)/BL than those fed the control, while no difference ($p > 0.05$) was observed between the other groups (Table 2). Broilers fed OCM at 2% or 4% with BL showed a reduced FCR (Table 2). The highest WG and the lowest FCR were observed in birds fed both OCM (4%) and BL (Table 2).

The abdominal fat was decreased ($p < 0.05$) in broilers fed OCM (2%), OCM (4%), OCM (2%)/BL, and OCM (4%)/BL diets without a difference to those fed OCM (2%) (Table 2).

Table 2. Effects of feeding *Bacillus licheniformis* (BL) or/and olive cake meal (OCM) on growth performance and organ weights in broilers.

Item	Control	OCM (2%)	OCM (4%)	BL	OCM (2%)/BL	OCM (4%)/BL	*p* Value		
							BL	OCM	BL × OCM
Initial body weight (g)	46 ± 0.57	46 ± 0.41	45.5 ± 0.64	45.5 ± 0.28	45.3 ± 0.48	45.75 ± 0.63	0.061	0.12	0.23
Body weight gain (WG, g/35 day)	2010 ± 27 ^{d}	2052 ± 13 ^{cd}	2090 ± 34 ^{bc}	2071 ± 11 ^{bcd}	2137 ± 32 ^{ab}	2180 ± 15 ^{a}	0.041	0.032	0.002
Feed intake (g/35 day)	3487 ± 50	3428 ± 21	3447 ± 96	3410 ± 15	3455 ± 9 ± 35	3433 ± 29	0.25	0.32	0.21
Feed conversion ratio (g feed/g WG)	1.735 ± 0.01 ^{a}	1.634 ± 0.02 ^{ab}	1.670 ± 0.03 ^{ab}	1.647 ± 0.02 ^{ab}	1.617 ± 0.03 ^{b}	1.575 ± 0.01 ^{b}	0.034	0.041	0.035
Mortality (%)	1.66 ± 0.02	1.66 ± 0.03	1.66 ± 0.02	1.66 ± 0.02	1.66 ± 0.03	1.66 ± 0.03	0.78	0.82	0.72
Organ weight (% body weight)									
Carcass	67.9 ± 1.6	67.3 ± 1.3	68.9 ± 1.4	68.8 ± 1	69.9 ± 161	70.7 ± 1.1	0.12	0.23	0.22
Breast muscle	22.71 ± 0.31	22.37 ± 0.89	22.78 ± 0.89	23.06 ± 0.46	22.82 ± 0.64	23.13 ± 0.88	0.34	0.25	0.31
Thigh muscle	16.36 ± 0.52	16.1 ± 0.48	16.28 ± 0.53	16.45 ± 0.46	16.37 ± 0.16	16.2 ± 0.33	0.32	0.24	0.44
Liver	2.24 ± 0.12	2.32 ± 0.12	2.38 ± 0.06	2.23 ± 0.11	2.35 ± 0.21	2.39 ± 0.14	0.52	0.61	0.34
Abdominal fat	2.08 ± 0.11 ^{a}	1.61 ± 0.22 ^{b}	1.61 ± 0.17 ^{b}	1.89 ± 0.17 ^{ab}	1.47 ± 0.12 ^{b}	1.42 ± 0.06 ^{b}	0.025	0.011	0.033

$^{a–d}$ Means within the same row with different superscripts differ ($p < 0.05$). Results are presented as means ± SEM.

3.2. Biochemical Parameters

Blood total protein, albumin, total cholesterol, HDL cholesterol, and ND titer were significantly influenced by OCM, BL, and their interaction ($p < 0.05$) (Table 3). Blood total protein increased by feeding both BL and OCM at 2% or 4% when compared with those fed the control or BL without a difference to OCM at 2% or 4% ($p > 0.05$) (Table 3). The albumin content increased in the BL and OCM (4%) groups with regard to the control ($p < 0.05$). Interestingly, the ND titer was influenced by OCM, BL, and their mixture ($p < 0.05$). Blood total cholesterol decreased in those fed OCM (4%), OCM (2%)/BL, and OCM (4%)/BL, while HDL cholesterol increased in those fed OCM (2%), OCM (4%), OCM (2%)/BL, and OCM (4%)/BL ($p < 0.05$) (Table 3). However, GOT and uric acid were not affected by the test diets ($p > 0.05$) (Table 3).

Table 3. Effects of feeding *Bacillus licheniformis* (BL) or/and olive cake meal (OCM) on plasma parameters in broilers.

Item	Control	OCM (2%)	OCM (4%)	BL	OCM (2%)/BL	OCM (4%)/BL	*p* Value		
							BL	OCM	BL × OCM
GOT (mg/dL)	346 ± 11	335 ± 9	327 ± 7	320 ± 6	335 ± 10	327 ± 4	0.23	0.51	0.43
Uric acid (mg/dL)	6.97 ± 0.34	6.47 ± 0.23	6.93 ± 0.23	6.5 ± 0.3	6.6 ± 0.33	6.47 ± 0.12	0.33	0.42	0.21
Total protein (mg/dL)	3.35 ± 0.07 ^{b}	3.63 ± 0.19 ^{ab}	3.65 ± 0.1 1^{ab}	3.33 ± 0.11 ^{b}	3.80 ± 0.09 ^{a}	3.85 ± 0.15 ^{a}	0.021	0.032	0.011
Albumin (mg/dL)	1.85 ± 0.06 ^{b}	2.05 ± 0.19 ^{ab}	2.12 ± 0.1 ^{a}	2.11 ± 0.05 ^{a}	2.00 ± 0.07 ^{ab}	2.00 ± 0.04 ^{ab}	0.001	0.021	0.002
ND titer	2.17 ± 0.48 ^{b}	3.67 ± 4.19 ^{a}	3.83 ± 0.31 ^{a}	3.33 ± 0.42 ^{a}	3.17 ± 0.17 ^{a}	3.50 ± 0.22 ^{a}	0.001	0.024	0.0021
Total cholesterol (mg/dL)	154 ± 3 ^{a}	140 ± 2 ^{ab}	139 ± 7 ^{b}	140 ± 5 ^{ab}	137 ± 6 ^{b}	131 ± 2 ^{b}	0.011	0.001	0.023
HDL-cholesterol (mg/dL)	80 ± 3 ^{b}	92 ± 2 ^{a}	93 ± 2 ^{a}	81 ± 3 ^{b}	93 ± 1 ^{a}	95 ± 1 ^{a}	0.025	0.034	0.029

$^{a–b}$ Means within the same row with different superscripts differ ($p < 0.05$). Results are presented as means ± SEM. Glutamic oxalacetic transaminase (GOT), Newcastle disease (ND), and high-density lipoprotein (HDL).

3.3. Muscle Fatty Acid Profiles

Muscle oleic, linoleic, and linolenic acids were influenced by OCM, BL, and their interaction ($p < 0.05$) (Figure 1; Table 4). Muscle oleic and linoleic acids increased significantly in broilers fed both OCM (4%) and BL, while linolenic acid increased in all groups except those fed BL and control diets ($p < 0.05$) (Figure 1). However, arachidonic acid was not affected by the test diets ($p > 0.05$).

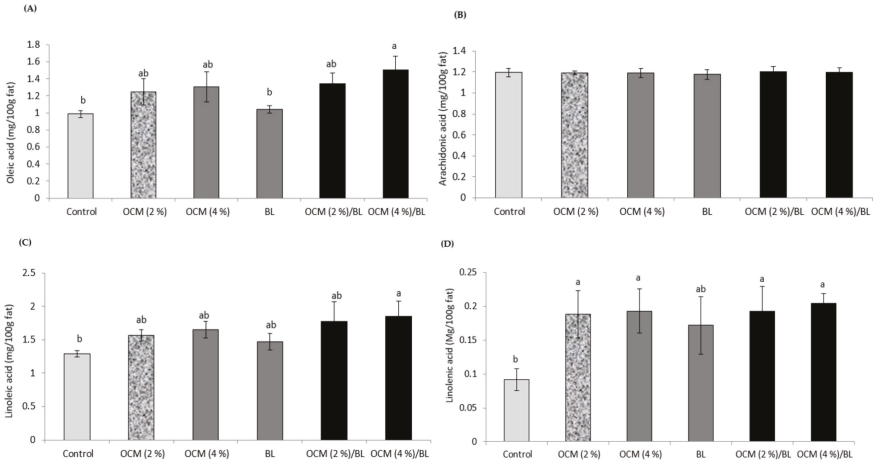

Figure 1. Effects of feeding *Bacillus licheniformis* (BL) or/and olive cake meal (OCM) on muscle fatty acids (oleic acid, arachidonic acid, linoleic acid, and linolenic acid) in broilers.

Table 4. Two-way ANOVA (*p*-value) of the muscle fatty acids (oleic acid, arachidonic acid, linoleic acid, and linolenic acid) in broilers fed *Bacillus licheniformis* (BL) or/and olive cake meal (OCM).

	Two-Way ANOVA (*p*-Value)		
	BL	**OCM**	**BL × OCM**
Oleic acid (mg/100g fat)	0.03	0.01	0.043
Arachidonic acid (mg/100g fat)	0.13	0.24	0.12
Linoleic acid (mg/100g fat)	0.012	0.02	0.031
Linolenic acid (Mg/100g fat)	0.041	0.012	0.042

3.4. Muscle Vitamin E and Liver MDA

Muscle vitamin E and liver MDA were significantly influenced by OCM, BL, and their interaction ($p < 0.05$) (Figure 2; Table 5). Vitamin E was increased by feeding BL or both OCM (4%) and BL ($p < 0.05$) (Figure 2A). Liver MDA was decreased by feeding BL or both OCM at 2% or 4% and BL ($p < 0.05$) (Figure 2B). The highest MDA level was found in broilers fed the control or OCM at 2% ($p < 0.05$) (Figure 2B).

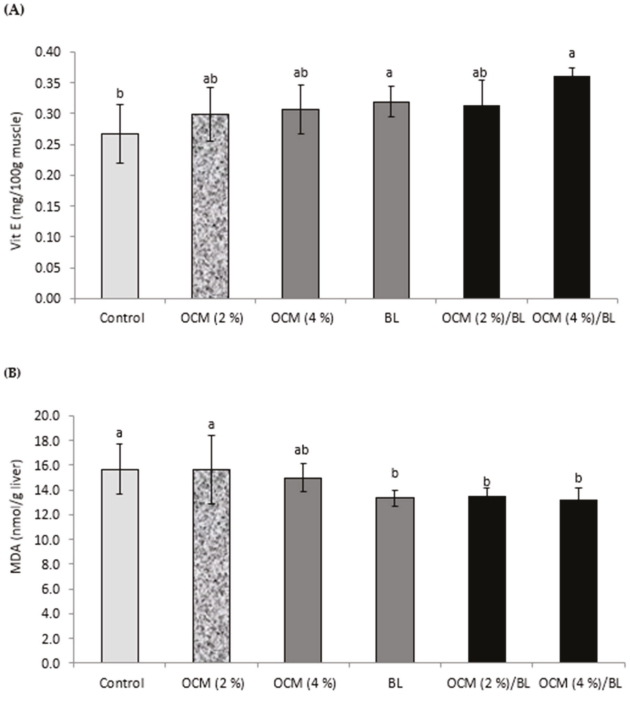

Figure 2. Effects of feeding *Bacillus licheniformis* (BL) or/and olive cake meal (OCM) on muscle vitamin E and liver malondialdehyde (MDA) in broilers.

Table 5. Two-way ANOVA (*p*-value) of the muscle vitamin E and liver MDA in broilers fed *Bacillus licheniformis* (BL) or/and olive cake meal (OCM).

	Two-Way ANOVA (*p*-Value)		
	BL	**OCM**	**BL × OCM**
Vit E (mg/100g muscle)	0.031	0.042	0.012
MDA (nmol/g liver)	0.001	0.032	0.0012

4. Discussion

Most of the recent studies concluded that growth performance was not affected by including up to 10% of OCM in poultry diets [3,6–9]. This study revealed that OCM was successfully included in the diet of broilers at 4%. Moreover, by adding 4% of OCM, broilers obtained better weight gain compared to the control group. The inclusion of up to 4% of OCM in the diet did not impair broiler feed efficiency (FI and FCR). As the above-mentioned level of OCM inclusion would not yet be sufficient for today's scenarios, the intensification of using a blend of the plant by-products has made it necessary to formulate the most cost-effective balanced feed with sound nutrition. This agrees with numerous studies that tested OCM in the diets of broilers [3,6,7,9]. The results showed enhanced growth parameters when broilers were fed OCM at 4%. However, chicks fed a diet with a high level of OCM (4%) with BL showed better growth performance and feed utilization. In the case of a high inclusion level (4%) with BL, the body weight was significantly higher than the other groups, which may result in a reduced feed conversion ratio (FCR). High digestive enzyme activity and feed palatability are also other factors that could increase feed efficiency and, accordingly, the growth of broilers [29]. Zhao et al. [11] reported that low feed intake and a low feed efficiency ratio (increased FCR) resulted from BL supplementation in the broilers' diet. In parallel, a significant role of this

probiotic bacterium on the growth, intestinal microbiota, and immunological responses in chicks has been demonstrated [14,18]. Probiotics may increase the growth and feed efficiency by increasing the secretion of amylase, protease, and lipase, which can increase the digestibility of nutrients in the animal's gut [21–24]. The improvement of growth performance by feeding OCM with BL appears to result from an increase in the feed efficiency of broiler chickens and metabolizable energy (ME) from the diet. The reason for this increase in ME could be due to the digestion of either raw starches or soluble and insoluble non-starch polysaccharide content in OCM, as this probiotic possesses the ability to digest raw starches and to produce cellulase and xylanase, which are required for the digestion of insoluble non-starch polysaccharides [30,31]. In addition, probiotics could improve the nutritional quality of soybean meal because the trypsin inhibitor contained in unprocessed soybean is degraded by BL [32].

The abdominal fat was lowered by including OCM and/or BL in the current study. Similarly, Al-Harthi [3] stated that abdominal fat was decreased by using OCM and/or yeast as probiotics in the broilers' diet. In this study, broilers' feed may elevate the body fat bulk, which may be the reason for the increasing abdominal fat level in broilers fed the control diet in comparison to OCM and/or BL. Probiotics are well known for their function in facilitating the gut absorption of essential nutrients to improve the growth and, accordingly, the general health status, which means reducing the accumulation of nutrients in the gut such as abdominal fat [33].

Blood biochemical indicators relate to some enzymes activity and protein levels in the blood [34] which can reflect the physiological and immunological status of the organism [35]. GOT levels in serum can reflect liver function. When the liver is damaged, the activity will be higher than the normal range [36]. The result of this study revealed that GOT was not influenced by test diets, which indicates the safe function of OCM and/or BL in the broilers' diet. Albumin is the highest protein in serum, which refers to increased antibody and immunity responses [37]. Compared to the control group, after the addition of OCM and/or BL, the changes of biochemical indices and blood routine indices were within the normal range, indicating that OCM and/or BL had no adverse effects on the liver, kidney, and other organs and muscles, as well as on the protein metabolism of broilers. This also proves that the addition of OCM and/or BL had no adverse effect on animal health. Furthermore, dietary OCM and/or BL supplementation in the present investigation elevated the level of total protein and ND titer, which can be attributed to the improved immunity of broilers. Olive oil is a potent immunomodulator that can improve immunity and generate more pathogen resistance [5].

By feeding OCM and/or BL, plasma HDL cholesterol concentration was increased, while plasma total cholesterol was decreased in broilers. Unfortunately, similar investigations concerning the inclusion of OCM and/or BL in broilers' diet are very scarce. Generally, OCM has been reported to decrease the total cholesterol and to increase HDL lipids due to its content of unsaturated and polyunsaturated fatty acids [3,38,39]. Similarly, using OCM in broilers' feed has been shown to result in low levels of total cholesterol [3,40]. The obtained results also revealed that the effect of BL on blood cholesterol levels depends on the level of OCM inclusion. The influence of probiotics in reducing the total cholesterol can be attributed to its role in breaking down the total lipids and bile acids to avoid the re-synthesis of cholesterol [24,33]. Similar results were obtained when chicks fed diets supplemented with different probiotic strains [3,41]. These studies stated that diets containing probiotic bacteria have a negative effect on plasma total cholesterol, but a positive effect on plasma high-density lipoprotein cholesterol (HDL-C) in chickens. Saleh et al. [3,41] reported that the mechanism underlying the cholesterol-lowering effect of probiotics could be due to the inhibition of 3-hydroxyl-3-methylglutaryl-coenzyme (HMG-CoA) reductase. In addition, probiotics might affect fat deposition by influencing the activities of hormone-sensitive lipase and malate dehydrogenase enzyme in adipose tissues [42].

The obtained results also revealed improved ND titer in broilers fed OCM and/or BL, which indicates an improved immunity and, consequently, resistance against infectious diseases. Balanced diets supplemented with reasonable functional feed additives usually can keep high immunity status

and resistance against infectious diseases in broilers [1,2]. Probiotics, on the other hand, are beneficial microorganisms that compete with the harmful bacteria in the animals' gut and provides the host high resistance against infectious diseases [43]. The muscle oleic, linoleic, and linolenic acids as unsaturated fatty acids were increased by OCM and/or BL in the broilers' diet in this study. The increased levels of muscle oleic, linoleic, and linolenic acids can be attributed to the high content of OCM from unsaturated and polyunsaturated fatty acids [38,39]. The increase in oleic, linoleic, and linolenic acids in the muscle is probably due to the intestinal activities of the probiotic. Srianta et al. [44] reported that probiotics produce linolenic acid. Furthermore, BL has the ability to produce desaturase, which changes saturated fatty acids to unsaturated fatty acids [45].

Regarding the muscle vitamin E, feeding OCM and/or BL increased the level of vitamin E in the muscle of broilers, which might be involved in reducing the lipid peroxidation process in broilers and, accordingly, in reducing the oxidation [24]. Oxidative emphasis normally happens when the creation and elimination of free radicals (ROS) are unbalanced, since the oxidative damage of cultured species is directly related to the quality of the diet [46,47]. Superoxide dismutase, glutathione peroxidase, and catalase are important scavengers of ROS, protecting the body tissues from oxidative stress damage [48]. Malondialdehyde (MDA) is a product of lipid peroxides and high levels of ROS, which can cause damage to the cell's DNA, protein, and cytoplasm [49,50]. Interestingly, broilers fed OCM and/or BL showed reduced MDA, confirming that the known antioxidant properties of this probiotic are not lost when administered orally in broilers. Like the current study, earlier reports revealed improved antioxidant response by feeding probiotics [11,51].

5. Conclusions

In conclusion, feeding olive cake meal with *B. licheniformis* improved growth performance, modified plasma lipid, and fatty acid profiles, as well as enhanced the health status of broiler chickens—these factors were probably influenced through improved feed efficiency and antioxidative response. Based on the results obtained, the use of BL to improve the nutritional value of OCM and to increase the feed utilization of OCM by broilers is recommended.

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animals

MDPI

Article

Effect of Dietary Supplementation of Biological Curcumin Nanoparticles on Growth and Carcass Traits, Antioxidant Status, Immunity and Caecal Microbiota of Japanese Quails

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Simple Summary: Nanoparticles such as nano-curcumin are easier to pass through cell membranes in organisms and interact rapidly with biological systems. Therefore, using nano-curcumin is one of the recommendations for improving the bioavailability of curcumin, which would increase its absorption. Thus, this study focused on effects of nano-curcumin levels on the growth, carcass yield, blood chemistry and caecal microbiota of growing quails. From our results, supplemental nano-curcumin had beneficial impacts on growth, lipid profile, antioxidant, immunity of quail, and reduction in pathogenic bacteria.

Abstract: This study was planned to evaluate the impact of different nano-curcumin levels on the growth rate, carcass, blood chemistry and caecal microbes of growing quail. A total of 270 Japanese quails at one-week-old were distributed to six equal groups; each group consisted of 45 unsexed birds with five replications (nine quails each). The 1st group was fed a basal diet, whereas the 2nd, 3rd, 4th, 5th and 6th groups were fed diets containing nano-curcumin (0.1, 0.2, 0.3, 0.4 and 0.5 g/kg diet, respectively). Nano-curcumin levels significantly increased ($p \leq 0.0001$) body weight at 3 weeks and 5 weeks of age. Body weight gain during 1–3, 3–5 and 1–5 weeks of age was significantly increased ($p < 0.0001$) in groups treated with nano-curcumin levels (except at 0.3 g/kg; 1–3 weeks) compared to control. During 1 to 5 weeks, feed intake was decreased ($p < 0.0001$) in birds receiving nano-curcumin (0.1, 0.3 and 0.4 g/kg) diets. The best values of feed conversion ratio were recorded for the 0.4 g nano-curcumin-treated group. Carcass traits were not affected Nano-curcumin levels. The inclusion of nano-curcumin (0.2, 0.3 or 0.5 g/kg) significantly increased serum TP ($p = 0.0004$), albumin ($p = 0.0078$) and globulin ($p < 0.0001$). Quails fed with nano-curcumin (0.2 g/kg) exhibited the highest SOD and GSH activities, serum IgG and IgM concentrations and complement values compared to control. The addition of any level of nano-curcumin in the quail diet also significantly improved the lipid profile. In conclusion, supplemental nano-curcumin had beneficial impacts on growth, lipid profile, blood constituents, antioxidant indices, and immunity of growing quail, as well as increasing counts of lactic acid bacteria and reducing pathogenic bacteria.

Keywords: biological nano-curcumin; growth; diet; immunity; antioxidant; pathogens; quail

1. Introduction

The general trend in the poultry industry is to provide a safer feed, to enhance physiological and productive indicators [1]. The effect of natural products on the capability of nutrients absorption in the gut is a major rationale for recent research. Several investigations have stated that plant derivatives included in poultry feeds deliver useful effects on performance, health, immune response and product quality [2–5]. One of these plant materials is curcumin. Curcumin is the principle active constituent of *Curcuma longa*. Curcumin has long been used in poultry feeds, owing to its favorable effects, including antimicrobial, antioxidant, anti-inflammatory, anti-inflammatory and immunostimulant properties [6,7]. Curcumin shows pharmacological efficacy and safety and contributes to the treatment of several diseases. It also improves the endogenous secretion of digestive enzymes [8] and reduces lipid peroxidation [9].

Curcumin can be used with nanotechnology that potentiates its useful effects. Nanoparticles are easier to pass through cell membranes in organisms and interact rapidly with biological systems [10]. Thus, using nano-curcumin is one of the recommendations for enhancing the bioavailability of curcumin, leading to an increase in its absorption [11]. It has been established that nano-curcumin displays improved bioavailability and distribution in the tissues [12]. The dietary supplementation of nanocurcumin displayed a significantly positive effect on performance [13]. Sayrafi et al. [14] clarified that the declined the liver enzyme activity following supplementation with nanocurcumin may be due to its antioxidant properties. Curcumin has an antioxidant function and adjusts the intestinal microbial composition [15]. Furthermore, Partovi et al. [16] stated that nanocurcumin at a level of 300 mg/kg diet can be used in poultry production as a good feed additive. However, the inclusion of nano-curcumin in quail diets during the growth period is still limited.

Thus, the purpose of the current study was to determine the effects of different nano-curcumin levels on the growth, carcass yield, lipid profile, blood constituents, and antioxidant and immunological indices, as well as the caecal microbiota, of growing quails.

2. Materials and Methods

All experimental procedures of the study were performed according to the Local Experimental Animal Care Committee and approved by the ethics of the institutional committee of Department of Poultry, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.

2.1. Source of Curcumin Nanoparticles

Curcumin nanoparticles in this study were synthesized from *Bacillus subtilis* LA4, which was isolated from soil samples that were collected from different sites next to the plant rhizosphere in Sharqia Governorate, Egypt [17–19]. Under the optimum conditions of temperature, pH, incubation time, and other parameters, curcumin nanoparticles were produced. The characterization of the curcumin nanoparticles using modern devices and technologies was also performed to learn the properties of the curcumin nanoparticles obtained from the *Bacillus subtilis* LA4 bacteria.

2.2. Biosynthesis of Curcumin Nanoparticles

For the biofabrication of curcumin nanoparticles using the tested bacterium, 250 mL conical flasks containing 20 mL of supernatant from bacterial culture were separately mixed with 30 mL of 100 mg (0.27 mM) aqueous solutions of filtered sterilized curcumin, following the method of [20,21] with some modification. Then, the reaction mixture flasks were placed at 160 rpm in a shaker incubator at 30 °C for 72 h to allow the reduction process to occur. Furthermore, a set of flasks containing 20 mL of NB and 30 mL of 0.27 mM curcumin solution were prepared to confirm that the biotransformation of curcumin nanoparticles was only mediated by the use of bacterial cell-free extract [21].

2.3. Antibacterial Activity of Curcumin Nanoparticles

Fresh LB medium was used in all experiments to recover bacteria by sub-culturing. A tiny part from an inoculum of each bacterium was mixed in 5 mL of nutrient broth and kept overnight at 37 °C. The pathogenic bacteria *Staphylococcus aureus* MTTC 1809, *Bacillus subtilis* MTCC 430, *Salmonella enterica* MTCC 1253 and *Pseudomonas aeruginosa* MTCC 741 strains were gained from Egyptian Microbial Culture Collection, Microbiological Resource Center (The Cairo MIRCEN: Ain Shams University, Cairo, Egypt), cultured on a nutrient agar plate, and kept in the NA slants at 4 °C. Overnight cultures in the nutrient broth were used for the laboratory studies. The antibacterial activity of curcumin nanoparticles was estimated using the disc diffusion method [22], which was presented by the National Committee for Clinical Laboratory Standards (NCCLS). The zone of inhibition was measured after a day of incubation at 30 °C or 37 °C. Bactericidal effects of curcumin nanoparticles were detected using a modified version of the method shown by NCCLS. The diluted bacterial culture (0.1 mL) was extended on the sterile NA plate. Dried discs of 6 mm diameter of Whatman filter paper No. 1 that had been previously soaked in curcumin nanoparticles were placed on the seeded plates against Gram-negative and Gram-positive bacteria [23–25]. The estimation of the MIC was obtained through the determination of the turbidity of the bacterial growth after a day of incubation. The inhibited concentration was 99% of bacterial growth, which was considered as the MIC estimate [24,26]. According to the standard method, the MBC values of the particles were measured, and the MBC estimate was determined through sub-culturing the MIC dilutions onto sterile Muller-Hinton agar plates incubated at 37 °C for one day.

2.4. Experimental Design and Diets

The study was carried out at the Poultry Research Farm, Department of Poultry, Faculty of Agriculture, Zagazig University, Egypt. At one week of age, we used 270 Japanese quails with an average body weight of 26.1 ± 0.08 g. Quail chicks were haphazardly distributed across six equal groups, each group consisting of 45 unsexed birds with five replications (nine birds each). Quails were reared in common type cage (90 × 40 × 40 cm) under the same conditions with 23 h light:1 h dark. Feed and water were opened throughout the experiment (five weeks). Birds received feeds in mash form according to their treatment. The dietary treatments were as follows: the 1st group was fed a basal diet without any supplementation (0 g/kg diet), whereas the 2nd, 3rd, 4th, 5th, and 6th groups were fed diets supplemented with 0.1, 0.2, 0.3, 0.4 and 0.5 g/kg of nano-curcumin, respectively. The basal diet was based on corn-soybean meal and contained 24% CP, 12.53 MJ/kg, 0.8 Ca, and 0.45 P, according to NRC [27].

2.5. Growth Performance and Carcass Measurements

All growth parameters [body weight (BW), body weight gain (BWG) feed intake (FI) and feed conversion ratio (FCR = g feed/g gain)] were measured at 1, 3 and 5 weeks of age. At 5-weeks-old, 24 quails were used for carcass examinations. All edible parts (gizzard, liver, heart, and carcass) were weighed and expressed as a % of the live BW before slaughter.

2.6. Microbiological Analysis

We collected the samples (~10 g) from the cecum content (five samples per each treatment) and separately transported them to a 250 mL Erlenmeyer flask containing 90 mL of sterile peptone (0.1% peptone) saline solution (0.85% NaCl) and blended the mixture well. The total bacterial count (TBC), total yeasts and molds count (TYMC), *Enterococci*, lactic acid bacteria count, Coliform, *E. coli* and *Salmonella* were recorded according to [28,29].

2.7. Blood Chemistry

After slaughter by sharp knife to complete bleeding, we collected the blood samples from 24 quails in sterilized tubes. We used the centrifuge (Janetzki, T32c, 5000 rpm, Wall-hausen, Germany) at 2000× *g* 15 min to separate the plasma. Using commercial kits from Biodiagnostic Company (Giza, Egypt),

we determined the level of albumin (ALB), total protein (TP), globulin (GLOB), A/G ratio, and the activity of alanine transaminase (ALT), lactate dehydrogenase (LDH), aspartate transaminase (AST), urea, creatinine, total cholesterol (TC), triglycerides (TG), very low-density lipoprotein (VLDL), high-density lipoprotein (HDL), and low-density lipoprotein (LDL). The levels of immunological parameters (IgG) and M (IgM) as well as complement (C3) were determined using kits from Spectrum Company (Cairo, Egypt). For the antioxidant parameters, using commercial kits and a spectrophotometer (Shimadzu, Japan), the content of reduced glutathione (GSH) and malondialdehyde (MDA), and the activity of superoxide dismutase (SOD) were determined in quail plasma.

2.8. Statistics

All of the statistical analyses were carried out using the SAS software (SAS Institute Inc., Cary, NC, USA). Data regarding growth, carcass, blood chemistry and microbiology traits were analyzed with one-way ANOVA using the post-hoc Tukey's test ($p < 0.05$).

3. Results

The antibacterial activity of synthesized curcumin nanoparticles was tested against *Staphylococcus aureus* MTTC 1809, *Bacillus subtilis* MTCC 430, *Salmonella enterica* MTCC 1253 and *Pseudomonas aeruginosa* MTCC 741. The antibacterial activity against G+ and G- bacteria at different concentrations of curcumin nanoparticles was performed using the disc diffusion method. The bacterial growth culture at 24 h (0.1 mL) was spread aseptically onto nutrient agar plates. Discs (6 mm) which were impregnated with one of each curcumin nanoparticle concentration were dispensed with a sufficient separation from each other so as to avoid the overlapping of the inhibition zones. The diameters of the inhibition zone around each disc were estimated. They showed good antimicrobial activity against all the tested bacteria, though the effect of curcumin nanoparticles was found to be more pronounced with *Staphylococcus aureus* MTTC 1809 and *Bacillus subtilis* MTCC 430 (Table 1) compared to other bacteria. The data in Table 1 show the susceptibility of four bacterial strains to five concentrations of curcumin nanoparticles, namely 100, 200, 300, 400 and 500 μg/mL. The data confirmed that, with increasing concentrations of curcumin nanoparticles, halo diffusion increased regardless of the bacterial strains tested—giving a maximum diameter of 31 mm for *Staphylococcus aureus* MTTC 1809 when 500 μg/mL was used. From the obtained results, it was also observed that curcumin nanoparticles display more efficient as antibacterial activity compared to normal curcumin. The MIC values were 90, 100, 200 and 220 μg/mL, respectively, when *Staphylococcus aureus* MTTC 1809, *Bacillus subtilis* MTCC 430, *Salmonella enterica* MTCC 1253 and *Pseudomonas aeruginosa* MTCC 741 were used. The MBC rates were 180 μg/mL, 200 μg/mL, 400 μg/mL and 440 μg/mL, respectively, for the same pathological bacterial isolates mentioned earlier (Table 2).

Table 1. Zone of inhibition produced by curcumin and curcumin nanoparticles.

Items	Curcumin (100 μg/mL)	Curcumin nanoparticles (μg/mL)					SEM	*p* Value
		100	200	300	400	500		
Staphylococcus aureus MTTC 1809	13.33 ^{e}	19.33 ^{d}	21.67 ^{d}	25.67 ^{c}	28.33 ^{b}	31.00 ^{a}	0.831	<0.0001
Bacillus subtilis MTCC 430	14.67 ^{e}	19.00 ^{d}	20.67 ^{d}	23.33 ^{c}	26.33 ^{b}	28.67 ^{a}	0.612	<0.0001
Salmonella enterica MTCC 1253	12.33 ^{e}	15.67 ^{d}	18.67 ^{c}	21.33 ^{b}	23.67 ^{ab}	26.00 ^{a}	0.793	<0.0001
Pseudomonas aeruginosa MTCC 741	11.33 ^{d}	13.67 ^{d}	17.33 ^{c}	19.67 ^{bc}	21.67 ^{ab}	23.33 ^{a}	0.882	<0.0001

$^{a–e}$: Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different ($p < 0.05$).

Table 2. The MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of the curcumin nanoparticles.

Pathogenic Bacteria	Curcumin Nanoparticles	
	MIC µg/mL	MBC µg/mL
Staphylococcus aureus MTTC 1809	90	180
Bacillus subtilis MTCC 430	100	200
Salmonella enterica MTCC 1253	200	400
Pseudomonas aeruginosa MTCC 741	220	440

3.1. Growth Performance and Carcass Yield

Results for growth performance are shown in Table 3. It was found that the nano-curcumin levels significantly increased ($p < 0.0001$) body weight at 3 weeks and 5 weeks of age. It was reported that the diet enriched with nano-curcumin at levels of 0.2 or 0.4 g/kg resulted in the best body weight. Body weight gain during all periods (1–3, 3–5 and 1–5 weeks of age) was increased ($p < 0.0001$) in the groups treated with nano-curcumin levels, except the BWG of the group fed nano-curcumin (0.3 g/kg), which did not significantly differ from the control between 1 and 3 weeks of age. Between 1 and 3 weeks, the FI was significantly lower ($p < 0.0001$) with the supplementation of nano-curcumin (0.3 g/kg) than in all other groups, while the highest FI was with the birds fed on a diet containing nano-curcumin (0.2 g/kg). During the period of weeks 3–5, quails fed 0.1, 0.2, 0.3 and 0.4 g nano-curcumin-treated diets consumed less feed ($p < 0.0001$) than the others. Between 1 and 5 weeks, the FI was decreased ($p < 0.0001$) in the birds that received nano-curcumin (0.1, 0.3 and 0.4 g/kg) diets compared with that of the control and other groups. In all periods, the quails fed nano-curcumin had better FCR ($p < 0.0001$) than the control quails, except those quails fed nano-curcumin (0.5 g/kg) did not significantly differ from the control between 3 and 5 weeks of age. Generally, the best value in FCR was recorded for the 0.4 g nano-curcumin-treated group. As shown in Table 4, the carcass traits of Japanese quail were not affected by variation in nano-curcumin levels.

Table 3. Growth performance of Japanese quail as affected by dietary nanocurcumin.

Items	Nano-curcumin Level (g/kg Diet)						SEM	*p* Value
	0	0.1	0.2	0.3	0.4	0.5		
Body Weight (g)								
1 week	26.00	26.15	26.10	26.22	26.15	26.17	0.075	0.5406
3 weeks	90.33 ^{c}	96.84 ^{b}	100.23 ^{ab}	90.87 ^{c}	102.20 ^{a}	98.45 ^{ab}	1.157	0.0001
5 weeks	173.08 ^{d}	181.63 ^{bc}	189.14 ^{a}	178.13 ^{c}	191.19 ^{a}	182.68 ^{b}	1.091	<0.0001
Body Weight Gain (g/day)								
1–3 weeks	4.59 ^{c}	5.05 ^{b}	5.29 ^{ab}	4.62 ^{c}	5.43 ^{a}	5.16 ^{ab}	0.083	0.0002
3–5 weeks	5.91 ^{d}	6.06 ^{c}	6.35 ^{a}	6.23 ^{b}	6.36 ^{a}	6.02 ^{c}	0.026	<0.0001
1–5 weeks	5.25 ^{d}	5.55 ^{bc}	5.82 ^{a}	5.43 ^{c}	5.89 ^{a}	5.59 ^{b}	0.039	<0.0001
Feed Intake (g/day)								
1–3 weeks	14.21 ^{bc}	13.91 ^{c}	15.42 ^{a}	13.22 ^{d}	13.76 ^{cd}	14.54 ^{b}	0.152	<0.0001
3–5 weeks	22.96 ^{a}	21.65 ^{b}	22.04 ^{b}	19.74 ^{c}	19.97 ^{c}	23.10 ^{a}	0.227	<0.0001
1–5 weeks	18.59 ^{a}	17.78 ^{b}	18.73 ^{a}	16.48 ^{c}	16.86 ^{c}	18.82 ^{a}	0.153	<0.0001
Feed Conversion Ratio (g/g)								
1–3 weeks	3.09 ^{a}	2.75 ^{d}	2.91 ^{b}	2.86 ^{bc}	2.53 ^{e}	2.82 ^{cd}	0.020	<0.0001
3–5 weeks	3.88 ^{a}	3.58 ^{b}	3.47 ^{b}	3.17 ^{c}	3.14 ^{c}	3.84 ^{a}	0.046	<0.0001
1–5 weeks	3.54 ^{a}	3.20 ^{c}	3.22 ^{c}	3.04 ^{d}	2.86 ^{e}	3.37 ^{b}	0.021	<0.0001

$^{a–d}$: Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different ($p < 0.05$).

Table 4. Carcass traits and relative organs of growing Japanese quail as affected by dietary nano-curcumin.

Items	Nano-curcumin Level (g/kg Diet)						SEM	*p* Value
	0	0.1	0.2	0.3	0.4	0.5		
Carcass %	76.83	76.28	78.21	76.53	75.52	75.45	1.561	0.8558
Liver %	2.23	2.48	2.12	2.54	2.73	2.74	0.180	0.2600
Gizzard %	2.63	2.52	2.74	2.64	2.44	2.37	0.204	0.8648
Heart %	0.98	1.17	1.12	1.08	1.05	0.94	0.100	0.6537
Giblets %	5.85	6.17	5.98	6.27	6.23	6.04	0.276	0.9506
Dressing %	82.67	82.45	84.19	82.8	81.75	81.5	1.705	0.9170

3.2. Blood Chemistry

The effects of dietary nano-curcumin on the liver and kidney function of quail are shown in Table 5. The inclusion of nano-curcumin (0.2, 0.3 or 0.5 g/kg) increased serum TP ($p = 0.0004$) and globulin ($p < 0.0001$) compared to the control and other groups. The group fed nano-curcumin (0.3 g/kg) had the highest serum albumin level ($p = 0.0078$). The A/G ratio in the group fed 0.2, 0.3 and 0.5 g nano-curcumin/kg diet was lower than that in the control and other groups ($p < 0.0001$).

Table 5. Liver and kidney function of growing Japanese quail as affected by dietary nano-curcumin.

Items	Nano-curcumin Level (g/kg Diet)						SEM	*p* Value
	0	0.1	0.2	0.3	0.4	0.5		
TP † (g/dL)	3.13 ^{b}	3.07 ^{b}	3.75 ^{a}	3.70 ^{a}	3.31 ^{b}	3.61 ^{a}	0.074	0.0004
ALB ‡ (g/dL)	1.24 ^{bc}	1.23 ^{bc}	1.14 ^{c}	1.36 ^{a}	1.31 ^{ab}	1.24 ^{bc}	0.028	0.0078
GLOB § (g/dL)	1.89 ^{c}	1.84 ^{c}	2.62 ^{a}	2.35 ^{b}	2.00 ^{c}	2.38 ^{b}	0.049	<0.0001
A/G ¶ (%)	0.65 ^{a}	0.67 ^{a}	0.44 ^{d}	0.58 ^{b}	0.66 ^{a}	0.52 ^{c}	0.015	<0.0001
AST †† (IU/L)	181.20 ^{a}	168.30 ^{b}	169.87 ^{ab}	164.20 ^{b}	161.15 ^{b}	180.70 ^{a}	3.107	0.0116
ALT ‡‡ (IU/L)	14.64 ^{b}	13.90 ^{b}	14.50 ^{b}	9.57 ^{c}	12.85 ^{b}	16.63 ^{a}	0.532	<0.0001
LDH * (IU/L)	196.40 ^{a}	157.85 ^{b}	165.60 ^{b}	190.07 ^{a}	167.80 ^{b}	206.70 ^{a}	5.159	0.0002
Creatinine (mg/dL)	0.34	0.31	0.33	0.34	0.36	0.37	0.017	0.3588
Urea (mg/dL)	6.82	6.55	6.84	7.22	7.07	7.09	0.128	0.0970

$^{a–d}$: Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different ($p < 0.05$). † TP: total protein; ‡ Alb: albumin § GLOB: globulin; ¶ A/G: albumin/globulin ratio; †† AST: aspartate aminotransferase and ‡‡ ALT: alanine aminotransferase. * LDH: lactate dehydrogenase.

The AST activity in the serum decreased ($p = 0.0116$) with the addition of dietary nano-curcumin (0.1, 0.3 or 0.4 g/kg) to the feed. Furthermore, the serum ALT activity of the birds fed nano-curcumin (0.3 g/kg) was lower ($p < 0.0001$) than of those in the control and the other groups. The LDH values of the birds fed rations enriched with nano-curcumin (0.1, 0.2 or 0.4 g/kg) were lower ($p = 0.0105$) than of those in the other groups. There was no significant difference in the serum urea and creatinine values between birds supplemented with nano-curcumin at all levels and the control group.

The response of quails to dietary nano-curcumin levels on the lipid profile is presented in Table 6. The addition of various levels of nano-curcumin in the quail feed significantly decreased the TC and LDL in the serum ($p < 0.0001$) compared to the control. The highest values of HDL ($p = 0.0308$) were recorded with the group fed a diet containing nano-curcumin (0.4 g/kg). The TG and VLDL values were significantly decreased ($p < 0.0001$) with the addition of nano-curcumin (0.2, 0.4 and 0.5 g/kg) compared with the control and other groups, but the highest values were recorded for the 0.1 g/kg level of nano-curcumin.

Table 6. Lipid profile of growing Japanese quail as affected by dietary nano-curcumin.

Items	Nano-curcumin Level (g/kg Diet)						SEM	*p* Value
	0	0.1	0.2	0.3	0.4	0.5		
TC † (mg/dL)	259.15 ^{a}	176.75 ^{cd}	195.45 ^{b}	182.95 ^{c}	172.00 ^{cd}	167.17 ^{d}	3.155	<0.0001
TG ‡ (mg/dL)	218.15 ^{b}	296.98 ^{a}	137.21 ^{d}	208.48 ^{b}	186.25 ^{c}	169.83 ^{c}	5.167	<0.0001
HDL § (mg/dL)	44.56 ^{b}	49.01 ^{ab}	51.15 ^{ab}	43.64 ^{b}	56.51 ^{a}	51.87 ^{ab}	2.188	0.0308
LDL ¶ (mg/dL)	170.97 ^{a}	68.35 ^{d}	116.86 ^{b}	97.61 ^{c}	78.24 ^{d}	81.33 ^{d}	3.960	<0.0001
VLDL¶¶ (mg/dL)	43.63 ^{b}	59.40 ^{a}	27.44 ^{d}	41.70 ^{b}	37.25 ^{c}	33.97 ^{c}	1.033	<0.0001

$^{a–d}$: Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different ($p < 0.05$). † TC: total cholesterol; ‡ TG: triglycerides; § HDL: high density lipoprotein; ¶ LDL: low density lipoprotein; ¶¶ LDL: very low density lipoprotein.

The results of the antioxidant and immunity indices are presented in Table 7. Quails fed with nano-curcumin (0.2 g/kg) exhibited the highest SOD and GSH activities. However, MDA concentrations of serum were decreased by the addition of dietary nano-curcumin levels. In Table 7, serum IgG concentrations were increased by nano-curcumin (0.1, 0.2 and 0.5 g/kg) supplementation compared to those of the control. However, serum IgM concentrations were also increased by nano-curcumin (0.1, 0.2, 0.3 and 0.4 g/kg) supplementation compared to those of the control. The values of complement 3 were significantly augmented in the group fed diets containing nano-curcumin (0.2, 0.3, 0.4 and 0.5 g/kg).

Table 7. Antioxidant and immunological indices of growing Japanese quail as affected by dietary nano-curcumin.

Items	Nano-curcumin Level (g/kg Diet)						SEM	*p* Value
	0	0.1	0.2	0.3	0.4	0.5		
SOD † (U/mL)	0.23 ^{b}	0.22 ^{b}	0.37 ^{a}	0.25 ^{b}	0.24 ^{b}	0.24 ^{b}	0.010	<0.0001
MDA ‡ (nmol/mL)	0.17 ^{a}	0.19 ^{a}	0.10 ^{c}	0.11 ^{c}	0.14 ^{b}	0.12 ^{c}	0.006	<0.0001
GSH ¶ (ng/mL)	0.25 ^{b}	0.23 ^{b}	0.39 ^{a}	0.26 ^{b}	0.25 ^{b}	0.28 ^{b}	0.012	0.0002
IgG §§ (mg/dl)	1.00 ^{c}	1.30 ^{ab}	1.45 ^{a}	1.15 b ^{c}	1.05 ^{c}	1.32 a^{b}	0.061	0.0047
IgM §§ (mg/dl)	0.65 ^{b}	1.01 ^{a}	1.09 ^{a}	1.01 ^{a}	1.08 ^{a}	0.54 ^{b}	0.059	0.0002
C3 ǂǂ (mg/dl)	128.00 ^{e}	138.50 ^{e}	177.50 ^{d}	199.50 ^{c}	311.50 ^{b}	405.00 ^{a}	3.657	<0.0001

$^{a–d}$: Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different ($p < 0.05$). † SOD: superoxide dismutase; ‡ MDA: malondialdehyde; § TAC: total antioxidant capacity; ¶ GSH: reduced glutathione; ǂǂ GPX: glutathione peroxidase; ǂǂ C3: complement 3; §§ IgG: immunoglobulin G.

3.3. Microbiological Aspects

Table 8 presents the effect of nano-curcumin on the caecal microbiota of quail. The significant reduction in TBC, TYMC and *Enterobacter* in the caecal microbiota of quail was observed following the supplementation of nano-curcumin levels. The coliform count in the caecal microbiota of quail was significantly decreased in those groups fed a diet containing nano-curcumin (0.1, 0.4 and 0.5 g/kg) compared to in the other groups. Furthermore, the supplementation of nano-curcumin (0.2, 0.4 and 0.5 g/kg) led to a reduction in the caecal *E. coli* count compared to the control and other groups. Quails fed diets supplemented with nano-curcumin (0.2, 0.3 and 0.4 g/kg) exhibited higher lactic acid bacteria colonization than those in the control and other groups. The *Salmonella* counts in the caecal microbiota of quails were significantly decreased in all of the groups fed diets containing nano-curcumin. Finally, the best caecal microbiota were observed in the groups fed nano-curcumin at levels of 0.2 and 0.4 g/kg.

Table 8. Caecal microbiota of growing Japanese quail as affected by dietary nano-curcumin.

Items	Nano-curcumin Level (g/kg Diet)						SEM	*p* Value
	0	0.1	0.2	0.3	0.4	0.5		
Microbiological Count (Log CFU/g)								
TBC	6.07 ^{a}	5.13 ^{d}	6.00 ^{b}	5.30 ^{c}	6.03 ^{b}	5.14 ^{d}	0.009	<0.0001
TYMC	5.88 ^{a}	4.82 ^{d}	5.74 ^{b}	5.78 ^{b}	5.12 ^{c}	4.88 ^{d}	0.025	<0.0001
Coliform	5.98 ^{a}	5.01 ^{c}	5.94 ^{a}	5.94 ^{a}	5.23 ^{b}	5.05 ^{c}	0.019	<0.0001
E. coli	5.94 ^{a}	5.86 ^{a}	4.96 ^{c}	5.90 ^{a}	5.20 ^{b}	5.01 ^{c}	0.025	<0.0001
Lactic acid bacteria	5.24 ^{c}	5.04 ^{d}	5.91 ^{b}	5.97 ^{b}	6.03 ^{a}	5.08 ^{d}	0.016	<0.0001
Enterobacter spp.	5.92 ^{a}	4.91 ^{d}	5.82 ^{b}	5.83 ^{b}	5.16 ^{c}	4.94 ^{d}	0.023	<0.0001
Salmonella spp.	6.46 ^{a}	3.60 ^{b}	2.13 ^{c}	1.36 ^{d}	0.98 ^{e}	0.26 ^{f}	0.067	<0.0001

$^{a–d}$: Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different ($p < 0.05$); TBC: Total bacterial count; TYMC: total yeast and molds count.

4. Discussion

Concerning the antimicrobial activity of curcumin nanoparticles against tested pathogenic bacteria, it was found [24] that when studying the effect of curcumin nanoparticles on four bacterial isolates, Gram-positive bacteria are more affected than Gram-negative bacteria. Furthermore, it was reported [24] that the antibacterial activity of nanocurcumin against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* demonstrated a broad-spectrum inhibitory effect against all microorganisms. The MICs of nano-curcumin for *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* were 100, 75, 250, and 200 µg/mL, respectively.

The trend of using nano-curcumin in poultry feed has recently been discussed and may be a possible approach to enhance the physiological and productive performance, and the health status, of poultry. The enhancement of curcumin bioavailability using nanotechnology techniques can increase its absorption [30], in turn boosting the poultry performance and health. Nanocurcumin can be used as a safe and natural feed additive to increase nutritional value [16]. Supplemental nanocurcumin displayed a positive impact on BW and FCR, which is in accordance with previous studies [13,31], which validated the affirmative effect of curcumin on the growth performance of birds. As for the present findings, it was indicated [32] that the addition of 10 mg of nano-encapsulated curcumin/kg diet improved the FCR of quail. It was also illustrated [33] that the addition of nano-curcumin in the drinking water of broiler chicks improves the body growth and FCR. Curcumin, when used as a functional molecule, can act as a growth promoter in poultry, and as a strong natural antioxidant in the improvement of performance [34]. Additionally, using curcumin in the diet encourages the secretion of bile acids and stimulates the proteases lipase, amylase trypsin and chymotrypsin enzymes [35]. The favorable impacts of curcumin on the growth of broilers might be due to boosted secretions of these enzymes. The improvement in the growth of birds fed diets containing curcumin may be due to improvements in the intestinal morphology of the birds [13]. Furthermore, this positive effect of curcumin might be attributed to its well-reported antibacterial, antioxidant and anti-inflammatory effects [36]. Finally, nanocurcumin can be used in the poultry industry as a potential, promising feed additive.

The results of the current study on the carcass yield of quail were in agreement with previous articles that studied the effects of turmeric or its extract on carcass traits, which were not affected. In Durrani et al. [31], higher breast and thigh weights, and a higher dressing percentage, were noticed in broilers fed a diet enriched with 5 g turmeric powder/kg compared with the control. A previous study [37] observed no improvement in the gizzard or liver following the application of *Curcuma longa* in the diet. Furthermore, it was stated [38] that the addition of turmeric rhizome extract (TRE) (100–300 mg/kg) had no significant influence on the dressing %. Moreover, it was found [39] that the gizzard and dressing % were not significantly influenced by dietary turmeric treatments.

The levels of blood ALT and AST reflect the health status of the liver. In the present study, these enzymes were significantly decreased following the addition of dietary nano-curcumin. Looking at previous research [14], it was clarified that supplementation of nanocurcumin (200 mg/kg) declined the serum AST enzyme level, and they attributed this reduction to the antioxidant properties of nano-curcumin. Furthermore, one study [40] reported that chickens fed a diet containing curcuma powder (5 g/kg) had the highest level of LDH, implying that curcuma might have a positive impact on liver enzymes. Moreover, it was highlighted [41] that a nano-curcumin level of 400 mg/kg diet displayed affirmative and consistent influences on the serum biochemical parameters.

Serum TG, TC, HDL and LDL concentrations are viewed as diagnostic markers in lipid metabolism. The present results indicated that the addition of nano-curcumin levels in the quail feed significantly decreased the lipid profile. In agreement with previous studies [42,43] it was stated that curcumin reduced the serum LDL cholesterol and triglycerides levels and improved the liver function. Dietary curcumin lessened blood cholesterol levels and encouraged the digestion of fat [44,45]. Research [40] has revealed that the dietary supplementation of turmeric in broiler chickens significantly diminished LDL-cholesterol and augmented HDL-cholesterol, but did not affect triglyceride levels. Moreover, it was indicated [43,46] that curcumin caused a reduction in TC, perhaps due to the inhibition of enzyme hepatic 3-hydroxyl-3-methyglutaryl CoA-reductase (HMGCR) activity, which is responsible for the production of TC in the hepatic tissues [47]. Furthermore, curcumin may decline the activity of the enzymes that act as rate-limiting enzymes in lipogenesis, such as acetyl-CoA carboxylase (the rate-limiting enzyme in fatty acids synthesis) [48].

Antioxidant ability is the key to the health and growth of poultry. Curcumin, the major antioxidative molecule of curcuma longa, is a powerful damper of oxygen species [49]. The results of the current study show that the groups fed with nano-curcumin exhibited high SOD and GSH activities. As shown by the present findings, nano-curcumin possesses a better antioxidant and biological activity than curcumin [50]. The antioxidant ability against peroxyl radicals was augmented in the nanocurcumin-supplemented group compared to the control [32]. The curcumin can alleviate the oxidative stress by modifying the hepatic nuclear transcription factors and decreasing lipid peroxidation in the muscle and serum of quail [51]. Curcumin helps to maintain the antioxidant status of the cells through suppressing oxidative enzymes, scavenging free radicals and prompting de novo glutathione synthesis [52]. Zhai et al. [15] illustrated that curcumin compounds could reduce the oxidative injury and disruption of lipid metabolism through modifying the cecum microbiota of ducks. The dietary supplementation of turmeric rhizome extract augmented the enzymatic activities of SOD and GSH-PX, and reduced the malondialdehyde concentration [38]. As a result, dietary curcumin reduces the production of reactive free radicals, leading to an increase in the antioxidant metabolites concentration in the poultry body. The inclusion of curcumin in the diet decreased the malondialdehyde concentration and boosted the activities of CAT, T-AOC, SOD, and GSH-Px compared to the control group [53]. Thus, curcumin can alleviate the negative impact of any stressful environmental condition.

Curcumin has been found to have numerous pharmacological activities, including antimicrobial, anti-inflammatory, antifungal, antiviral and antioxidant activities [54]. The highest values of immunoglobulins in the present study were obtained from birds fed a diet containing nano-curcumin. Similar results have been observed in other studies, which pointed out that a diet with a nano-curcumin level of 400 mg/kg displayed the best immune response compared to the control group [41]. Emadi et al. [55] showed that the immunologlobulins (IgA, IgM and IgG) of chickens were significantly increased by dietary turmeric. Furthermore, the turmeric plant has been proven to be a powerful immunomodulatory factor that can improve the activation of B and T cells, neutrophils and macrophages cells [56]. Thus, it can be said that nano-curcumin can be an appropriate alternative to synthetic antioxidants, perhaps due to the improvement of the antioxidant metabolites of birds, which may boost the bird's immunity.

The present study found that the supplementation of nano-curcumin in quail diets reduced harmful bacteria and boosted useful bacteria. Curcumin, the main bioactive component of turmeric,

was found to possess antibacterial activities [57]. Curcumin could modify the gut microbial balance, improving the intestinal integrity [58]. El-Rayes et al. [39] illustrated that the dietary supplementation of turmeric as source of curcumin led to an increase in counts of lactic acid bacteria and a reduction in pathogenic bacteria (*S. aureus*, *E. coli* and total coliform bacteria) compared to in the control group. Gupta et al. [59] described that the extracts of *C. longa* inhibited the growth of pathogenic bacteria. Curcumin had an inhibitory effect against many pathogenic bacteria and decreased the population of harmful gut bacteria [60].

5. Conclusions

The findings of our study demonstrated the positive effects of dietary nano-curcumin supplementation on the growth, lipid profile, blood constituents, immunity and antioxidant indices of quails. The best values in feed efficiency were achieved when quails were fed with 0.4 g nano-curcumin in their diets. Furthermore, the best values in immune response and antioxidant indices were observed in the 0.2 g nano-curcumin-treated group.

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Green Tea and Pomegranate Extract Administered During Critical Moments of the Production Cycle Improves Blood Antiradical Activity and Alters Cecal Microbial Ecology of Broiler Chickens

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Simple Summary: Since the European Union's (EU) antibiotic ban in 2006, interest in natural feed additives has largely increased. Natural feed additives are used to prevent diseases and promote growth in chickens, supporting animal health and modulating the development of the gut microflora during stressful situations. In the present study, a bioactive compound from plants belonging to the class of phytobiotics was assessed for its effects on production performance, antiradical activity and gut microflora in broiler chickens. The obtained results show how the tested compound is able to exert beneficial effects on the antiradical activity and gut microbial ecology of birds, even though the chickens' performance was unaffected.

Abstract: Phytobiotics are usually tested in feed and throughout the production cycle. However, it could be beneficial to evaluate their effects when administered only during critical moments, such as changes in feeding phases. The aim of the trial was to investigate the effect of a commercial plant extract (PE; IQV-10-P01, InQpharm Animal Health, Kuala Lumpur, Malaysia) on growth performance, blood antiradical activity and cecal microbiome when administered in drinking water to broiler chickens during the post-hatching phase and at each change of diet. In the experiment, 480 1-day-old male broiler chicks were assigned to two groups in a 50-day trial. Broilers received drinking water (C) or drinking water plus PE (T) at a rate of 2 mL/L on days 0 to 4, 10–11 and 20–21. PE did not affect performance and water intake, while total antiradical activity was improved ($p < 0.05$). A greater abundance of lactic acid bacteria (false discovery rate (FDR) < 0.05) was found in the T group and the result was confirmed at a lower taxonomic level with higher Lactobacillaceae abundance (FDR < 0.05). Our findings suggest that PE administration during critical moments of the production cycle of broiler chickens may exert beneficial effects at a systemic level and on gut microbial ecology.

Keywords: broiler chickens; phytobiotics; green tea; pomegranate; drinking water; antiradical activity; cecal microbiota

1. Introduction

In 2006, the European Union banned the use of antimicrobial growth promoters in animal nutrition [1]. This decision led to the result that antimicrobials, other than coccidiostats and histomonostats, were no longer allowed as feed additives [2]. As such, antibiotic alternatives

designed to maintain productivity and health became the focus of much research [3,4]. At the present moment, different molecules, compounds, bioactive substances, and active principles have been investigated and are still under investigation [5]. Among them, several classes of feed additives are now available, including probiotics [6,7], prebiotics [8,9], organic acids [10,11], and phytobiotics [12]. Although the benefits of such additives have been proven in most cases, there is still a lack of clarity on their effects, as evidenced by some contrasting results in different trials.

Considering the available feed additive classes, the use of phytobiotics in poultry nutrition could represents a valuable tool [13]. Phytobiotics, also known as botanicals, are plant-derived products that are a natural source of bioactive compounds [14]. Supplementation with phytobiotics for broiler chickens has shown beneficial effects on animal production and the quality of animal-derived products [13]. However, their mechanism of action remains to be elucidated, and different hypotheses have been proposed, in which the antioxidant properties seem to play a major role [15]. Phytobiotics in fact are rich in polyphenolic compounds, which can support the antioxidative capacity by counteracting the harmful effects of free radicals generated during stressful situations, finally resulting in improved general health and better performance of the animals [16].

Phytobiotics were also found to be able to modulate gut microflora [14,17] and its development, which plays an important role in production performance and overall health [18]. It is indeed recognized that the first microbial population colonizing the gut could impact an animal's entire life span [19]. In this view, the chance to modulate the gut microflora in chickens via a nutritional approach is of particular interest, especially during critical moments of their life, such as the post-hatching phase.

Among phytobiotics, green tea and pomegranate extracts have been shown to improve broiler productivity and antioxidant status [20–22], as well as modulate the intestinal microflora [20,23]. Green tea (*Camelia sinensis*) has been widely studied in humans and animals due to its numerous biofunctional properties, including antioxidant, antiviral and anticoccidial activity [24,25]. Most of these properties are ascribed to the high levels of polyphenolic compounds, among which catechins are the most represented group [26]. Similarly, pomegranate (*Punica granatum*) also possesses biofunctional properties, such as antioxidant and anti-inflammatory properties, antimicrobial activity and anticancerogenic effects [27]. A recent in vitro study by Jain et al. [28] showed that the simultaneous use of different plant extracts, including green tea and pomegranate, led to a synergistic enhancement of antioxidant activity. However, combined administration of green tea and pomegranate has not yet been tested in animal nutrition. Green tea and pomegranate have also been demonstrated to affect the intestinal microbiota [23,25], promoting beneficial bacteria in the intestinal tract [29,30].

Until now, the majority of in vivo studies in poultry have investigated the effects of administering phytobiotics in the feed and for the entire rearing period, while the effect of their inclusion in drinking water was scarcely investigated. Phytobiotics supplementation in drinking water might represent a valuable way to perform targeted interventions, limited to the critical moments of the production cycle (e.g., limited number of days during post-hatching phase and transitions between feeding phases). This route of administration could sustain the health of animals when it needs to be supported and boosted, rather than being used for the whole rearing period through the feed. This could then be turned into a smaller amount of phytobiotics used per rearing cycle, with economic advantages in terms of production cost.

To the best of our knowledge, at the present moment no literature is available on the addition of pomegranate to drinking water for poultry. Only two studies considered the effects of including green tea in drinking water [31,32], and neither of these accounted for treatment only during specific critical moments of the rearing cycle. In both these trials, in fact, tea supplementation was performed consecutively from 3 to 10 weeks of age [32] or for a total of 42 days [31]. Kaneko et al. [32] outlined linearly reduced growth performance with an increasing concentration of tea extract, while Rowghani et al. [31] reported improved growth performance following supplementation with 3 mL/L of green tea extract.

The aim of the trial was to evaluate the effect of including a commercial plant extract based on green tea leaves and pomegranate rinds in drinking water on the growth performance, antiradical activity and cecal microbial ecology of broiler chickens during specific critical moments of the rearing cycle.

2. Materials and Methods

2.1. Animals and Housing

The trial was performed at the Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy), using 1-day-old male broiler chicks (ROSS 308) obtained from a commercial hatchery (Avicola Alimentare Monteverde, Rovato, BS, Italy). At hatching, all chickens were vaccinated against Marek's disease, Newcastle disease, infectious bronchitis, and coccidiosis. The chickens were housed in floor pens (2.9 m^2) on new shavings of white wood in two identical climate-controlled rooms. Water and feed were provided ad libitum. Room temperature was 35 °C for the first 3 days, then decreased weekly by 2 °C to a final temperature of 21 °C at the end of the trial. The study period lasted from the day of hatch until day 50. All procedures were reviewed and approved by the Animal Care and Use Committee of the University of Milan (OPBA_92_2016).

2.2. Experimental Design

A total of 480 1-day-old ROSS 308 male broiler chickens were randomly allocated to two experimental groups of 12 pens each at a stocking density of 20 birds/pen. Each experimental room housed six randomly distributed pens per treatment, in order to reduce any environmental effects.

All animals received the same diets (Table 1) formulated to meet the nutrient requirements established by the National Research Council (NRC, 1994).

Table 1. Feed ingredients and nutrient composition of basal diets (as-fed basis).

Ingredients (g/kg feed)	Starter Diet	Grower Diet	Finisher Diet
	0–10 days	11–20 days	21–50 days
Corn	550.5	574.0	616.7
Soybean meal (48% crude protein)	373.0	341.0	292.0
Soybean oil	30.0	43.0	53.0
Dicalcium phosphate	25.0	25.0	21.0
Calcium carbonate	7.0	4.5	5.0
Mineral + vitamin premix †	5.0	5.0	5.0
Sodium chloride (NaCl)	4.0	4.0	4.0
$_{DL}$-Methionine ($_{DL}$-Met)	3.2	1.8	1.6
$_{L}$-Lysine-HCl ($_{L}$-Lys-HCl)	2.3	1.7	1.7
Nutrient values of diets, analysed (g/kg)			
Dry matter (g)	877.7	878.2	878.0
Crude protein (g)	229.7	215.1	195.0
Ether extract (g)	56.3	69.4	79.8
Ash (g)	68.2	64.04	58.6
Calcium (Ca; g)	10.0	9.1	8.1
Phosphorus (P; g)	8.7	8.5	7.6
Nutrient values of diets, calculated (g/kg)			
Metabolizable energy (kcal/kg)	3002.5	3099.9	3200.1
Lysine (Lys)	10.0	8.3	7.6
Metionine + cysteine (Met + Cys)	6.4	4.9	4.4

† Provided the following per kg of diet: vitamin A, 11,250 IU; vitamin D_3, 5000 IU; vitamin E, 60 mg; $MnSO_4 \cdot 1H_2O$, 308 mg; $ZnSO_4 \cdot 1H_2O$, 246 mg; $FeSO_4 \cdot 1H_2O$, 136 mg; $CuSO_4 \cdot 5H_2O$, 39 mg; KI, 2.4 mg; Na_2SeO_3, 657 µg; 6-phytase EC 3.1.3.26, 750 FTU; endo-1, 4-beta-xylanase EC 3.2.1.8, 2250 U.

Diets were provided by Agricom International (Pognano, BG, Italy) according to a three-phase feeding program, in crumbled form for starter and grower phases (0–10 and 11–20 days, respectively),

and pelleted form for finisher phase (21–50 days). All experimental diets were formulated and manufactured using the same lots of ingredients and without antibiotics or coccidiostats. Collected feed samples were analysed before the beginning of the trial to determine the content of dry matter (method 930.15), crude protein (method 984.13), ether extract (method 920.39A), ash (method 942.05), Ca (method 968.08), and P (method 946.06) following the relevant Association of Official Analytical Chemists methods of analysis [33].

Experimental treatments consisted of including (treated, T) or not including (control, C) a plant extract (PE) in the drinking water at a dosage of 2 mL/L. Treated birds received PE from 0 to 4 days of the trial and on days 10, 11, 20, and 21, corresponding to the beginning of the trial and the start of the second and third feeding phases. PE was included in one graduated tank for each pen to determine water intake during the treatment period.

The PE was composed of green tea leaves (*Camellia sinensis*) and pomegranate rinds (*Punica granatum*) (IQV-10-P01, InQpharm Animal Health, Kuala Lumpur, Malaysia). During the entire trial, water was provided ad libitum via automatic nipple cup drinker, except during the three treatment periods, when it was provided in graduated plastic tanks placed in each pen. During the trial, growth performance was evaluated at the beginning, at each feed change and at the end of the experiment. On day 50, one representative broiler chicken from each pen was selected and sacrificed; dressing percentage was calculated and blood and cecal content were collected for total antiradical activity assay and gene sequencing, respectively.

2.3. Growth Performance and Water Intake

Body weight (BW) and feed intake (FI) of the broilers were determined on a pen basis at 0, 10, 20, and 50 days of age. Mortality was recorded daily together with the BW of dead birds to calculate mortality percentage and correct productive performance results. Water intake was determined on a pen basis during PE administration on days 0–4, 10–11 and 20–21 as the difference between offered and residual water. At the end of the trial, one representative animal was selected from each pen based on pen average BW and sacrificed.

Dressing percentage was calculated by dividing eviscerated weight by live weight. Breast muscle was then removed and weighed, and breast muscle yield was calculated as percentage of eviscerated weight.

2.4. Total Antiradical Activity

Blood samples were collected from sacrificed broiler chickens on day 50 in 10 mL vacutainer tubes containing EDTA (Venoject®, Terumo Europe NV, Leuven, Belgium) and stored at 4 °C for determination of total blood antiradical activity. Blood samples were processed within 3 h of sampling and analysed in the next 24 h after collection by a Kit Radicaux Libres biological test (KRL, Laboratories Spiral, Dijon, France) following the user protocol. The results were expressed as time (in minutes) required to achieve 50% of maximal haemolysis (half-haemolysis time, HT_{50}), which references whole blood and red blood cell (RBC) resistance to a standardized free-radical attack generated from the thermal decomposition of a 27 mmol/L solution of 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) at 37 °C [34–36].

2.5. Cecal Microbiota

Cecal contents were collected from sacrificed broiler chickens to perform 16S rRNA gene sequencing. Cecal contents were removed and placed into a sterile tube (Sarstedt, Nümbrecht, Germany), snap-frozen in liquid nitrogen and stored at –80 °C. Bacterial DNA was isolated from cecal contents using the Exgene™ Stool DNA mini kit (Geneall Biotechnology Co., Ltd., South Korea) starting with 200 µg samples following the manufacturer's procedure. The extracted DNA was quantified using Synergy HTX (Biotek, Winooski, VT, USA) with a final concentration ranging from 3–10 ng/uL. Variable regions V3–V4 of the 16S rRNA were amplified by Polymerase Chain Reaction (PCR) with universal

primers for prokaryotes [37]. Amplicon sequencing was carried out on an Illumina MiSeq 300PE platform to obtain raw paired-end reads 2 × 300 bp (BMR Genomics, Padova, Italy). The 16S sequencing data were processed and analysed using CLC Genomics Workbench version 12.0 and CLC Microbial Genomics Module version 4.1 (CLC bio, Arhus, Denmark). The paired-end reads were merged into one high-quality representative by default settings of CLC Workbench (mismatch cost = 1, minimum score = 40, gap cost = 4, maximum unaligned end mismatches = 5). The CLC pipeline was used for primer and quality trimming (trim using quality scores = 0.05; trim ambiguous nucleotides: maximum number of ambiguities = 2; discard reads below length = 5). The SILVA reference database [38] was used for sequence alignment, and sequences were binned into operational taxonomic units (OTUs) based on 97% similarity. The OTU table was further filtered by removing OTUs with low abundance (minimum combined abundance = 10), to get a final abundance table for each sample. The phylogenetic tree was constructed using a maximum likelihood phylogeny tool based on multiple sequence alignment of the OTU sequences (100 most abundant OTUs) generated by the multiple sequence comparison by log-expectation (MUSCLE) tool [39] in the workbench. The maximum likelihood phylogeny tool determines the probability of sequences in the tree, using neighbour joining as the construction method and the Jukes–Cantor model as a nucleotide substitution model. The OTU table was used to calculate alpha diversity indices such as Chao1 and Shannon's indices.

2.6. Statistical Analysis

A completely randomized design was used. Growth performance was analysed using Statistical Analysis System software (SAS version 9.4; SAS Institute Inc., Cary, NC, USA) applying a MIXED procedure for repeated measurements and accounting for the effects of treatment, time and treatment × time interaction. Total weight gain (TWG), total feed intake (TFI), total feed conversion ratio (TFCR), water intake, carcass characteristics, and KRL measurements were analysed using one-way analysis of variance (ANOVA) to compare the means of the two groups using the GLM procedure of SAS. Mortality rate was analysed by PROC FREQ of SAS over the trial period.

The pen represented the experimental unit for growth performance parameters, while the broiler represented the experimental unit for carcass characteristics and KRL measurements. All numerical data in tables are presented as least-square means (LSMeans) accompanied by standard error of the mean (SEM) values. Differences between groups were considered statistically significant at $p < 0.05$, whereas a trend for a treatment effect was noted for $0.05 < p < 0.10$.

To determine diversity shared among communities in the cecal microbiome of the samples, beta diversity (both weighted and unweighted UniFrac) was calculated in the CLC Workbench (CLC bio, Aarhus, Denmark) and significance was measured by permutational multivariate analysis of variance (PERMANOVA). MicrobiomeAnalyst tool [40] was used for further relevant statistical analysis. During the analysis, the OTUs that did not meet the following parameters were removed: minimum number of counts 1, 5% prevalence in the sample and 1% of samples below the standard deviation. Log transformation was used as a normalization method for downstream analysis, which also includes differential abundant analysis at different taxon levels, performed by the metagenomeSeq package (v3.10, Bioconductor) [41]. Differentially abundant taxa were determined at a false discovery rate (FDR) < 0.05.

3. Results

3.1. Growth Performance, Water Intake, Carcass Characteristics and Total Antiradical Activity

Body weight, weight gain, FI, and FCR are shown in Table 2. The administration of PE in drinking water did not affect growing performance of treated broilers during the different rearing phases ($p > 0.05$). In the same way, no significant differences were seen for mortality rate, dressing or breast percentage. Pen water intake was not influenced by the treatment in the first 4 days of hatching

(C: 3.42 L vs. T: 3.36 L; $p = 0.84$), and on days 10–11 (C: 5.75 L vs. T: 7.72; $p = 0.86$) and 20–21 (C: 11.53 L vs. T: 11.75 L; $p = 0.44$) of the trial.

Table 2. Effects of plant extract supplementation on growth performance parameters and carcass characteristics of broilers. Data shown as LSMeans ± SEM.

Item	Groups		SEM	*p*-Value		
	C	T		Treatment	Time	Treatment × Time
No. Birds/Pen	**20**	**20**				
BW (kg/pen) [1]						
0 day	0.883	0.872	0.842	0.469	<0.001	0.638
10 day	6.195	6.215				
20 day	18.312	18.332				
50 day	74.892	73.106				
Gain (kg/pen) [1]						
0–10 days	5.312	5.342	1.075	0.445	<0.001	0.533
11–20 days	12.117	12.117				
21–49 days	56.580	54.774				
TWG	74.008	72.233	1.667	0.460		
FI (kg/pen) [1]						
0–10 days	6.393	6.343	0.819	0.276	<0.001	0.294
11–20 days	18.102	18.158				
21–49 days	122.808	120.863				
TFI	147.302	145.363	1.257	0.287		
FCR (kg/kg) [1]						
0–10 days	1.20	1.19	0.035	0.721	<0.001	0.689
11–20 days	1.50	1.50				
21–49 days	2.18	2.23				
TFCR	2.00	2.02	0.036	0.613		
Mortality (%)	3.33	5.83		0.190		
Carcass characteristics						
No. birds [2]	12	12				
Dressing (%)	75.59	76.83	0.56	0.133		
Breast (%)	21.41	22.41	0.66	0.293		

Note: $p < 0.05$ considered significantly different, $0.05 < p < 0.1$ considered tendency. SEM: standard error of the mean; BW: body weight; TWG: total weight gain; FI: feed intake; TFI: total feed intake; FCR: feed conversion ratio; TFCR: total feed conversion rate. C: animals receiving no supplementation; T: animals receiving 2 mL/L green tea and pomegranate extract in drinking water at days 0–4, 10–11 and 20–21. [1] Corrected for mortality; mortality and BW of dead birds were recorded daily to calculate mortality percentage and correct productive performance results. [2] One representative animal from each pen was selected based on pen average BW.

The effects of PE on total antiradical activity are shown in Table 3. Including PE in drinking water during critical moments of the broiler's rearing cycle significantly improved the total antiradical activity, in both whole blood (HT_{50} blood, $p < 0.01$) and RBCs (HT_{50} RBC, $p < 0.05$).

Table 3. Effects of plant extract supplementation on total antioxidant activity. Data shown as LSMeans ± SEM.

	Groups		SEM	*p*-Value
Item	**C**	**T**		
No. birds [1]	12	12		
HT_{50} whole blood, min.	69.17	76.52	4.91	<0.001
HT_{50} RBC, min.	56.72	61.28	3.45	0.023

Note: $p < 0.05$ considered significantly different. HT_{50}: time (minutes) required to achieve 50% of maximal haemolysis; RBC: red blood cell. C: animals receiving no supplementation; T: animals receiving 2 mL/L green tea and pomegranate extract in drinking water at days 0–4, 10–11 and 20–21. [1] One representative animal from each pen was selected based on pen average BW; blood samples were obtained at slaughter.

3.2. Cecal Microbiota

Sequencing of amplicons resulting from the amplification product of PCR for variable regions V3–V4 of the 16S rRNA by PCR was performed to investigate the treatment effect on cecal microbiome. Details of sequence read and OTU counts are provided in the supporting materials (Figure S1).

No statistical differences ($p > 0.05$) were seen in alpha diversity measured by bias-corrected Chao1 and Shannon's indices between C and T groups. Similarly, for beta diversity, no statistical differences ($p > 0.05$) were observed in PERMANOVA (unweighted and weighted UniFrac) between the experimental groups.

Relative abundance at different taxon levels (phylum, order, class) is shown in Figure 1. Firmicutes was found to be the most abundant phylum in both experimental groups, accounting for 69.47% in the C group and 68.65% in the T group. Bacteroidetes emerged as the second most abundant phylum, with 20.94% in the C group and 25.55% in the T group. Proteobacteria were the third phylum, with 8.49% in the C group and 4.84% in the T group. At the class level, Clostridia was the most abundant taxon in both experimental groups, followed by Bacteroidia, Gammaproteobacteria and Bacilli (Figure 1).

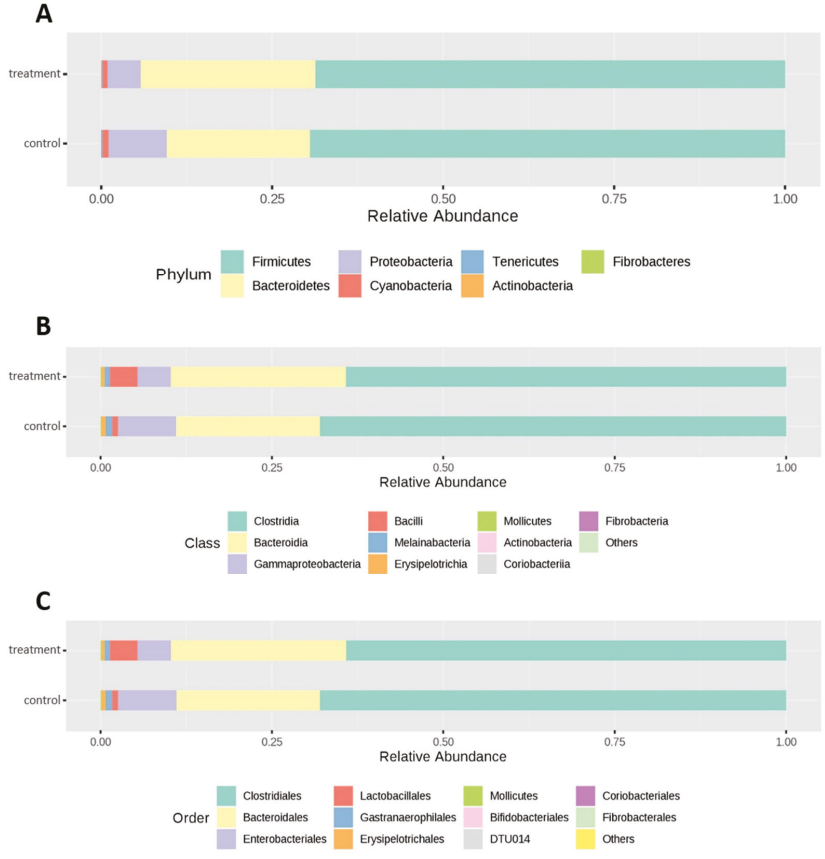

Figure 1. Relative abundance in control and treated groups at different taxon levels: (**A**) phylum, (**B**) class and (**C**) order. Classes and orders with counts <10 are merged and reported as "others".

Differential abundant analysis was performed to find the significantly different (FDR < 0.05) taxon between the two groups (C and T). No significant differences were found at the phylum level. At the class level, Bacilli were significantly higher in the T group with respect to the C group.

Similarly, at the order level, Lactobacillales showed significantly (FDR < 0.05) greater abundance in T animals compared to C animals. At the family level, Lactobacillaceae and Peptococcaceae were significantly more abundant in the T group compared to the C group (FDR < 0.05). Clostridiaceae_1 tended (FDR = 0.06) towards higher abundance in the T group compared to the C group. At the genus level, *Roseburia* was found to be significantly higher in the T group compared to the C group (FDR < 0.05). On the contrary, *Shuttleworthia* was found to be significantly (FDR = 0.04) higher in the C group. *Lactobacillus_ambiguous_taxa*, *Christensenellaceae_R7_ambiguous_taxa* and *Tyzzerella_3* tended (FDR = 0.06) to be higher in the T group compared to the C group. A list of significantly differentially abundant taxa based on *p*-value (<0.05) is given in Supplementary Table S1.

4. Discussion

Recently, phytobiotics gained increasing attention as a replacement for antimicrobial growth promoters to enhance growth performance and improve animal health [42,43]. The positive effects of phytobiotics have been associated with high polyphenolic content, which can counteract the effect of free radical generation [15], and their ability to modulate gut microflora composition, leading to increased performance [14,22].

In the present study, the lack of positive results as expected might be due to the administration route, the dosage applied, or the duration of supplementation. Generally, supplementation of poultry with green tea and pomegranate extracts was shown to improve broiler productivity [20–22]. However, nearly all studies reporting positive effects on growth performance administered the compounds in the feed and for the entire rearing period. To the best of our knowledge, only two studies investigated the single administration of green tea extract to broiler chickens in drinking water, while no data are available on pomegranate or combined supplementation. Rowghani et al. [31] outlined improved growth performance after administration of green tea extract in drinking water at a rate of 3 mL/L, while Kaneko et al. [32] observed a linear reduction of body weight and feed intake with increased concentration of Japanese tea from 6.25 g/L to 25 g/L. In our trial, the lower dosage of green tea and pomegranate mixture was chosen on the basis of the synergistic activity previously evidenced between green tea and other plants, including pomegranate that was able to enhance antioxidant activity in vitro [28]. Finally, the administration of PE for only a few days rather than the total length of the trial could have contributed to the lack of expected results. This is in contrast with the results we obtained in a previous study on post-weaning piglets, with a similar experimental design, which led to an increase in average daily weight gain during the last week of the experimental period [44].

Besides these aspects, a large body of literature highlights the high variability of the efficacy of phytobiotics in improving animal performance and carcass characteristics. This can be explained by the different biological potential of the phytobiotics tested, accounting for the extraction procedure, the part of the plant used, the geographic origin, and the harvest season [45]. According to our findings, Farahat et al. [46] observed no effect on carcass characteristics with different amounts of green tea extract in feed, while Erener et al. [47] and Hamady et al. [20] reported improved carcass characteristics following the administration of green tea and pomegranate extract, respectively.

The administration of PE significantly increased the total antiradical activity of whole blood and RBCs, confirming the beneficial effect of PE in improving antioxidant defences of animals. This result can be attributed to the high polyphenol content of both green tea and pomegranate extract, which is able to prevent reactive oxygen species (ROS) generation and the damage they induce. The proposed mechanism of action for polyphenols is that after being absorbed in the gut, they are bound by blood cells, mainly erythrocytes, leading to enhanced total antioxidant-scavenging capacity of the blood [48]. The antioxidant effects of PE were recently confirmed by Rao et al. [49], who observed reduced lipid peroxidation and increased glutathione peroxidase activity after supplementation with pomegranate peel meal in broiler chickens. Similarly, including green tea extract in the poultry diet increased the glutathione-reduced level in the liver and significantly decreased the malondialdehyde level of meat tissue [46].

The development of intestinal microbiota in poultry plays an important role in production performance and overall health [18], and phytobiotics, including green tea and pomegranate, have been proven to be effective in its modulation [20,23]. It is recognized that colonization of the gut microbiota in critical moments of life could have an impact on an animal's entire life span [19]. Among the critical moments, the post-hatching phase is one of the most important, since it is when the first gut colonization occurs [50]. Several studies have shown that early gut microflora modulation can affect health and productivity in later stages of a broiler's life [36,51]. The post-hatching phase, however, is not the only critical moment in defining the gut microbiota composition. The microbial population can also be affected by changes in the diet, with regard to the feed form or its chemical composition [52]. To the best of our knowledge, the present study is the first to investigate the effects of a targeted intervention with phytobiotics at critical moments of the production cycle. Our results show that the administration of PE during the post-hatching phase and changes in the feeding phase did not impact the cecal microbiota composition, keeping the microbial profile in line with the diet used in general practice. The gut microbial population observed in this study was indeed aligned with what was reported by Wei and colleagues [53]. In this review, the authors described the cecal microbial composition of adult birds, reporting Firmicutes as the most abundant phylum, followed by Bacteroidetes and Proteobacteria.

Although the microbial profile was not different between the two experimental groups, relative abundance differences were noted at different taxonomic levels (class, order and family), suggesting a beneficial modulation of gut microflora by PE. In accordance with our findings, Saeed et al. [29] observed higher relative abundance of Bacilli in the ileum and jejunum of broiler chickens following supplementation with L-theanine, an amino acid extracted from green tea [29]. In our study, animals receiving PE showed greater relative abundance of lactic acid bacteria compared to the control group. This result was confirmed at the family level, where Lactobacillaceae and Enterococcaceae were found to be more abundant in T broilers. Also, at the genus level, *Lactobacillus* showed a tendency to be higher in the T group. These findings are of particular interest because lactic acid bacteria are recognized for their beneficial effect in the intestine, regulating the composition of intestinal microflora, developing intestinal immunity and promoting gut health [54]. Lactobacilli can indeed protect against the colonization of pathogenic bacteria through the acidification of the lumen and the production of bacteriocins [55,56].

Besides modulating lactic acid bacteria, PE supplementation also determined some differences at the genus level. *Roseburia_ambiguous_taxa* was found to be significantly higher in animals receiving PE. *Roseburia* genus is a commensal saccharolytic bacteria that produces SCFAs and has been proposed in human medicine as probiotic for restoration of beneficial flora [57]. In addition, a lower abundance of *Shuttleworthia* was observed in the T group. Information about this genus is limited, but a study reported that enrichment of *Shuttleworthia* in the ceca of male broiler chickens was associated with high body weight [58], which was not evidenced in our study.

5. Conclusions

The administration of PE in drinking water during the post-hatching phase and at changes between feeding phases can improve total blood antiradical activity and may positively affect the gut microbial ecology of adult broiler chickens by increasing the relative abundance of lactic acid bacteria, with no effect on performance parameters.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/5/785/s1. Figure S1: (A) Mean number of 16S rRNA sequence reads and (B) OTU counts detected in cecal samples of broilers in treated (T) and control (C) groups. Table S1: Significantly different taxa according to p-value (≤ 0.05) shown by differential abundance analysis between the two experimental groups.

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Associations Between *IGF1, IGFBP2* and *TGFß3* Genes Polymorphisms and Growth Performance of Broiler Chicken Lines

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Simple Summary: The main goal of breeding programs for broiler chickens is to increase growth rate and breast and thigh muscles weight. The candidate gene approach is a powerful technique for genetically improving performance traits in chickens. We studied the associations of the single nucleotide polymorphisms of three genes involved in protein synthesis, glucose metabolism and cell proliferation (*IGF1, IGFBP2, TGFβ3*) with performance traits in the Hubbard F15 and Cobb E chicken lines. Based on our results, it can be concluded that the *TGFβ3* gene could be used as a candidate gene marker for chicken growth traits.

Abstract: Marker-assisted selection based on fast and accurate molecular analysis of individual genes is considered an acceptable tool in the speed-up of the genetic improvement of production performance in chickens. The objective of this study was to detect the single nucleotide polymorphisms (SNPs) in the *IGF1, IGFBP2* and *TGFß3* genes, and to investigate their associations with growth performance (body weight (BW) and average daily gain (ADG) at 14, 21, 28, 35 and 42 days of age) and carcass traits in broilers. Performance (carcass) data (weight before slaughter; weights of the trunk, giblets, abdominal fat, breast muscle and thigh muscle; slaughter value and slaughter percentage), as well as blood samples for DNA extraction and SNP analysis, were obtained from 97 chickens belonging to two different lines (Hubbard F15 and Cobb E) equally divided between the two sexes. The genotypes were detected using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) methods with specific primers and restrictase for each gene. The statistical analysis discovered significant associations ($p < 0.05$) between the *TGFβ3* SNP and the following parameters: BW at 21, 28 and 35 days, trunk weight and slaughter value. Association analysis of BWs (at 21, 28 and

35 days) and SNPs was always significant for codominant, dominant and overdominant genetic models, showing a possible path for genomic selection in these chicken lines. Slaughter value was significant for codominant, recessive and overdominant patterns, whereas other carcass traits were not influenced by SNPs. Based on the results of this study, we suggested that the *TGFβ3* gene could be used as a candidate gene marker for chicken growth traits in the Hubbard F15 and Cobb E population selection programs, whereas for carcass traits further investigation is needed.

Keywords: chicken; SNP; *IGF1*; IGFBP2; TGFß3; Hubbard F15; Cobb E; growth; meat

1. Introduction

With the growth of the human population [1], the total amount of meat consumed increases at a global level worldwide [2]. Meat consumption rose worldwide from 23.08 kg per person per year in 1961 to 43.22 kg per person per year in 2013 [2]. Chicken meat is one of the most consumed types of meat worldwide [3].

Growth performance and carcass traits are the most important economic traits in broiler chicken production, and are controlled by a number of genes [4]. Growth is a complex process that involves the regulated coordination of a wide range of neuroendocrine pathways [5]. Therefore, it is very difficult to achieve rapid genetic improvement in these traits using only traditional selection methods. The growing knowledge of the structure and function of the chicken genome can be beneficial, and can lead to the recognition of causal genes and the development of new selectable molecular markers.

Although the *Gallus gallus* (chicken) genome was first sequenced as early as 2004 [6], it still required further improvements [7–9]. The newest version of the chicken genome assembly (Gallus_gallus-5.0; GCA_000002315.3), built from combined long single molecule sequencing technology, finished bacterial artificial chromosomes (BACs) and improved physical maps, was presented in 2017 [10]. Since the methodological approach has improved, the originally reported size of the chicken genome has increased from 1.05 [6] to 1.23 Gb, which has contributed to the increased number of genes observed [10]. Initial assemblies have been found insufficient for the more complete discovery of allelic contributions to complex traits [10], leading to ongoing efforts to improve the quality of the chicken reference genome [8,11].

However, the genetic improvement of polygenic traits, including growth performance and meat production, can be accomplished by marker-assisted selection that is more accurate in estimating the animal's genetic value [12]. The molecular markers linked to quantitative trait loci (QTLs) are not affected by environmental conditions. Therefore, they could increase the speed and effectiveness of animal breeding progress. As soon as the relationship between a DNA polymorphism and an important trait is revealed, the DNA marker may be used [13]. The candidate gene approach has become a powerful technique for the genetic improvement in chicken breeding programs, and can result in increased efficiency in detecting the required production performance traits [4].

The main objectives of the strategy in commercial broiler breeding programs include increasing growth rate and breast muscle weight, reducing abdominal fat content, improved feed efficiency and increased fitness. The relationships between these individual production traits are very complex and some of them are very difficult to measure. Therefore, the use of molecular marker-assisted selection (MMAS) is necessary. In case that the favorable allele is rare, a larger positive impact can be expected [14].

The purpose of the present study was to identify polymorphisms and evaluate the association between polymorphisms in three studied genes—*IGF1* (insulin-like growth factor 1), *IGFBP2* (insulin-like growth factor binding protein 2) and *TGFβ3* (transforming growth factor β)—with growth performance and meat production in chickens from two broiler lines: Hubbard F15 and Cobb E. The biological functions and interdependence of these genes are shown in Figure 1.

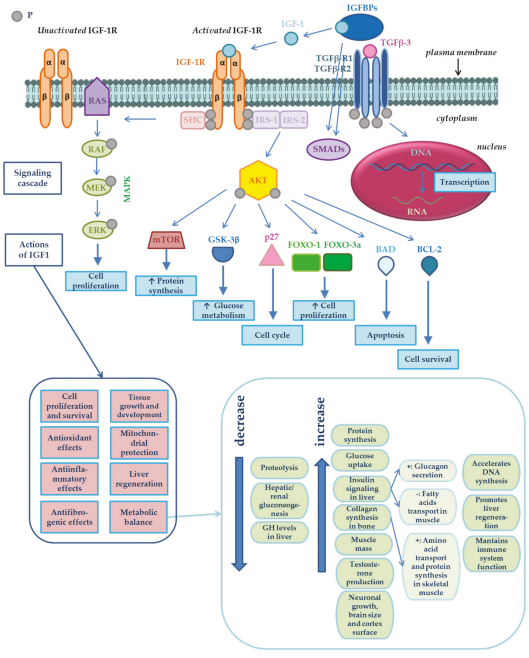

Figure 1. Signaling cascade of insulin-like growth factor 1 (IGF-1) and its potential impacts in metabolism, its interactions with transforming growth factor β3 (TGF-β3) and the biological functions of the *IGF1*, *IGFBP2* and *TGFß3* genes. IGF-1 bioavailability is modulated by IGF binding proteins (IGFBPs) [15]. IGF-1 action is mediated by its binding to its receptor [16], the type 1 insulin-like growth factor receptor (IGF-1R). IGF-1R is a heterotetramer composed of two extracellular α subunits and two transmembrane β subunits, as shown in Figure 1. α subunits are cysteine-rich regions, whereas β subunits possess a tyrosine kinase domain, which constitutes the signal transduction mechanism [16]. Tyrosine phosphorylation activates a signaling cascade [17]. IGF-1 has autocrine, paracrine [18,19] and endocrine effects [18]. IGF-1 binds to its receptor (IGF-1R) in the cell membrane, resulting in autophosphorylation and the recruitment of the adaptor proteins–insulin receptor substrate IRS-1, IRS-2, and the proto-oncogene tyrosine-protein kinase (SRC) homology and collagen protein (SHC). The serine/threonine kinase (AKT) is activated by the 3-phosphoinositide-dependent protein kinase-1 (PDK1) and by the mammalian target of rapamycin (mTOR)-containing complex mTOR-C2, leading to the phosphorylation at threonine 308 and serine 473, respectively. Activated AKT regulates downstream signaling molecules such as tuberous sclerosis protein 1/2 (TSC-1/2), which inhibit mTOR-C1 complex and regulate the ribosomal protein S6 kinase 1/2 (S6K-1/2) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EB-P1) phosphorylation, FOXO transcription factors, glycogen synthase kinase-3β (GSK-3β), p27, BCL-2 antagonist of cell death (BAD), and BCL-2. These substances are involved in some cellular processes such as protein synthesis, glucose metabolism and cell survival. SHC activation induces the activation of the RAS/mitogen-activated protein (MAP) kinase pathway, resulting in enhanced cell proliferation [15]. Activation of IRS induces the activation of intracellular RAF/MEK/ERK/RAS and PI3K signaling pathway. The first mentioned pathway mediates mitosis, and the second one mediates metabolism and cell growth effect through AKT [20]. After the ligand (IGF-1) binds to its receptor (IGF-1R), PI3K is activated, cell proliferation is promoted by activating the mitogen-activated protein kinase (MAPK) cascade, and apoptosis is blocked by inducing the phosphorylation and the inhibition of proapoptotic proteins such as BAD [21]. The protein IGFBP-2 encoded by the gene of the same name is able to control the biological actions of IGFs [22] and TGFß [23] in vivo via the endocrine, autocrine or paracrine pathways. The protein TGFß-3 encoded by the *TGFβ3*gene controls the growth, proliferation and differentiation of cells, cell motility and apoptosis. TGFß-3 plays an essential role in the development of skeletal muscles. It also can suppress the formation of tumors [24]. Adapted from [15,25–27] based on other works: [21,28–53].

The insulin-like growth factor 1 gene (*IGF1*) has been identified as a biological candidate gene for growth, body composition, metabolic and skeletal characteristics, and is also a positional candidate gene for growth and fat deposition in chicken [54]. This gene is involved in growth of various tissues such as muscle and bones [55]. The chicken *IGF1* gene was mapped to 165.95 cM on chromosome 1 (GGA1). In a broiler-layer F_2 population used to map body weight (BW) QTL by a genome scan, a QTL affecting BW at 6 weeks of age has been detected at 160 cM (confidence interval (CI) 114 to 180 cM) on chicken GGA1 [56]. In the same F_2 cross, a QTL at 150 cM (CI from 100 to 182 cM) on GGA1 affecting abdominal fat weight (AFW) has been ascertained [57].

The bioavailability of the insulin-like growth factors (IGFs) is regulated by a family of structurally conserved insulin-like growth factor binding proteins (IGF-binding proteins; IGFBPs) [58–60]. IGFBPs selectively bind to IGF-1 and IGF-2 proteins but do not bind to insulin [61]. More than 99% of IGF molecules circulate in blood serum as complexes with these specific and high affinity-binding proteins. Although IGF-binding protein 3 (IGFBP-3) is a main component and binds over 75% of the circulating IGF [62], IGFBP-2 is sensitive to dietary protein level, and may play a substantial role in the modulation of the growth-promoting effect of circulating IGF-1 by creating the IGF-1-IGFBP-2 complex in chickens [63]. IGFBP-2 is the predominant IGFBP in serum for different species [64]. IGFs, IGFBPs and IGFBP proteases are the major regulators of somatic growth and cell proliferation [65]. IGFBP-2 controls the biological actions of IGFs [22] and TGFs [23] in vivo via endocrine [22,23], autocrine [23] or paracrine mechanisms [22], and affects the growth and development of animals [66]. IGFBP-2 might indirectly affect adipocyte differentiation by controlling IGF [67] and TGF-β biological actions in fat tissue [68].

The transforming growth factor β (TGF-β) belongs to a large family of multifunctional growth factors [69], with important regulatory roles in embryonic and adult development [70], such as morphogenesis, development and differentiation [69]. Polypeptide growth factors of TGF-β family regulate a number of cellular processes such as cell proliferation, differentiation, migration, adhesion and apoptosis [70]. TGF-β plays a key role in maintaining both bone and articular cartilage homeostasis [71].

2. Materials and Methods

2.1. Experimental Population—Animals

The chicken hatching eggs were produced and the experiment was performed in the testing station of broilers (fattening test No. 1148) at the state-owned enterprise International Testing of Poultry, Ustrasice (Czech Republic). After hatching, 50 chickens from each of the two broiler lines Hubbard F15 and Cobb E were stalled in windowless air-conditioned hall with deep bedding and controlled light mode (Table 1). Stocking density was 6.1 chicks/m^2. The hall was disinfected with Virkon before the chickens were stored. The chickens were watered by automatic dropper drinking basins and fed with three feed mixtures, differently for particular period of fattening, from tube feeders *ad libitum*. Hypermangan solution was applied to the water in the first days of age. The composition (contents of main nutrients) in individual complete feed mixtures BR1, BR2 and BR3 for fattening broiler chickens up to the 10th, 35th and 42nd day of age, respectively, are shown in Table 2.

Table 1. Light regime in the chicken house.

Day	The Proportion of Light and Darkness Within 24 h	
	Light (h)	Dark (h)
1–7	23	1
8–37	18	6
38	20	4
39	22	2
40–42	23	1

Table 2. The content of nutrients in feeding mixtures for broilers (BR1, BR2, BR3) in different periods of the experiment.

Nutrient	Feeding Mixture *		
	BR1	BR2	BR3
	Period		
	1st–10th Day	11th–35th Day	36th–42nd Day
Crude protein (%)	22.01	19.86	18.50
Fat (%)	3.85	5.76	7.64
Lysine (%)	1.16	1.22	1.07
Methionine (%)	0.56	0.56	0.51
Calcium (%)	0.91	0.84	0.81
Phosphorus (%)	0.60	0.54	0.47
Vitamin A (IU/kg)	15,300	12,300	10,300
Vitamin D3 (IU/kg)	5,000	5,000	5,000
Metabolizable energy (MJ/kg)	12.64	13.07	13.59

* The feeding mixtures were produced in ZZN Pelhrimov, a.s., according to given recipes.

2.2. Phenotypic Data

Body weight (BW) was measured at 14, 21, 28, 35 days and before slaughter at 42 days of age. The mortality during experiment was 3% (sudden death syndrome). Chickens were slaughtered at 42 days of age and the slaughter analysis was performed. The carcass traits, such as weight of trunk, giblets, abdominal fat, breast muscle (with and without skin) and thigh muscle (with and without skin), as well as slaughter value and slaughter percentage were investigated. The slaughter value was calculated as the ratio between the weight of the carcass (trunk weight) and the weight at 42 days of age before slaughter, and the slaughter percentage was calculated as the ratio between the sum of weight of the trunk and giblets and the weight at 42 days.

2.3. SNP Genotyping

2.3.1. DNA Extraction

Genomic DNA for genotyping assays was extracted from whole blood samples, which were collected from 97 chickens at 42 days of age before slaughtering. Blood was taken from *vena ulnaris* into 1.5 mL EDTA-treated microtubes. For extraction of genomic DNA, chelex 100 was used, and the concentration and purity of genomic DNA were verified by spectrophotometer Shimadzu BioSpec-nano (Shimadzu Corporation, Kyoto, Japan).

2.3.2. Optimalization of PCR-RFLP Assay

Polymerase chain reaction (PCR) was performed for all assays in total volume 25 µL mixture containing 1 µL genomic DNA, 10 pmol of each primer and 12.5 µL PPP Master Mix (Top-Bio, s.r.o., Vestec, Czech Republic). Sequences of sets of primer pairs for all three gene polymorphisms used in PCR assays are shown in Table 3.The primers for the *IGF1* genotyping were designed according to Moody et al. [72]. The thermal profile included pre-denaturation at 94 °C for 2 min followed by 30 cycles 94 °C for 30 s, 67 °C for 30 s and 72 °C for 50 s, with a final extension of 72 °C for 7 min. Thermocycler BIOER Life ECO (Hangzhou Bioer Technology Co,. Ltd., Bin An Rd, Hi-tech (Binjiang) District, Hangzhou, China) was used for DNA amplification. SNP of *IGF1* gene was detected after digesting PCR product with *Hinf*I restriction endonuclease (Fermentas, Vilnius, Lithuania) at 37 °C overnight. For detection of *IGFBP2* genotypes PCR amplification was done using primer set by Li et al. [73]. Amplification was performed under following conditions: pre-denaturation at 94 °C for 2 min followed by 30 cycles 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 7 min. PCR products were digested (restriction fragment length polymorphism–RFLP) with *Eco72*I restriction endonuclease (New England Biolabs, Ipswich, MA, USA) at 37 °C overnight. The PCR primers designed by Li et al. [69] were applied for *TGFβ3*. The PCR reaction conditions were the same as for *IGFBP2*, except the annealing temperature which was 58 °C. Gene fragments

were subjected to digestion by *Bsl*I restriction enzyme (New England Biolabs, Ipswich, MA, USA) at 37 °C overnight.

Table 3. Primers used in polymerase chain reaction (PCR) assay.

Gene		Primer Sequence	Product Length (bp)	Restriction Enzyme	Restriction Enzyme Production Size (bp) *
IGF1 *SNP/site* *A>C/Promoter and 5′UTR*	forward [a] reverse [a]	5′-CATTGCGCAGGCTCTATCTG-3′ 5′-TCAAGAGAAGCCCTTCAAGC-3′	813	*Hinf*I	*AA: 378+244+191* *AC: 622+378+244+191* *CC: 622+191*
IGFBP2 *intron 2 C1032T (accession number AY 326194)*	forward [b] reverse [b]	5′-GTCCCAGATAAACCTTGCT-3′ 5′-GCTGGCAAGGGGTCTG-3′	367	*Eco72*I	*AA*: 367 *AB*: 367+265+102 *BB*: 265+102
TGFβ3 *A C/A SNP at base 2,833 (accession number X60091)*	forward [c] reverse [c]	5′-TCAGGGCAGGTAGAGGGTGT-3′ 5′-GCCACTGGCAGGATTCTCAC-3′	294	*Bsl*I	*AA:* 145+75+74 *AB:* 145+125+75+74 *BB:* 125+75+74+20

[a] Moody et al. (2003) [72], Zhou et al. (2005) [54]. [b] Li et al. (2006) [73]. [c] Li et al. (2003) [69]. * All possibilities of fragments.

2.3.3. Electrophoresis

The PCR products were visualized by 2% and restriction patterns by 3% agarose gel electrophoresis and ethidium bromide staining. The ENDURO™ 250 V power supply (Labnet International, Inc., New York, USA) and the HU13 midi horizontal gel electrophoresis unit (Scie-Plas Ltd., Cambourne, Cambridge, UK) were used for DNA electrophoresis. Syngene™ Ingenius 3 Manual Gel Documentation System (Syngene) was used for photo-documentation.

2.4. Statistical Analysis

Associations of three different polymorphisms of *IGF1*, *IGFBP2* and *TGFβ3* genes with growth characteristics and carcass data in 97 chicken belonging to two different lines (Hubbard F15 and Cobb E), equally divided between two sexes, were studied. Genotypes were tested for Hardy-Weinberg equilibrium (HWE) using a chi-square (χ^2) test in R/SNPassoc Package (R Development Core Team). Whole-Genome association analyses were performed assuming five different genetic models (inheritance patterns) using R/SNPassoc Package (R Development Core Team): codominant, dominant, recessive, overdominant and log-additive effect. The level of significance was tested at the nominal 5% significance level after correcting for the number of tests performed (Bonferroni correction). Phenotypes were represented by the carcass data collected on poultry, whereas line and sex were included in the model as fixed effects. Hardy-Weinberg equilibrium (HWE) was calculated and tested using χ^2 test at the 0.05 level of statistical significance.

Another statistical analysis was performed using box and forest plots. Average slopes of growth curve and total integrals for the weight sum of the trunk, giblets, abdominal fat, breast and thigh muscles at 42 days of age (at the slaughter of chickens) in both chicken lines, for both sexes and for all genotypes observed, were evaluated using the laboratory information system Qinslab (Prevention Medicals, Studenka, Czech Republic).

3. Results

The *IGF1/Hinf*I PCR-RFLP analysis of 97 DNA samples obtained from chicken belonging to broiler lines Hubbard F15 and Cobb E showed only two from three genotypes, namely *AA* (378 + 244 + 191 bp), and *AC* (622 + 378 + 244 + 191 bp), as shown in Figure 2. The *AA* homozygotes (73.20%) predominated over heterozygotes (26.80%)–Table 4, Figure 3. No *CC* homozygous individuals were detected on either of the two broiler lines. Correspondingly, the frequency of allele *A* is much higher (86.60%) than allele *C* (13.40%) in the investigated chicken population, as is evident from Table 4.

In contrast, in *IGFBP2/Eco72*I polymorphism, all three genotypes (*AA, AB*, and *BB*) were found, however, *BB* (265 + 102 bp) homozygotes showed very low frequency (4.12%). The most represented genotype was *AB* (367 + 265 + 102 bp) with a frequency of 56.70%.

Also, in *TGFß3*/*BslI* SNP, all three genotypes were detected, with the highest observed genotypic frequency in heterozygotes (42.27%) followed by 36.08% and 21.65% in *AA* (145 + 75 + 74 bp) and *BB* (125 + 75 + 74 + 20 bp), respectively.

Only for IGFBP2 frequencies in total population, Hardy-Weinberg equilibrium (HWE) was identified: $p < 0.01$.

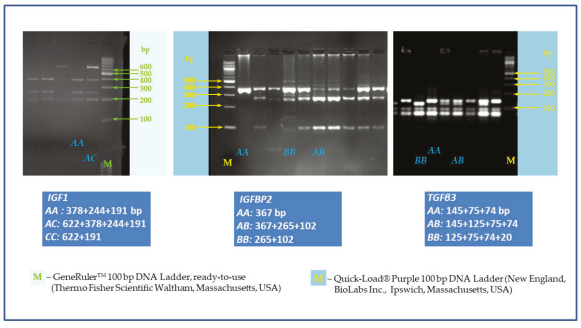

Figure 2. The restriction fragment length polymorphism (RFLP) patterns for *IGF1* (*AA:* 378 + 244 + 191 bp; *AC:* 622 + 378 + 244 + 191 bp; CC–it was not detected), *IGFBP2* (*AA*: 367 bp; *AB*: 367 + 265 + 102 bp; *BB:* 265 + 102 bp) and *TGFß3* (*AA*: 145 + 75 + 74 bp, *AB*: 145 + 125 + 75 + 74 bp; *BB*: 125 + 75 + 74 + 20 bp). Agarose 2%, 120 V, 60 min, Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer. M–marker.

Table 4. Genotype and allele frequencies of *IGF1*, *IGFBP2* and *TGFß3* genes in the chicken population.

Broiler Line	Gene	Genotype Frequencies [a]			Allele Frequencies [a]		χ^2 Test HWE [b] (*p*-Value)
Hubbard F15	*IGF1*	*AA*	*AC*	*CC*	*A*	*C*	
		28 (56.00)	22 (44.00)	nf	78 (78.00)	22 (22.00)	0.0908
	IGFBP2	*AA*	*AB*	*BB*	*A*	*B*	
		20 (40.00)	27 (54.00)	3 (6.00)	67 (67.00)	33 (33.00)	0.1998
	TGFß3	*AA*	*AB*	*BB*	*A*	*B*	
		15 (30.00)	20 (40.00)	15 (30.00)	50 (50.00)	50 (50.00)	0.1639
Cobb E	*IGF1*	*AA*	*AC*	*CC*	*A*	*C*	
		43 (91.49)	4 (8.51)	nf	90 (95.74)	4 (4.26)	1.00
	IGFBP2	*AA*	*AB*	*BB*	*A*	*B*	
		18 (38.30)	28 (59.57)	1 (2.13)	64 (68.09)	30 (31.91)	**0.0172***
	TGFß3	*AA*	*AB*	*BB*	*A*	*B*	
		20 (42.55)	21 (44.68)	6 (12.77)	61 (64.89)	33 (35.11)	1.00
Total	*IGF1*	*AA*	*AC*	*CC*	*A*	*C*	
		71 (73.20)	26 (26.80)	0 (0)	168 (86.60)	26 (13.40)	0.2066
	IGFBP2	*AA*	*AB*	*BB*	*A*	*B*	
		38 (39.18)	55 (56.70)	4 (4.12)	131 (67.53)	63 (32.47)	**0.0050***
	TGFß3	*AA*	*AB*	*BB*	*A*	*B*	
		35 (36.08)	41 (42.27)	21 (21.65)	111 (57.22)	83 (42.78)	0.2125

[a] The numbers in brackets are percentage frequencies (relative frequencies). [b] HWE–Hardy-Weinberg equilibrium; * statistically significant ($p < 0.05$); nf–not found.

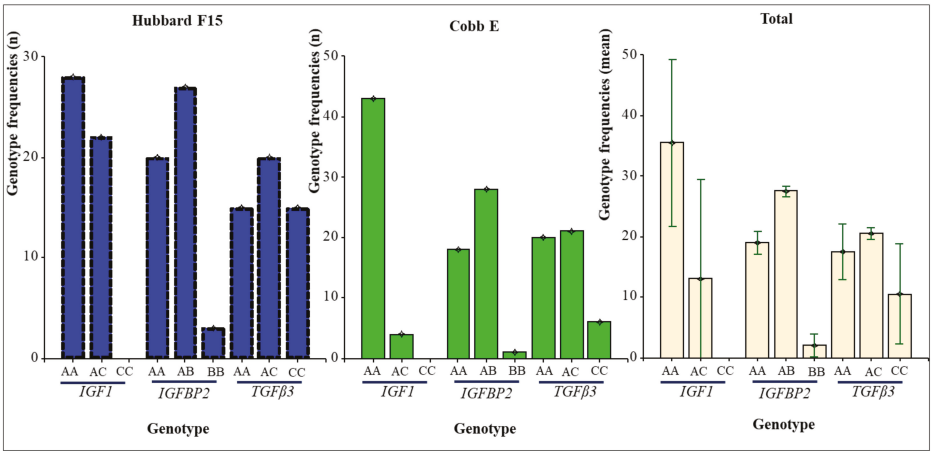

Figure 3. Genotype distribution of individual genes in both chicken lines.

Tables 5–7 show average values of growth performance and carcass traits in both chicken lines, according to individual genotypes. The highest average BW at 42 days was achieved in the Cobb E line, with the *AC* genotype of *IGF1* (2967.50 g), *BB* genotype of *IGFBP2* (3170.00 g) and the *AB* genotype of *TGFβ3* (3104.29 g). The highest average breast muscle (without skin) was found in a Cobb E chicken with an *AC* genotype of *IGF1* (629.25 g), a *BB* genotype of *IGFBP2* (753.00 g) and an *AB* genotype of *TGFβ3* (653.62 g). The Cobb E chicken with an *AC* genotype of *IGF1* (52.50 g) and a *BB* genotype of *IGFBP2* (63.00 g) also had the highest average abdominal fat weight (AFW); whereas for *TGFβ3*, the highest AFW was found in the *AA* genotype (54.95 g). The highest average thigh muscle (with skin) were measured in a Cobb E line chicken with an *AC* genotype of *IGF1* (519.75 g), a *BB* genotype of *IGFBP2* (552.00 g) and an *AB* genotype of *TGFβ3* (538.72 g). On the contrary, the lowest average values of BW at 42 days and AFW showed in the Hubbard F15 line chicken with an *AA* genotype of *IGF1* (2585.00 and 35.32 g, respectively), a *BB* genotype of *IGFBP2* (2413.33 g and 30.33 g, respectively) and an *AA* genotype of *TGFβ3* (2541.33 g and 31.53 g). The lowest breast muscle (without skin) showed the Hubbard F15 line chicken with an *AA* genotype of *IGF1* (501.75 g), an *AA* genotype of *IGFBP2* (498.75 g) and an *AA* genotype of *TGFβ3* (494.33 g). The lowest thigh muscle (with skin) was observed in a Hubbard F15 line chicken with an *AA* genotype of *IGF1* (470.68 g) and a *BB* genotype of *IGFBP2* (445.00 g) and in a Cobb E line chicken with a *BB* genotype of *TGFβ3* (458.67 g).

Table 5. The average growth performance and carcass traits in the chicken population (according to *IGF1* genotypes).

Parameter	Line					
	Hubbard F15			Cobb E		
	Genotype			Genotype		
	AA (n = 28)	*AC (n = 22)*	*CC*	*AA (n = 43)*	*AC (n = 4)*	*CC*
	Mean ± SD; CI					
BW at 14 days *	421.07 ± 32 433; 408	428.18 ± 31 442; 414	-	473.24 ± 40 485; 461	462.50 ± 43 530; 394	-
BW at 21 days *	840.00 ± 69 866; 813	865.00 ± 67 894; 835	-	933.25 ± 96 962; 903	940.00 ± 113 1120; 760	-
BW at 28 days *	1393,21 ± 137 1446; 1340	1436.82 ± 114 1487; 1386	-	1558.84 ± 182 1614; 1502	1552.50 ± 204 1877; 1227	-
BW at 35 days *	1913.21 ± 183 1984; 1842	1947.27 ± 161 2018; 1876	-	2103.95 ± 240 2178; 2030	2152.50 ± 281 2600; 1705	-
BW at 42 days before slaughter *	2585.00 ± 298 2700; 2469	2588.64 ± 265 2706; 2471	-	2919.77 ± 318 3018; 2822	2967.50 ± 415 3627; 2308	-

Table 5. *Cont.*

Parameter	Line					
	Hubbard F15			Cobb E		
	Genotype			Genotype		
	AA (n = 28)	*AC (n = 22)*	*CC*	*AA (n = 43)*	*AC (n = 4)*	*CC*
	Mean ± SD; CI					
Trunk weight *	1798.00 ± 190 1871; 1724	1817.45 ± 188 1901; 1734	-	1989.60 ± 224 2058; 1920	2060.25 ± 289 2520; 1600	-
Giblets weight *	156.79 ± 18 163; 149	161.45 ± 14 168; 155	-	169.14 ± 18 174; 163	165.50 ± 21 199; 132	-
Abdominal fat weight *	35.32 ± 9 38; 32	35.68 ± 13 41; 30	-	51.47 ± 12 55; 47	52.50 ± 17 80; 25	-
Breast muscle with skin *	542.18± 53 562; 521	558.00± 64 586; 529	-	668.81± 84 694; 643	684.50± 106 853; 515	-
Breast muscle without skin *	501.75 ± 50 521; 482	517.09 ± 61 544; 489	-	615.98 ± 83 641; 590	629.25 ± 98 712; 530	-
Thigh muscle with skin *	470.68 ± 61 494; 447	475.14 ± 55 499; 450	-	499.98 ± 60 519; 584	519.75 ± 94 669; 371	-
Thigh muscle without skin *	421.39 ± 55 442; 400	426.77 ± 53 450; 402	-	441.88 ± 60 460; 423	464.25 ± 90 608; 320	-
Slaughter value **	69.66	70.22	-	68.14	69.42	-
Slaughter percentage ***	75.74	76.48	-	73.94	75.01	-

BW–average body weight; SD–standard deviation; CI–confidence interval (95%); * (g); ** slaughter value = weight of trunk/BW at 42 days * 100 (%); *** slaughter percentage = weight of trunk+ weight of giblets/BW at 42 days * 100 (%).

Table 6. The average growth performance and carcass traits in the chicken population (according to *IGFBP2* genotypes).

Parameter	Line					
	Hubbard F15			Cobb E		
	Genotype			Genotype		
	AA (20)	*AB (27)*	*BB (3)*	*AA (17)*	*AB (27)*	*BB (1)*
	Mean ± SD; CI					
BW at 14 days *	419.50 ± 33 435; 403	427.78 ± 32 440; 415	423.33 ± 15 461; 385	468.82 ± 39 489; 449	474.81 ± 43 492; 458	460.00
BW at 21 days *	839.50 ± 72 873; 805	864.07 ± 66 890; 838	810.00 ± 62 965; 655	927.65 ± 95 976; 879	932.22 ± 103 973; 892	1010.00
BW at 28 days *	1392.50 ± 128 1452; 1333	1436.67 ± 128 1487; 1385	1326.67 ± 90 1552; 1101	1535.29 ± 184 1630; 1441	1554.81 ± 184 1628; 1482	1750.00
BW at 35 days *	1894.00 ± 179 1978; 1810	1967.78 ± 164 2032; 1902	1800.00 ± 125 2110; 1489	2096.47 ± 232 2216; 1977	2097.41 ± 256 2199; 1996	2300.00
BW at 42 days before slaughter *	2578.50 ± 320 2728; 2428	2611.85 ± 257 2713; 2510	2413.33± 240 3009; 1818	2891.76 ± 344 3069; 2716	2912.59 ± 311 3036; 2789	3170.00
trunk weight *	1791.20 ± 206 1887; 1695	1827.41 ± 178 1898; 1757	1721.33 ± 157 2110; 1333	1958.06 ± 226 2074; 1842	1996.22 ± 228 2086; 1906	2251.00
giblets weight *	159.45 ± 19 168; 151	159.44 ± 15 165; 154	149.33 ± 18 194; 105	166.53 ± 20 177; 156	168.74 ± 18 176; 162	194.00
abdominal fat weight *	35.55 ± 11 41; 30	36.00 ± 9 40; 32	30.33 ± 18 75; (-14)	50.59 ± 13 57; 44	51.26 ± 12 56; 46	63.00
breast muscle with skin *	539.65 ± 64 569; 510	555.85 ± 56 578; 533	552.00 ± 33 634; 470	651.24 ± 74 689; 613	676.11 ± 90 712; 641	809.00
breast muscle without skin *	498.75 ± 60 527; 471	515.59 ± 53 537; 495	509.67 ± 36 600; 420	598.82 ± 72 636; 562	622.37 ± 89 658; 587	753.00
thigh muscle with skin *	471.80 ± 63 501; 442	476.33 ± 56 498; 454	445.00 ± 52 575; 316	488.06 ± 65 522; 455	503.33 ± 61 527; 479	552.00
thigh muscle without skin *	423.25 ± 59 451; 396	426.74 ± 52 447; 406	400.33 ± 49 522; 278	431.24 ± 63 464; 399	445.56 ± 62 470; 421	487.00
slaughter value **	69.57	69.99	71.36	67.77	68.51	71.01
slaughter percentage ***	75.78	76.12	77.54	73.53	74.31	77.13

BW–average body weight; SD–standard deviation; CI–confidence interval (95%); * (g); ** slaughter value = weight of trunk/BW at 42 days * 100 (%); *** slaughter percentage = weight of trunk + weight of giblets/BW at 42 days * 100 (%).

Table 7. The average growth performance and carcass traits in the chicken population (according to *TGFβ3* genotypes).

Parameter	Line					
	Hubbard F15			Cobb E		
	Genotype			Genotype		
	AA (15)	*AB (20)*	*BB (15)*	*AA (20)*	*AB (21)*	*BB (6)*
	Mean ± SD; CI					
BW at 14 days *	420.67 ± 40 443; 398	433.00 ± 24 444; 422	416.00 ± 30 433; 399	462.00 ± 38 480; 444	484.76 ± 41 503; 466	463.33 ± 34 499; 428
BW at 21 days *	842.67 ± 72 883; 803	866.00 ± 65 896; 836	839.33 ± 72 879; 799	888.50 ± 93 932; 845	980.00 ± 91 1021; 939	923.33 ± 50 976; 871
BW at 28 days *	1399.33 ± 122 1467; 1332	1420.50 ± 117 1475; 1366	1414.67 ± 154 1499; 1330	1453.00 ± 167 1531; 1375	1664.76 ± 149 1733; 1597	1536.67 ± 125 1668; 1405
BW at 35 days *	1884.67 ± 171 1979; 1790	1938.00 ± 165 2015; 1861	1958.67 ± 187 2062; 1855	1986.00 ± 240 2098; 1874	2241.91 ± 190 2328; 2155	2046.67 ± 176 2231; 1862
BW at 42 days before slaughter *	2541.33 ± 255 2682; 2400	2597.00 ± 302 2738; 2456	2618.00 ± 292 2780; 2456	2778.50 ± 312 2924; 2633	3104.29 ± 270 3227; 2982	2780.00 ± 208 2998; 2562
trunk weight *	1773.27 ± 175 1870; 1676	1825.40 ± 209 1923; 1728	1814.73 ± 177 1913; 1717	1896.95 ± 223 2001; 1793	2129.19 ± 181 2211; 2047	1857.00 ± 136 1999; 1715
giblets weight *	159.00 ± 15 168; 150	158.15 ± 19 167; 149	159.60 ± 15 168; 151	161.45 ± 19 170; 153	177.43 ± 16 185; 170	163.33 ± 14 178; 148
abdominal fat weight *	31.53 ± 7 35; 28	37.05 ± 13 43; 31	37.33 ± 10 43; 32	54.95 ± 14 62; 48	48.90 ± 9 53; 45	49.50 ± 15 66; 33
breast muscle with skin *	533.67 ± 59 566; 501	555.15 ± 68 587; 523	556.60 ± 41 580; 534	648.30 ± 86 689; 608	710.48 ± 72 743; 678	601.83 ± 51 656; 548
breast muscle without skin *	494.33 ± 54 525; 465	515.55 ± 66 546; 485	513.27 ± 39 535; 491	598.60 ± 86 639; 559	653.62 ± 73 687; 621	551.00 ± 52 605; 497
thigh muscle with skin *	469.00 ± 63 504; 434	476.75 ± 63 506; 447	470.80 ± 49 498; 444	475.65 ± 61 504; 447	538.72 ± 51 562; 516	458.67 ± 30 490; 428
thigh muscle without skin *	421.47 ± 56 453; 390	429.10 ± 61 458; 401	418.93 ± 45 444; 394	419.65 ± 61 448; 391	477.05 ± 54 502; 452	407.83 ± 32 441; 375
slaughter value * *	69.80	70.34	69.44	68.26	68.62	66.93
slaughter percentage * * *	76.08	76.43	75.57	74.08	74.34	72.81

BW–average body weight; SD–standard deviation; CI–confidence interval (95%); * (g); ** slaughter value = weight of trunk/BW at 42 days * 100 (%); *** slaughter percentage = weight of trunk + weight of giblets/BW at 42 days * 100 (%).

By means of statistical software analysis, the relationships between SNPs and individual traits were identified. The fixed effects included in the "whole" model were sex and line, and the *p*-values obtained were adjusted by the number of tests using Bonferroni correction.

Only the *TGFβ3* SNP (Table 8) resulted in statistical significance for the following parameters: body weight at 21, 28 and 35 days, trunk weight and slaughter value. The *p* values were significant for codominant, dominant and overdominant genetic models, with the exception of the slaughter value, which was not significant for the dominant inheritance pattern.

Figure 4 shows average slopes of growth curve (14–42 days of age) in individual genotypes of both chicken line and sexes.

Figure 5 represents total integrals for the sum of the trunk, giblets, abdominal fat, breast and thigh muscles at 42 days of age in all genotypes observed.

Table 8. Results of statistical analysis for testing association between *TGFβ3* polymorphism and growth performance and carcass traits in the chicken population.

Parameter	Genetic Model				
	Codominant	**Dominant**	**Recessive**	**Overdominant**	**Log-Additive**
BW at 14 days	ns	ns	ns	ns	ns
BW at 21 days	**−0.013**	**0.014**	0.606	**0.005**	0.199
BW at 28 days	**−0.004**	**0.002**	0.847	**0.004**	**0.035**
BW at 35 days	**−0.004**	**0.001**	0.560	**0.008**	**0.016**
BW at 42 days before slaughter	ns	ns	ns	ns	ns
trunk weight	**−0.010**	**0.013**	0.535	**0.003**	0.215
giblets weight	ns	ns	ns	ns	ns
abdominal fat weight	ns	ns	ns	ns	ns
breast muscle with skin	ns	ns	ns	ns	ns
breast muscle without skin	ns	ns	ns	ns	ns
thigh muscle with skin	ns	ns	ns	ns	ns
thigh muscle without skin	ns	ns	ns	ns	ns
slaughter value	**−0.015**	0.646	**0.015**	**0.014**	0.306
slaughter percentage	ns	ns	ns	ns	ns

BW–body weight; ns–no significant SNP after Bonferroni correction. Statistical significances at significance level 0.05 are highlighted in bold.

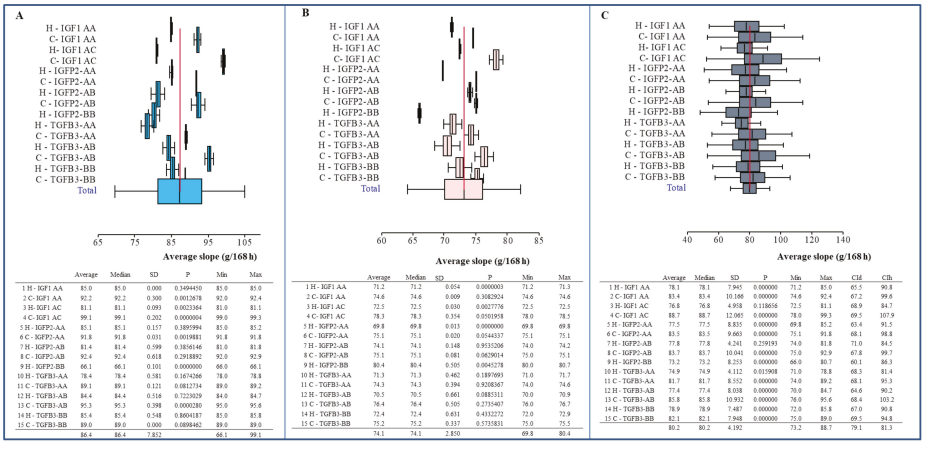

Figure 4. Forest plot of body weight (g) of chicken (at 14–42 days of age) of both lines in individual genotypes. (**A**) Males; (**B**) females; (**C**) total. Statistical characteristics of individual variants: average (n), median, standard deviation (SD), *p*-value (compared to average value), minimum (min) and maximum (max) value. Symbol H is Hubbard F15; C is Cobb E. The line segments represent confidence interval–CI (95%).

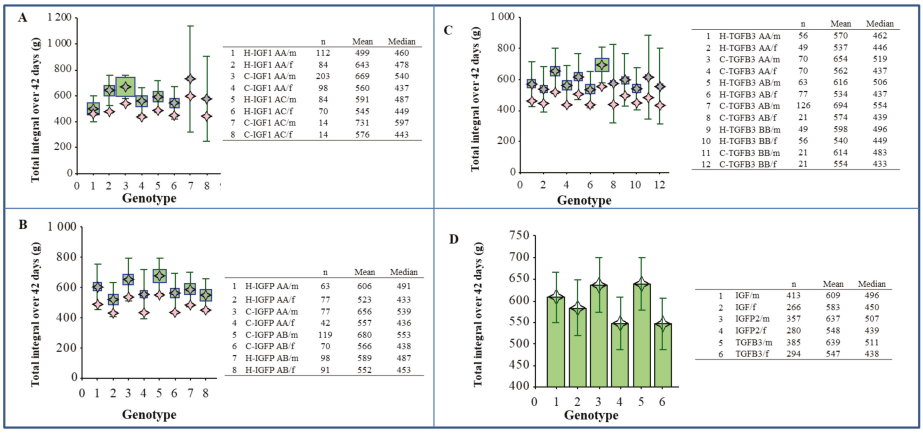

Figure 5. The weight sum of the trunk, giblets, abdominal fat, breast muscle with and without skin and thigh muscle with and without skin. *IGF1* gene with genotype *AA* and *AC* (**A**), *IGFBP2* with genotype *AA* and *AB* (**B**), *TGFβ3* with genotype *AA*, *AB* and *BB* (**C**). The pink symbol—median, the grey symbol—mean, square indicates the weight (number of samples). A comparison of individual genotypes (**D**). Statistical characteristics of individual variants: number (n), mean and median. The line segments represent confidence interval–CI (95%); m—males, f—females.

4. Discussion

The study of candidate genes is one of the primary methods to determine whether specific genes are related to economically important traits in farm animals [69]. We performed genotyping of the SNP of three genes linked to consumer-priced characteristics in chicken meat.

One of the main hormones required to normal growth process and muscle development is insulin-like growth factor 1 (IGF-1) [74]. The chicken *IGF1* gene consists of four exons and three introns, spanning more than 50 kb on chromosome 1 [75].

IGF1 encodes the same-name protein (IGF-1), which has a similar molecular structure to insulin [76] and induces insulin-like metabolic effects in muscle and adipose tissues [65]. This protein plays an important role in the proliferation, differentiation and metabolism of myogenic cell lines in chickens [76]. IGF-1 is one of the three ligands (insulin, IGF-1, IGF-2) belonging to the IGF system, which also includes three cell surface binding receptors (InsR, IGF-1R, IGF-2R), and insulin-like growth factor binding proteins (IGF binding proteins, IGFBPs) and IGFBP protease [77]. In addition, the IGF-1 protein is a potent mitogen and an essential stimulus for the differentiation of adipocytes [78]. The production and secretion of IGF-1 is affected by age, nutritional status, and several hormones [79]. The predominant source of IGF-1 is the liver and some other tissues, including muscle, brain and kidney [80].

IGF-1 binds to the type 1 insulin-like growth factor receptor (IGF-1R), which plays a critical role in signaling cell survival and proliferation [21]. However, IGF-1 can also bind, albeit with lower affinity, to the insulin receptor [16], regulating some metabolic functions [25].

Insulin-like growth factors (IGFs) provide essential signals for the control of embryonic, as well as postnatal development in vertebrates [81]. In addition to the growth hormone (GH), IGF-1 is one of the two main hormones required to support normal growth in chicken. Optimal growth requires a "set-point" concentration of both IGF-1 and triiodothyronine (T_3) in blood circulation. Pituitary GH plays a role in controlling the circulating levels of both IGF-1 and T_3 [74]. IGFs stimulate hepatic glycogen, increase DNA synthesis and promote tissue growth in chicken [82]. The highest level of *IGF1 mRNA* expression was detected in the chicken liver. High levels of *IGF1 mRNA* (10%–30% of the value in the liver) were expressed in spleen, lung and brain of chickens. *IGF1 mRNA* expression was

also observed in other extrahepatic tissues such as the kidney, heart, intestine, thymus and muscle of chickens, but these expression levels were less than 4% of that in the liver [83].

The abundant expression of *IGF1* gene was detected in the liver of normal chicken, but no *IGF1 mRNA* expression was found in that organ of dwarf chicken [83]. The expression of hepatic *IGF1 mRNA* level and circulating IGF-1 concentration were significantly higher in chicken with a high growth rate, compared to the line with low growth rate, supporting the hypothesis of its stimulatory effect during post-hatching growth of chickens as stated by Beccavin et al. [84]. The liver is the main site of IGF-1 production during post-hatch growing stages of chicken as described by Kita et al. [85].

IGF-1 is significantly altered by the genotype, suggesting a pivotal role in the control of growth rate in broiler chickens [84].

The SNPs within the chicken *IGF1* promoter were reported by numerous previous studies [54,86–92] but according to the author's best knowledge, no research work on gene constitution of *IGF1* SNP in the Hubbard F15 and Cobb E lines has been reported until now.

Genotype frequency analysis indicated that the *AA* genotype (73.20%) was of higher frequency than the *AC* (26.80%) and *CC* (0%) genotypes in both chicken lines, which is consistent with another study [92]. For the other two genes (*IGFBP2, TGFβ3*), the predominance of heterozygous (*AB*) genotypes was detected. Interestingly, in Hubbard F15, a distribution of both allele in *TGFβ3* was identical (Table 4).

With regards to the genotype frequencies of *IGF1/Hinf*I gene polymorphism, from the three known restriction patterns, only two genotypes were detected: *AA* and *AC*, with an almost three times higher prevalence of *AA* homozygotes over heterozygotes. The *CC* homozygous genotype was not found in either chicken line, which is consistent with the finding of Moe et al. [90], which reported an absence of the *CC* genotype in two commercial broiler strains (Chunky and Cobb). The noticeable predominance of allele *A* (86.60%) over allele *C* (13.40%) (Table 4) observed in our study is in conspicuous accordance with previous studies. As Moe et al. [90] have shown, allele *C* occurs especially in native chickens, for example in nine Japanese native chicken breeds (Chabo, Ehime-jidori, Gifu-jidori, Koeyoshi, Koshamo, Mikawa, Satsuma-dori, Engie and Tokuchijidori), the frequency of this allele to be 1.0. Our finding of low incidence of *C* allele in broiler chicken also corresponds with the results of genotyping performed by Anh et al. [4], who observed the *CC* genotype of *IGF1* gene with very low frequencies (0.13 to 0.15) in all populations of crossbreds from commercial parent stock broilers with four Thai synthetic chicken lines (the Kaen Thong, Khai Mook Esarn, Soi Nin, and Soi Pet). The *IGF1* SNP gene constitution of these four Thai synthetic chicken lines was studied by Promwatee et al. [92], who found that the *AA* genotype had a considerably lower frequency than the *AC* and *CC* genotypes in all chicken lines except Soi Noi, in which the *AA* and *CC* genotypes were similar. With the exception of Soi Nin, there was the predominance of the *C* allele—the frequency of the *A* allele was lower than that of C in all lines except Soi Noi, where both allelic frequencies were the same. This indicates that allele *C* is evidently typical for native chicken breeds. A higher frequency of allele *A* than that of allele *C* in commercial broiler stocks compared to native chicken can be explained as a result of selection effect on growth traits [90].

It can be concluded that the incidence of a higher *A* allele frequency over *C* allele in the *IGF1* locus observed in our study could be a result of a long-term selection strategy applied in the populations of chosen broiler lines that are the subject of this study.

Various studies reported associations between *IGF1* polymorphism and growth traits in chickens. Zhou et al. [54] and Amills et al. [86] reported that polymorphism of the *IGF1* gene in the promoter and 5′-untranslated region (5′-UTR) was directly associated with chicken growth rate. Bian et al. [89] found that haplotypes based on three *IGF1* polymorphisms (c.-366A > C, c.528G > A and c.*1024C > T–in 5′-flanking, exon 3 and 3′-flanking regions of *IGF1*) were associated with BW traits.

In our study, *AC* genotype of *IGF1* evinced the highest average BW at 42 days in both chicken lines. This genotype also corresponded with a higher average AFW, breast muscle weight (with or without skin), thigh muscle (with or without skin), slaughter value and slaughter percentage in both lines. On the contrary, the *AA* genotype of *IGF1* was associated with the average lowest BW at 42 days,

trunk weight, AFW, breast and thigh muscles, slaughter value and slaughter percentage in both lines. However, no significant difference was identified.

These results are inconsistent with the study of Zhou et al. [54], which observed that broiler line with fragment sizes of 378, 244 and 191 bp (*AA* genotype) showed greater improvement of marketable BW. Additionally, in Thai native chickens, the *AA* genotype resulted in a higher BW compared to the *AC* and *CC* genotypes [93]. Promwatee et al. [92] found, in two synthetic lines (Khai Mook Esarn, Soi Pet), the association between the *AA* genotype and BW at 8 and 12 weeks of age and average daily gain (ADG) at 0–12 and 0–14 weeks. On the contrary, in the Soi Nin synthetic line, BW at 8 and 12 weeks and ADG at 0–12 weeks were associated with the *AC* genotype. In the fourth synthetic line (Kaen Thong), no significant association was found [92]. In Thai native chickens (Chee), the *IGF1* gene was significantly associated with BW at 12 and 16 weeks of age, and ADG during 0–12 and 0–16 weeks of age [94].

IGFBP-2 binds to insulin-like growth factors [64]. IGFBP-2 is the predominant binding protein produced during adipogenesis of white preadipocytes [95]. IGFBP-2 is secreted by white adipocytes and contributes to the prevention of diet-induced obesity [96]. The circulating IGFBP-2 level was significantly and negatively correlated with fasting plasma glucose, triglycerides, low-density lipoprotein (LDL) cholesterol, IGF-1, IGF-2 and insulin C-peptide [97].

IGFBP-2 regulates a broad spectrum of physiological processes involved in growth, development, and differentiation [73]. Both inhibitory and stimulatory effects of IGFBP-2 on cell proliferation have been reported [98]. IGFBP-2 plays an important role in growth and fat metabolism [64]. IGFBP-2 is the predominant IGF binding protein produced during adipogenesis, and is known to increase the insulin-stimulated glucose uptake in myotubes [99]. IGFBP-2 stimulates glucose uptake in a phosphatidylinositol-3-OH kinase (PI3K)-dependent manner. Adipocytes treated with insulin and IGF-1 for 30 min exhibited a significant ($p < 0.001$) increase in PI3K phosphorylation when compared with the control cells. Similarly, IGFBP-2 induced a significant increase in PI3K phosphorylation in 3T3-L1 adipocytes treated for either 30 min ($p < 0.01$) or 24 h ($p < 0.001$). Similarly, IGFBP-2 induced a noticeable increase in AKT phosphorylation in 3T3-L1 adipocytes treated for either 30 min ($p < 0.05$) or 24h ($p < 0.01$) [99]. IGF-1 significantly ($p < 0.001$) increased, whereas insulin failed to induce ($p > 0.05$) AMP-activated protein kinase (AMPK) phosphorylation in 3T3-L1 adipocytes. Similarly, the treatment of adipocytes with IGFBP-2 for either 30 min or 24 h induced a significant ($p < 0.001$) increase in AMPK phosphorylation [99].

Among the seven IGFBPs, IGFBP-2 is the main binding protein secreted by differentiating white preadipocytes, indicating a potential role in the development of obesity. Overexpression of IGFBP-2 was associated with decreased susceptibility to obesity and improved insulin sensitivity [78]. IGFBP-2 expression was associated with fat mass percentage ($p < 0.02$). It was demonstrated that IGFBP-2 is expressed by subcutaneous abdominal adipocytes of obese individuals and that the expression elevated with increasing adiposity and reducing insulin sensitivity [100].

The main functions of IGFBPs are: (1) acting as carrier proteins for circulating IGF-1 and controller of its flow from the vascular space to tissues; (2) increasing IGF-1 half-life and regulating its metabolic clearance [101]; (3) modulating the interaction between IGF-1 and its receptor, and thus indirectly controlling IGF-1 biological activity [102]; (4) modulating IGF-1 in target tissues, inhibiting or activating its specific actions: cell proliferation, differentiation, survival and migration [62,103–105]; and (5) providing a specific localization pool of IGF-1, because IGFBPs can associate with cell membranes or the extracellular matrix (ECM) [106]. Moreover, some IGFBPs can possess some biological effects outside the IGF-1 signaling pathways, such as apoptosis induction and proliferation/inhibition in some tumors [105].

The *IGFBP2* gene has a total length of 32 kb and it is composed by four exons, 2.0 kb (rat) and 1.6 kb (human) *mRNAs* are generated, and the mature protein is approximately 31 kDa and 36 kDa in rats and humans, respectively [107]. The chicken *IGFBP2* gene spans to more than 38 kb on chromosome 7 (GGA7), consists of four exons, and presents similar organization compared with rats and humans.

The chicken *IGFBP2* gene is expressed in a majority of tissues, such as liver, muscle, kidney, heart, ovary, brain, intestine and other tissues [108]. *IGFBP2* gene expression was downregulated in the visceral white adipose tissue of mice, and its circulating levels were reduced in obese mice [96]. Eckstein et al. [109] reported that IGFBP-2 level negatively affected bone size and mineral content in mice, suggesting it was an important regulator of bone biology in vivo.

As for another gene necessary for growth and development processes, the analysis of *IGFBP2/Eco72*I gene polymorphism in the present study showed all three known genotypes (*AA*, *AB*, and *BB*), with an obvious predominance of the heterozygous genotype (56.70%). However, *BB* (265 + 102 bp) homozygotes showed very low frequency (4.12%). The most represented genotype *AB* (367 + 265 + 102 bp) had a similar frequency to the heterozygotes (53.21%) detected by Li et al. [73], who found almost identical frequencies of both homozygotes (*AA* 22.96%, *BB* 23.83%).

The predominance of *A* allele over *B* allele in *IGFBP2* locus in our study may be, similarly to *IGF1* locus, a long-term selection strategy employed in these chicken populations.

The study of Li et al. [73] indicated that chicken *IGFBP2* gene intron 2 C1032T (accession number AY 326194) polymorphism was associated with growth and body composition traits in an F_2 population. Moreover, the *IGFBP2* gene was found to be highly expressed in abdominal fat [73]. QTL for fat deposition was mapped between the marker brackets *LEI0064* and *ROS0019* (75 kb to 27 Mb) on *GGA7* in the chicken linkage map [57], which covers the chicken *IGFBP2* gene (23 to 24 Mb). In Thai native chickens (Chee), the *IGFBP2* gene was significantly associated with body weight at 4 weeks of age, ADG during 0–4 weeks of age and breast width at 16 weeks of age [94].

An excessive abdominal fat in chickens is undesirable and is therefore sought to be reduced, in order to improve the quality of the final product. The IGFBP-2 could inhibit the biological actions of IGF in vivo via endocrine or paracrine mechanisms [22] and indirectly control adipocyte differentiation by regulating the actions of IGF [67]. The structure and function of the *IGFBP2* gene has been analyzed in detail, however, the association of this gene with growth features in chickens has been little studied [66].

In our study, heterozygous genotype *AB* of *IGFBP2* resulted—in both chicken lines—in a higher average BW at 42 days, trunk weight, AFW, breast and thigh muscles, slaughter value and slaughter percentage compared with *AA* genotype (Table 6). On average, chickens with the *IGFBP2-BB* genotype grew slower and simultaneously deposited less fat in the body. These differences, however, were not statistically significant. The lowest breast muscle (without skin) was observed in the Hubbard F15 line chicken with an *AA* genotype of *IGFBP2* (498.75 g).

The findings of higher BW and AFW in heterozygotes in our research are not consistent with the findings of the study of Li et al. [73], which found that F_2 chicken homozygous for the *B* allele (*IGFBP2-BB*) had a higher AFW than birds of the other two genotypes.

The results point to the potential identification of *IGFBP2* as a candidate gene for altering the growth rate and abdominal fat [73]. Reduced growth was associated with increased hepatic *IGFBP2 mRNA* expression and elevated serum IGFBP-2 levels [22], further suggesting IGFBP-2 as a negative growth regulator in vivo [73].

TGF-βs are represented in birds and mammals by three isoforms of secreted cytokines TGF-β1, TGF-β2 and TGF-β3 [70]. The research of Li et al. [69] supported the broad effects of *TGFβ* genes on the growth and development of chickens. Recently, eight from 17 polymorphic sites of the *TGFβ3* gene [53 (T → C), 1653 (C → T), 1755 (A → G), 3343 (C → T), 3540 (C → T), 4786 (C → T), 7263 (C → T) and 7471 (G → A)] have been significantly related to reproduction traits, indicating these polymorphic sites as potential assistant selection markers for improvement of reproductive capacity of Liboyaoshan chicken [110].

The *TGFβ3* gene could be a marker for genetically improving duration fertility in hens. In the recent study performed by Gu et al. [111], four SNPs were identified in intron 1 of *TGFβ3*, and were significantly associated with the duration of fertility in hens ($p < 0.05$). In addition, they identified multi-copy and copy number variants (CNVs) in chicken *TGFβ3*, and later determined significant

associations between *TGFβ3* CNVs and duration fertility in hens. Specifically, the *TGFβ3* copy number exhibited a significant positive correlation with its expression ($p < 0.05$).

A significant association between the *TGFβ3-Bsr*I polymorphism and mortality between 14 and 42 days in broiler chickens was reported by Ye et al. [112].

In addition, significant effects of *TGFβ3-Bsr*I polymorphism on the cecum content *Salmonella enteritidis* bacterial load were found [113], which could have been of great importance, especially in commercial broiler chicken farms. A moderate association ($p < 0.17$) was found between the *TGFβ3-Bsr*I sire allele and antibody response to the *S. enteritidis* vaccine [113]. Polymorphism in the restriction site of *TGFβ3-Bsr*I was associated with *S. enteritidis* burden. The heterozygote *A/C* had the highest *S. enteritidis* burden in the cecum, spleen and liver compared with the other two genotypes ($p < 0.01$). The *C/C* genotype of *TGFβ3*showed the lowest bacterial burden for Village Chickens, whereas in Red Junglefowl, the *A/A* genotype exhibited the lowest *S. enteritidis* colonization [114]. *Salmonella enterica* serovar Enteritidis infection is a common concern in poultry production for its negative effects on growth, as well as food safety for humans [114].

In the study of Li et al. [69], the *TGFβ3* polymorphism in broilers crossed with Leghorn was associated with traits of growth and body composition, such as BW, ADG, breast muscle weight, abdominal fat and spleen weight. In our study, for the *TGFβ3* gene, *AB* genotype was the most common in both chicken lines. The allele A was identified as a dominant allele in Cobb E (64.89%), whereas in Hubbard F15, the frequency of both alleles was identical. This finding is different from another study analyzing *TGFβ3* genotypes in breeder hens [115], where the allele *B* was a dominant allele at *TGFβ3* locus, due to it having the highest frequency (0.81).

For the *TGFβ3* gene, different tendencies were observed in the association of the *A* and *B* alleles with the traits observed within both chicken lines. In Cobb E, the *AB* genotype of *TGFβ3* resulted in the highest average BW at 42 days (3104.29 g). The highest AFW had Cobb E chicken with *AA* genotype of *TGFβ3* (54.95 g). On the contrary, in Hubbard F15, the highest average BW at 42 days and AFW were observed in chickens with the *BB* genotype (2618.00 g, 37.33 g, respectively).

The highest average breast muscle (without skin) was found in the *AB* genotype of *TGFβ3* in both lines (in Cobb E chicken: 653.62 g and in Hubbard F15: 515.55 g). The highest average thigh muscle (with skin) was also found in the *AB* genotype of *TGFβ3* in both lines, with the highest average value in Cobb E (Cobb E: 538.72 g, Hubbard F15: 476.75 g).

On the contrary, the lowest average value of BW at 42 days was observed in the *AA* genotype of *TGFβ3* in both lines (in the Hubbard F15 line chicken with the *AA* genotype: 2541.33 g and in Cobb E in individuals with the *AA* genotype: 2778.50 g). The lowest AFW was found in the chicken with the *AA* genotype in the Hubbard F15 line (31.53 g), and in Cobb E in birds with the heterozygous genotype *AB* (48.90 g). The lowest breast muscle (without skin) was in the Hubbard F15 line chicken with the *AA* genotype of *TGFβ3* (494.33 g) and in Cobb E in the birds with the *BB* genotype (551.00 g). The lowest thigh muscle (with skin) was observed in the Hubbard F15 line chicken with the *AA* genotype (469.00 g), and in the Cobb E line chicken with the *BB* genotype of *TGFβ3* (458.67 g).

Association analysis showed that *Bsl*I genotypes of *TGFβ3* are related to some performance traits (Table 8). The statistical analysis revealed a significant association of *TGFβ3* with BW at 21, 28 and 35 days and trunk weight in the codominant (negative value), dominant and overdominant (positive values) genetic model, and with slaughter value in codominant (negative value), recessive and overdominant (positive values) genetic model.

The average slopes of the growth curve (14–42 days of age) constructed according to the line, genotype and sex (Figure 4) was confirmed to be a result of linear growth, as well as lineage and sex differences in body weight. Constructing the graphs of total integrals for the weight sum of the trunk, giblets, abdominal fat, breast and thigh muscles at 42 days of age (at the slaughter of chickens) in both chicken lines (with a separate evaluation of both sexes) and all genotypes observed (Figure 5) showed interesting tendencies. However, no statistically significant dependence was observed, despite apparent differences.

5. Conclusions

The presented study demonstrated that the point mutation can affect chicken growth, and confirmed some significant associations between SNP and growth traits. Based on these findings, it can be concluded that the *TGFβ3* gene could be applied as a candidate gene marker for chicken growth traits in the Hubbard F15 and Cobb E broiler line population selection program. However, further association analysis will be required to clarify the effects of this marker on growth and production traits in the broiler chicken population.

Author Contributions: All authors participated in the manuscript. B.H. performed genetic analyses and wrote manuscript, K.V. optimized laboratory methodology, performed genetic analyses and participated in writing manuscript, R.K. evaluated experimental data and critically revised the manuscript, R.B. evaluated genetic analyses, J.K. collected samples and revised manuscript, V.C. expertly revised the manuscript, F.K. and C.F. revised manuscript, V.M. and H.H. were responsible for running and conducting the experiment. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

ADG	average daily gain
AFW	abdominal fat weight
AKT	serine/threonine kinase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
BACs	bacterial artificial chromosomes
BAD	BCL-2 antagonist of cell death
BCL-2	antiapoptotic B-cell lymphoma/leukemia-2
bp	base pairs
BW	body weight
CI	confidence interval
CNVs	copy number variants
cM	centimorgan
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FOXO	Forkhead box, class O; subfamily of Forkhead transcription factors
Gb	giga base pairs
GGA1, GGA7	Gallus gallus autosome 1, Gallus gallus autosome 7
GH	growth hormone
GSK-3β	glycogen synthase kinase-3β
HWE	Hardy-Weinberg equilibrium
IGF	insulin-like growth factor
IGF-1	insulin-like growth factor 1
IGF1	insulin-like growth factor 1 gene
IGF-1R	the type 1 insulin-like growth factor receptor
IGF-2R	the type 2 insulin-like growth factor receptor

IGFBP	insulin-like growth factor binding protein
IGFBP-2, IGFBP-3	insulin-like growth factor binding protein 2, 3
IGFBP2	insulin-like growth factor binding protein 2 gene
InsR	insulin receptor
IRS	insulin receptor substrate
IU	international units
kb	kilobase pairs
LDL	low-density lipoprotein
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
Mb	megabase pairs
MEK	mitogen-activated protein kinase kinase 7 (MAP2K7)
MMAS	molecular marker-assisted selection
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTOR-C1, mTOR-C2	complexes of mTOR
p27	a protein of 27 kDa that regulates the cell cycle
PI3K	phosphatidylinositol-3-OH kinase
PCR	polymerase chain reaction
PDK1	3-phosphoinositide-dependent protein kinase-1
QTL	quantitative trait locus
RAF	proto-oncogene serine/threonine-protein kinase
RAS	a protein playing a key role in signal transduction of cell growth and differentiation
RFLP	restriction fragment length polymorphism
S6K-1/2	ribosomal protein S6 kinase 1/2
SD	standard deviation
SHC	the SRC homology and collagen protein
SMADs	proteins that are the main signal transducers for receptors of TGFß
SNP	single nucleotide polymorphism
SRC	proto-oncogene tyrosine-protein kinase
T_3	triiodothyronine
TBE buffer	Tris-borate-EDTA buffer
TGFß	transforming growth factor β
TGFß3	transforming growth factor β3 gene
TSC-1/2	tuberous sclerosis protein $\frac{1}{2}$
4E-BP1	eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1

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Effects of Dietary Inclusion of Bilberry and Walnut Leaves Powder on the Digestive Performances and Health of Tetra SL Laying Hens

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Simple Summary: In poultry, diet composition influences growth performance, egg production, as well as digestion. In this study, the effects of dietary additives obtained from bilberry and walnut leaves powder on the digestive performances of Tetra SL hens were evaluated by histologic and morphometric analyses of the intestinal mucosa as well as by the enzymatic activity measurements of alpha-amylase, invertase, maltase, and trypsin correlated with cecum microbiota.

Abstract: The purpose of this study was to examine the effects of dietary inclusion of two additives at the final concentration of 0.5% bilberry (E1) and 1% walnut (E2) leaves powder in the basal diet on digestive health of hens. A total number of 90 Tetra SL hens were divided into two experimental groups (E1 and E2) and one control group (C) consisting of 30 hens each. After four weeks, 10 hens of each group were sacrificed and tissue samples and intestinal content were taken from the duodenum, jejunum, and cecum in order to perform histological, enzymatic, and microbiota analyses. In groups E1 and E2, the histological analysis showed a significant increase of villus height, resulting probably in increased absorption of nutrients in duodenum and jejunum. A decrease in the specific activity of alpha-amylase and trypsin in E1 and E2 for both duodenum and jejunum compared to the control one was also recorded. In addition, the maltase and invertase specific activity in duodenum increased, a tendency that was kept for maltase but not for invertase in jejunum. The cecal microbiota of E1 and E2 individuals was characterized by an increase of *Firmicutes* and *Lactobacilli* and a decrease of *Enterobacteriaceae*. In conclusion, our results indicate that bilberry and walnut leaves additives in feed may improve the health status of the poultry gastrointestinal tract.

Keywords: bilberry leaves; walnut leaves; laying hens; digestive enzyme activities; nutrition

1. Introduction

Tetra SL is a brown egg-laying hybrid widely used for its excellent internal and external egg quality as well as for efficient and long-term egg production. Animal performance improvement is the most important detail from an economic point of view, especially in the livestock industry. Composition of diets given to hens [1] influences growth performance, egg production, as well as digestion. In poultry nutrition, energy suppliers and proteins are the most important feed constituents after water. Cereals provide about 70% of energy, whereas other sources supply the rest. Recently, phytogenic feed additives received attention as alternatives to prebiotics, probiotics, and antibiotics in laying hen nutrition. Some herbal feed inclusions were shown to improve performance, immunity, and antioxidant status in laying hens [2]. Other ones did not affect body weight but improved egg production; weight; and quality regarding yolk color, cholesterol, and malondialdehyde compared to the control [3]. Furthermore, bilberry and walnut leaves powder included in the diet of laying hens increased the antioxidant properties of egg yolks [4] but did not affect other performance indicators.

The avian gastrointestinal tract is divided into nine discrete segments: the oral cavity, esophagus, crop, proventriculus, gizzard, small intestine, ceca, large intestine, and the cloaca. The small intestine is the major site for digestion and absorption of nutrients and influences the rates of energy intake, feeding behavior, and energy allocation [5]. The electrolytes together with digestive enzymes secreted by pancreas and intestinal glands and those produced by mucosal cells are responsible for the hydrolysis of macronutrients. The initial digestion of feed is carried out by pepsin and pancreatic proteases, peptidases, lipase, and amylase [6,7].

Currently, there are limited scientific data regarding the relationship between the nutritional quality of feed used in poultry farming and digestive enzymes [8,9]. Bird [10] determined the distribution of trypsin and amylase in different segments of duodenum in Leghorn chickens. Thus, the first three quarters of the duodenum have a content of 45% trypsin, with 55% in the last part, and for amylase, the content is 23% in the first part, most of the activity of amylase in duodenum being in the last third, almost from the point where the pancreatic ducts communicate with the duodenum [11].

Functional anatomical and histological characteristics of the avian gastrointestinal tract are critical to their feed conversion efficiency. To facilitate maximal absorption of dietary components, the intestinal mucosa is highly convoluted and specialized. The epithelium is folded into villi and the epithelial cells have apical microvilli, forming a brush border observed by optical microscopy. These infoldings increase the small intestinal surface area for absorption by about 600-fold, resulting in a higher capacity for nutrient absorption [12]. Intestinal morphology (villus height and crypt depth) changes in response to exogenous agents. Deeper crypts indicate faster tissue turnover as they contain progenitor cells. Intestinal mucins are high molecular weight glycoproteins secreted by goblet cells. In chickens, mucins are observed to be extensively expressed by goblet cells in the colon and small intestine [13].

Also, the gastrointestinal tract of poultry has a diverse and complex microbiota that plays a significant role in the digestive process and the absorption of nutrients, maintaining immune system development and pathogen exclusion, which are vital for improvement of digestion, health, and growth performance [14]. Poultry microbiota composition depends on many factors including the exposure to growth environmental, intestinal segment, and diet [15]. The esophagus, crop, and cloaca are considered semi-aerobic environments, allowing mixed communities of aerobes, micro-aerobes and facultative anaerobes, including members of the α, β, and γ-Proteobacteria. The internal sections of the gastrointestinal tract located between the crop and cloaca are dominated by obligate or facultative anaerobes, including members of the *Firmicutes* and *Proteobacteria* [16].

Obviously, the improvement of poultry growth performance depends on intestinal health and consequently on microbiome composition. An appropriate microbiota is favored by different vegetal additives. In this context, a method to improve animal performance is the use of vegetal feed additives, which have beneficial effects in livestock production as well as in health and nutrition of animals, which might arise from activation of feed intake and digestive secretions. These have also antimicrobial, antiviral, antioxidant, and immunomodulatory properties [17,18].

Starting from the fact that plants with high phenolic content have strong antioxidant power [19], and due to the few data regarding the use of plant leaves as additives in the diets of laying hens and their nutritional assessment, in this study, we have chosen to use bilberry and walnut leaves powder. *Vaccinium myrtillus* is a species of the genus *Vaccinium* from the family *Ericaceae* [20]. Fruit and aerial parts of plant are known as a natural source of food and drink due to their richness in nutritional and antioxidant compounds and can also be integrated into food supplements and pharmaceuticals for preventing urinary tract infections [21] and cerebral vascular accidents [22]. Bilberry has several effects such as prevention or even reversal in a considerable degree of age-related object memory decline of rats [23] and antioxidant [24,25], anti-inflammatory, anticancer, anti-neurodegenerative, and cardioprotective effects [26,27] due to their phenolic compounds, including proanthocyanidins, flavonoids, stilbenoids, phenolcarboxylic acid derivatives, and flavonol glycosides [20].

Juglans regia L. belonging to the *Juglandaceae* family is the most well-known member, representing an important species of deciduous trees. Walnut leaves have been considered as a beneficial source of health, with important amounts of phenolic compounds [28], and have been intensively used in traditional medicine [29] for the treatment of venous insufficiency and hemorrhoids and for their antidiarrheal, anthelmintic, astringent, keratolytic, antifungal, hypoglycemic, hypotensive, and sedative effects [29]. Also, they have high content in flavonoids, alkaloids, saponins, steroids, and tannins [30].

Previously, it has been shown that walnut leaves administration reduced the proliferation of *Clostridium perfringens* in chickens [31]. Also, Mousavi et al. [32] showed that supplementation of broiler chicken diet with a green husk of walnut powder improved the function of the immune system. The goal of this study was to examine the consequences of the dietary inclusion effects of additives obtained from bilberry and walnut leaves powder on the microbiota from cecum correlated with digestive performances of Tetra SL hens by histologic and morphometric analyses of the intestinal mucosa as well as the enzymatic activities measurements. As far as we know, this is one of the first studies regarding the correlation between dietary inclusion of some plant leaves and the health of poultry gastrointestinal tract.

2. Materials and Methods

2.1. Plant Material and Antioxidant Capacity

Phytochemical characterization of plant material and leaf samples of bilberry and walnut were obtained from local pharmacies (S.C. Stefmar productie S.R.L, Râmnicu Vâlcea, Romania). The leaf extracts of bilberry and walnut were prepared according to the method described by Coșarcă et al. [33]. A mass of 1 g of dried vegetable material was mixed with 40 mL distilled water and heated at 90 °C for 45 min with shaking. The suspension was then centrifuged at 2370× *g*, and the supernatant was stored at −20 °C until analysis was done [34].

The total polyphenol content in extracts was quantified according to the Folin–Ciocalteu method as described previously [35]. A sample of 50 µL was homogenized with 250 µL of 1/10 diluted Folin–Ciocalteu reagent and incubated for 1 min at room temperature. Then, a volume of 750 µL of 7.5% (w/v) Na_2CO_3 solution was added. The mixture was brought to a final volume of 5 mL and then incubated in the dark for 2 h at 25 °C. At the end, the optical density was measured at 760 nm using distilled water as a blank. The concentration of polyphenols was calculated using a gallic acid calibration curve. The results were expressed in milligrams of gallic acid (GAE) per gram of dried weight (d.w.) (mg GAE/g dw).

The antioxidant capacity of leaf extracts of bilberry and walnut was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to the method of Burits and Bucar [36]. Different leaf extract concentrations were mixed with 0.04% DPPH at a ratio of 1:100. After 30 min of incubation in the dark at room temperature, the absorbance of samples was measured at 517 nm using a FlexStation 3 multi-mode microplate reader (Molecular Devices LLC, San Jose, CA, USA) [37].

Oxygen radical absorbance capacity (ORAC) assay was performed according to the method of Davalos et al. [38]. A volume of 20 μL of extract or phosphate buffer (for blank) was incubated with 120 μL of 70 nM fluorescein for 15 min in darkness at 37 °C. The peroxyl radical was generated by adding 60 μL of 12 mM 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), which was freshly prepared before each test. After 80 min of incubation in darkness at room temperature, the fluorescence intensity (FL) was recorded (excitation wavelength at 485 nm; emission wavelength at 520 nm) for 80 min at intervals of one minute using the FlexStation 3 multi-mode microplate reader (Molecular Devices LLC, San Jose, CA, USA). In parallel, a standard curve was prepared with Trolox (6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid) at concentrations ranging 0–100 μM (0, 12.5, 25, 50, 75, and 100 μM) [37].

2.2. Hens and Experimental Treatments

A total of 90 Tetra SL laying hens (aged 32 weeks) was assigned into two experimental groups (E1 and E2) and one control group (C) with 30 birds each (ten replicates each; 3 birds/replicates; and a total of 30 cages) and housed in an experimental hall under controlled environmental conditions (temperature, humidity, and ventilation) in 3-tier batteries and 16 h/24 h light regimen. Feed and water were offered ad libitum during the experiment. The corn–soybean meal basal diet (2800 kcal/kg metabolizable energy (ME) and 17.8% crude protein (CP) was the same for all groups as described by Untea et al. [4], which contained 30% corn; 31.46% wheat; 4% gluten, 21.2% soybean meal; and 1.46% vegetable oil and other components per kg. Unlike the diet formulation for group C, the experimental diets included two different herbal feed additives as follows: 0.5% bilberry leaves (E1) and 1% walnut leaves (E2). Diet formulations were calculated in agreement with the feeding requirements of laying hens as given by National Research Council [39]. After four weeks, 10 hens of each group (randomly selected from each cage) were slaughtered with the approval (case no. 5148/10.08.2018) of the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Balotești, Romania (Ethical Committee no. 52/30.07.2014).

Performance parameters regarding feed intake, feed conversion ratio, egg production, egg weight, and laying percentage were monitored on the experimental period. Herbal feed additives used did not influence the production performances of the birds except for the weight of the eggs, the results being presented previously by Untea et al. [4] as a part of this study. Also, the antioxidant stability of egg yolk was increased due to higher concentrations of lutein and zeaxanthin, that is very important for human nutrition [4].

2.3. Sample Collection for Analyses

The small intestine from each individual was collected, and intestinal content and tissue samples were taken from the duodenum and jejunum in order to perform enzymatic and histologic analyses. The intestinal contents from duodenum jejunum and cecum were also collected. The total protein extracts were obtained by homogenization of 1 g tissue in 10 mL phosphate buffer, pH 7.4. The suspensions were kept one hour at 4 °C and centrifuged at 9600× *g* for 10 min. The resulting supernatants were used for the determination of maltase and invertase activities. Also, the activities of alpha-amylase and trypsin were measured in the intestinal content, whereas the microbiological analyses were performed on the cecal content.

2.4. Light Microscopy Examination

The intestinal segments were immersed in 4% paraformaldehyde in phosphate buffered saline (PBS) solution and dehydrated in a graded series of ethanol. Finally, each specimen was embedded in paraffin and cut into 4-μm sections using a microtome. Hematoxylin and Eosin (H&E) (Merck, Darmstadt, Germany) stain was performed. Ten villi and crypts of Lieberkühn from duodenum and jejunum segments of each bird were measured using an optical microscope (Olympus BX43, Tokyo, Japan), a camera (30 XC Olympus, Tokyo, Japan), and image analysis software (Olympus Cell

Sense Dimension, Tokyo, Japan). For measurement of villus height and widths of crypt, mucosa segments were randomly selected from each cross section. A total of 10 villus heights (measured from the tip of the villus to the villus–crypt junction) and the depth of 10 crypts (measured from the crypt–villus junction to the base of the crypt) from cross sections of each individual were analyzed.

2.5. Enzymatic Analysis

2.5.1. Maltase Activity Assay

Maltase (EC 3.2.1.20) activity was determined using the Maltase assay kit instructions from My BioSource (San Diego, CA, USA). For each sample, an appropriate control was prepared. A volume of 25 µL from sample was mixed with 50 µL substrate (maltose) and incubated for 20 min at 37 °C. Then, the reaction was stopped by 25 µL stop solution and the mixture was centrifuged at 4000× *g* for 10 min. The supernatant was mixed with 200 µL of chromogenic agent, homogenized, and incubated for 15 min at 37 °C. The absorbance was measured using the Flex Station 3 Multi-Mode Microplate Reader (Molecular Devices LLC, San Jose, CA, USA) automatic plate reader at 505 nm. The enzymatic activity was calculated using a calibration curve with glucose. The results obtained were expressed in U/mg of protein.

2.5.2. Invertase Activity Assay

The enzymatic activity of invertase (EC 3.2.1.26) was evaluated according to the Invertase assay kit instruction (code MAK 118; Sigma-Aldrich, Darmstadt, Germany). Briefly, over a volume of 40 µL from each sample was added 5 µL of sucrose solution in each well. Then, the mixture was incubated for 20 min at room temperature. After this interval, a volume of 90 µL reaction mixture containing enzyme and dye reagent was added and incubated for 20 min in dark. The absorbance was measured at the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices LLC, San Jose, CA, USA) automatic plate reader at 570 nm. As a standard, a 100 µM glucose solution was used. The results obtained were expressed in U/mg of protein.

2.5.3. Alpha-Amylase Assay

Alpha-Amylase (EC 3.2.1.1) activity was determined according to Bernfeld [40]. The amount of reducing sugars released from soluble starch was measured using an alkaline 3, 5-dinitrosalicylic acid (DNS) reagent. Therefore, 100 µL of 20 mM Na_2HPO_4–NaH_2PO_4 (pH 6.9) buffer and 100 µL of 1% soluble starch solution were mixed with 5 µL of sample and incubated for 10 min at 25 °C. The reaction was stopped by addition of 200 µL of DNS reagent. The reaction mixture was heated at 100 °C for 4 min. The reducing groups were quantified at 546 nm with a FlexStation 3 Multi-Mode microplate reader. One unit of activity represented the amount of enzyme that released one µmole of maltose in one minute at 25 °C. Enzyme activity was expressed as specific activity (units per gram of protein).

2.5.4. Trypsin Assay

Trypsin (EC 3.4.21.4) activity was determined according to the method described by Hummel [41] using *N*-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) as substrate. The change in absorbance at 247 nm was measured for 10 min at 25 °C on a FlexStation 3 Multi-Mode microplate reader (Molecular Devices LLC, San Jose, CA, USA). The reaction mixture contained 216.6 µL of 50 mM Tris-HCl (pH 8.0) buffer, 30 µL of 10 mM TAME, and 8.66 µL of sample. One unit of trypsin activity was defined as the amount of enzyme hydrolyzing one micromole of TAME in one minute at 25 °C. Enzyme activity was expressed as specific activity (units per gram of protein).

2.6. Protein Determination

Protein concentration was determined by Bradford method [42] using bovine serum albumin as standard.

2.7. Microbiota Characterization

The Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) technique was used to analyze the changes induced by the different diets at the microbiota level. Microbial DNA extraction was performed using a commercial kit (AllPrep PowerViral DNA/RNA Kit, Qiagen, Hilden, Germany). Briefly, 250 mg of cecal content was subjected to treatment with cell lysis matrix (PowerBead Tubes, Glass 0.1 mm, Qiagen, Hilden, Germany) as well as enzymatic digestion that resulted in nucleic acid isolation. Nucleic acids were subsequently subjected to purification (based on MB Spin Columns, according to the manufacturer instructions). Microbial DNA concentration and purity were spectrophotometrically quantified. The concentration of all DNA samples was adjusted to 3 ng/µL in DNAse and RNAse free water. The relative abundance of microorganisms in cecal DNA was measured by qRT-PCR on Applied Biosystem ViiA7. The total number of bacteria in the samples was quantified using universal primers for 16S rRNA. qRT-PCR reactions were performed using SYBR Green Master mix (Applied Biosystems) and specific primers for different taxa (all primers were selected from literature, and their sequences are shown in Table 1) [43–46]. Each PCR reaction included 200 nM forward and reverse primer, 9 ng DNA, and 2xSYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The samples were incubated at 95 °C for 5 min and then amplified by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s.

Table 1. Primer sequences used for microbiota characterization.

Taxonomic Target	Primer Sequence
Eubacteria	UniF340 ACT CCT ACG GGA GGC AGC AGT UniR514 ATT ACC GCG GCT GCT GGC
Lactobacilli	LabF362 ACG AGT AGG GAA ATC TTC CA LabR677 CAC CGC TAC ACA TGG AG
Enterobacteriaceae	Uni515F GTG CCA GCM GCC GCG GTAA Ent826R GCC TCA AGG GCA CAA CCT CCA AG
Bacteroidetes	Bact934F GGAACATGTGGTTTAATTCGATGAT Bact1060R AGCTGACGACAACCATGCAG
Firmicutes	Firm934F GGAGCATGTGGTTTAATTCGAAGCA Firm 1060R AGCTGACGACAACCATGCAC

2.8. Statistical Analysis

Statistical analysis of data was performed with GraphPad Prism software (Version 6, GraphPad, San Diego, CA, USA) using one-way ANOVA, followed by Bonferroni's post hoc test. For histology, enzymology, and microbiota experiments, the number of replicates was n = 10. All values are expressed as mean ± standard deviation (SD) of three replicates, and the data were considered statistically significant only if the *p*-values were less than 0.05.

3. Results

3.1. Composition Analysis of Vegetable Additives

The antioxidant potential of each leaf extract was estimated using three methods: total polyphenols content measurement, DPPH free-radical scavenging activity, and ORAC assay (Table 2).

Table 2. Phytochemical characterization of plant material.

Parameters	Bilberry Leaves		Walnut Leaves	
	Average	**SD**	**Average**	**SD**
TPC (μg GAE/g d.w.)	392.644	±12.531	192.025	±10
DPPH (% inhibition)	84.807	±1.24	57.589	±3.17
ORAC (μmol Trolox/g d.w.)	328.908	±7.21	175.700	±4.5

TPC: Total polyphenolic content; GAE: Gallic Acid Equivalents; d.w: dry weight; DPPH: the antioxidant capacity using the 2,2-diphenyl-1-picrylhydrazyl radical; ORAC: Oxygen radical absorbance capacity value. All data are reported as mean plus or minus standard deviation (SD), (n = 3).

According to our data, the total polyphenol content is almost double in the bilberry leaves compared with the walnut leaves (Table 2).

Also, the extract from bilberry leaves exhibited an 84.8% DPPH scavenging activity, confirming that this feed additive acts as a free radical scavenger. At the same time, it was an effective scavenger of the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical, according to the ORAC test. The antioxidant activity of walnut leaves extract was lower, presenting a 57.59% DPPH scavenging activity and almost half capacity to counteract the ABTS radical in comparison with bilberry leaves.

3.2. Histology of the Duodenum

The duodenal villi were lined by a simple columnar epithelium, followed by a *lamina propria* made by connective tissue rich in vascular network, which absorbs the digestive products, and a *muscularis mucosa*, which underlies the base of crypts. The epithelium has a normal aspect, consisting of absorptive cells, called enterocytes, and individual goblet cells, which secrete mucin, for protection and lubrication of the intestinal contents. Brunner's glands were not present in duodenum (Figure 1).

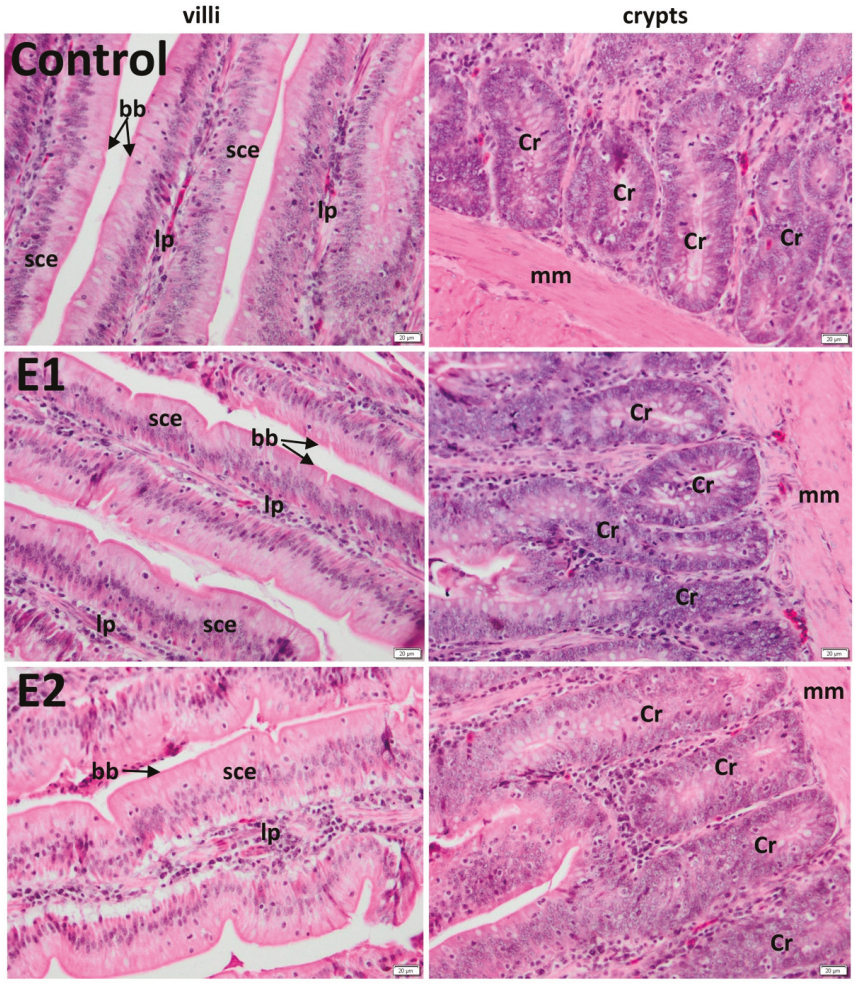

Figure 1. Effect of bilberry and walnut leaves supplementation diet on duodenum morphology of laying hens: The basal diet served as the control, and different levels of herbal feed additives were supplemented to the basal diet as follows: 0.5% cranberry leaves (E1) and 1% walnut leaves (E2). Sce: simple columnar epithelium; lp: lamina propria; cr: crypt; mm: muscularis mucosa. bb: brush border; H&E (Hematoxylin and Eosin) stain; (n = 10); n: number of replicates.

3.3. Histology of the Jejunum

Histology of the jejunum has a normal aspect (Figure 2).

The average lengths of villi of duodenum were 891.55 µm (control), 1158.84 µm (E1), and 1263.44 µm (E2), whereas the average widths of crypts of duodenum were 189.94 µm (control), 189.49 µm (E1), and 189.46 µm (E2) (Table 3).

Also, the average lengths of villi of jejunum were 905.98 µm (control), 1258.04 µm (bilberry leaves E1), and 1248.7 µm (walnut leaves E2). Moreover, the average widths of the crypts of jejunum were 209.51 µm (control), 210.26 µm (E1), and 211.39 µm (E2) (Table 3). Villus height (Table 3) was significantly higher for the experimental groups compared to control ($p < 0.001$).

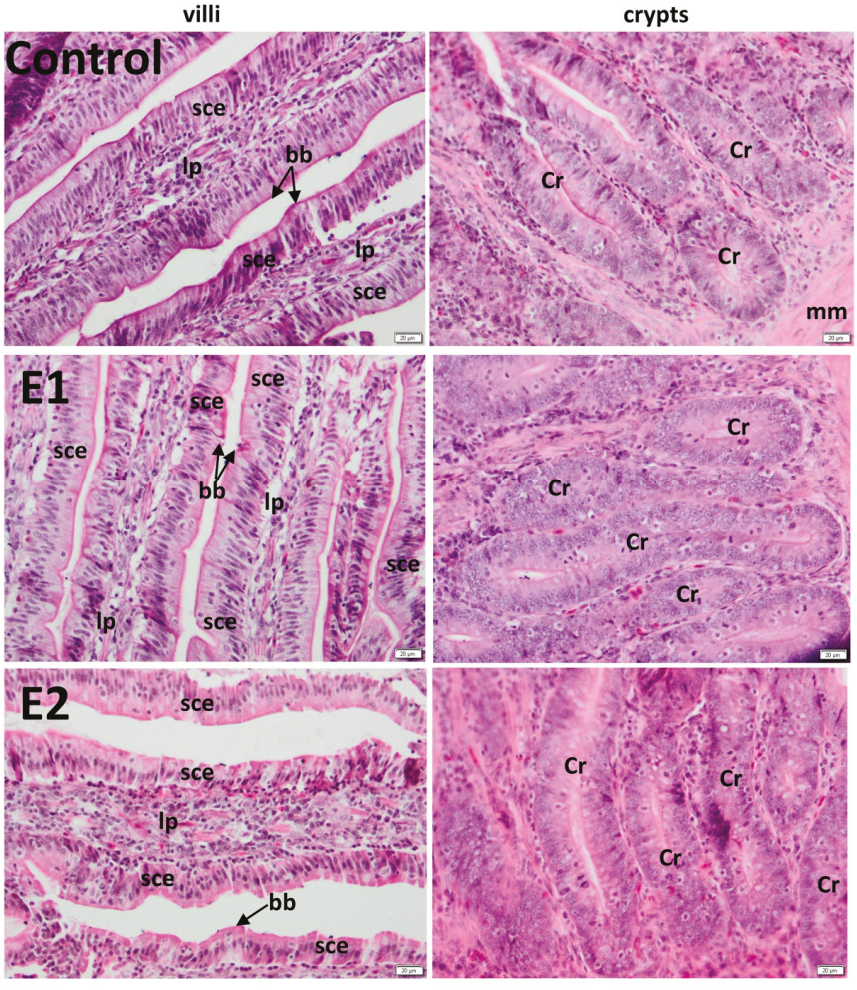

Figure 2. Effect of bilberry and walnut leaves supplementation diet on jejunum morphology of laying hens: The basal diet served as a control, and different levels of herbal feed additives were supplemented to the basal diet as follows: 0.5% cranberry leaves (E1) and 1% walnut leaves (E2). Sce: simple columnar epithelium; lp: lamina propria; Cr: crypt; mm: muscularis mucosa. bb: brush border; H&E (Hematoxylin and Eosin) stain; (n = 10); n: number of replicates.

Table 3. Measurements of the villi length and widths of crypt for the control and experimental groups.

	Duodenum		Jejunum	
Group	**Villus (μm)**	**Crypt (μm)**	**Villus (μm)**	**Crypt (μm)**
C	891.55 ± 15	189.94 ± 11	905.98 ± 27	209.51 ± 27
E1	1158.84 ± 20 ***	189.49 ± 11 ns	1258.04 ± 21 ***	210.26 ± 44 ns
E2	1263.44 ± 23 ***	189.46 ± 14 ns	1248.7 ± 18 ***	211.39 ± 37 ns

C group: basal diet/control group; E1 group: basal diet with 0.5% bilberry leaves and E2 group: basal diet with 1% walnut leaves. All data are reported as mean values ± standard deviation (SD) and statistical significance, where ns $p > 0.05$, *** $p \leq 0.001$; (n = 10); n: number of replicates.

3.4. Intestinal Enzymes Activities

In duodenum, the activity of maltase (Table 4) increased insignificantly by 157% for E1 by 264% for E2 compared to control. In jejunum, the same enzymatic activity decreased by 86% in the case of the E1 group, while for E2, it was increased by 330% (Table 4) compared to control. Regarding the specific activity of invertase in duodenum, it was unmodified in group E1 whereas, in group E2, it increased significantly by almost 11.61% compared to the control (Table 4). In contrast, the administration of basal diet enriched with 1% walnut leaves decreased significantly by 8.89% in jejunum. The basal diet supplemented with 0.5% bilberry leaves (E1) determined an increase of invertase specific activity at the jejunum level compared to the control level.

Table 4. Influence of dietary source on enzymatic specific activity (U/mg protein) of maltase, invertase, alpha-amylase, and trypsin of duodenum and jejunum of laying hens.

Intestinal Segment	Group	Maltase (U/mg)	Invertase (U/mg)	Alpha-Amylase (U/mg)	Trypsin (U/mg)
Duodenum	C	443.87 ± 92.65	8.68 ± 3.49	208.69 ± 23.63	131.33 ± 52.29
	E1	600.63 ± 306.37 ns	7.98 ± 7.37 ns	54.89 ± 10.91 *	52.43 ± 8.95 ***
	E2	707.39 ± 245.02 ns	20.29 ± 6.68 ***	63.43 ± 23.74 ns	42.95 ± 1.75 ***
Jejunum	C	559.18 ± 25.19	25.69 ± 2.22	18.14 ± 5.5	98.01 ± 8.17
	E1	473.00 ± 64.34 ns	32.23 ± 5.96 ns	14.06 ± 3.88 **	61.68 ± 28.34 *
	E2	889.09 ± 79.07 ns	16.80 ± 3.43 ***	15.37 ± 3.85 ns	71.24 ± 16.66 *

The basal diet served as a control (C), and different levels of herbal feed additives were supplemented to the basal diet as follows: 0.5% bilberry leaves (E1) and 1% walnut leaves (E2). All data are reported as mean values ± standard deviation (SD) and statistical significance, where ns: $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; (n = 10); n: number of replicates.

Our experimental data revealed a decrease in the specific activity of alpha-amylase in experimental groups for both duodenum and jejunum compared to the control. Furthermore, the enzymatic activity of trypsin from both intestinal segments was insignificantly decreased and below the control group level in all experimental groups.

3.5. Intestinal Microbiota

The modification of the diet recipe caused the appearance of changes in the intestinal microbiota. These were evident at the phylum level as well as at the bacterial population level. In general, *Firmicutes* and *Bacteroidetes* are the most abundant phyla, followed by *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Fusobacteria* [47]. The major phyla that make up the gut microbiota (*Bacteroidetes* and *Firmicutes*) have been analyzed. The administration of the commercial recipe enriched with bilberry leaves (E1) or walnut leaves (E2) led to a moderate decrease in the level of *Bacteroidetes* and an increase in the abundance of *Firmicutes* (Table 5).

Table 5. The relative abundance of *Firmicutes, Bacteroidetes, Lactobacillus* sp, and *Enterobacteriaceae* phyla as determined by The Real-Time Quantitative Reverse Transcription PCR (qRT-PCR): Eubacteria 16S rRNA was used for normalization.

Group	*Bacteroidetes*	*Firmicutes*	*Lactobacillus* sp.	*Enterobacteriaceae*
C	1.15 ± 0.39	0.90 ± 0.41	1.11 ± 0.59	0.73 ± 0.33
E1	1.09 ± 0.21 ns	1.45 ± 0.52 ns	1.26 ± 1.57 **	0.005 ± 0.01 **
E2	0.97 ± 0.17 ns	1.56 ± 0.35 ns	4.55 ± 1.34 **	0.058 ± 0.05 **

C: control group; E1 group: basal diet with 0.5% bilberry leaves and E2 group: basal diet with 1% walnut leaves. All data are reported as mean values ± standard deviation (SD) and statistical significance, where ns: $p > 0.05$, ** $p \leq 0.01$; (n = 10); n: number of replicates.

Generally, the personalization of diet formulas aims to induce beneficial modifications of the microbiota by increasing the number of bacteria with probiotic potential (e.g., lactobacilli) and by decreasing the level of potentially pathogenic bacteria (pathobionts) such as *Enterobacteriaceae*. In this

context, we quantified the abundance of lactobacilli and members of the *Enterobacteriaceae* family in isolated cecal samples from hens fed with different dietary formulas. For both analyzed groups, a significant decrease of the level of *Enterobacteriaceae* was observed as well as a statistically significant increase of the level of lactobacilli (Table 5).

4. Discussion

The poultry industry is one of the most dynamic animal industries. Feed efficiency and high performance of birds as well as the quality of eggs are the crucial goals in poultry production. In this context, the quality of diet along with environmental conditions and health of birds need to be considered to achieve these goals [48].

Nowadays, different herbal additives have been investigated for antioxidant, antimicrobial, and anti-inflammatory activities; growth-promoting effects; and egg quality. The beneficial impact of herbal additives could be due to the polyphenolic composition, which influences sugar digestion and absorption of nutrients in the small intestine [49]. Higher antioxidant protection of bilberry leaves compared to walnut ones could be conferred by the raised polyphenol content that can neutralize free radicals and can inhibit the propagation of free-radical reactions [50,51].

In the current study, histological evaluation showed that bilberry and walnut leaves powder supplementation of diets have exerted beneficial effects in the duodenum and the jejunum morphology, materialized in significant increases of villus height, resulting probably in increased adsorption of nutrients.

This hypertrophy of villi and, by default, of their epithelial cells resulted in an increased surface area and capacity of absorption [52], corresponding to raised activities of intestinal enzymes [53,54] probably due to feed supplementation with bilberry and walnut leaves powder. Previously, it has been stated that villi height and crypt depth ratio in the small intestine has a direct influence on the absorptive function [55]. These have been observed in chickens after the administration of zeolite [56], L-glutamine [57], and clinoptilolite [55].

In poultry, the activity of digestive enzymes located in the brush border membrane of enterocytes plays a significant role in the physiological processes occurring in the digestive tract that depend on nutritional feeding and characteristics of diet [5]. Previously, it has been shown that activities of digestive enzymes are affected by the amount, composition, and regime of food intake during the growing phase [11]. Several studies have shown that the activities of proteases in the intestinal juice are modified according to the amount of protein in the diet, while the activities of amylase and lipase depend on the content of food in carbohydrates and lipids as substrates for their activity [7].

According to our data, a strong negative correlation between the total polyphenol content and a decreased alpha-amylase and trypsin activities both in duodenum and jejunum was observed (Table 2).

The alpha-amylase catalyzes hydrolysis of the internal α-1, 4-glycosidic linkages in starch, generating glucose and maltose. In our study, the activity of alpha-amylase is decreased in both experimental groups E1 and E2 for duodenum and jejunum compared to control. Considering that amylase activity is diminished, probably oligosaccharides escaped digestion in the small intestine and reached the cecum, where they were fermented by microbiota producing short chain fatty acids [58] and other aminated compounds with beneficial impact on hens. There are three main short chain fatty acids, namely acetate, propionate, and butyrate, that represent signaling molecules with epigenetic impact [59].

Trypsin is responsible for protein and peptides degradation into amino acids. The peptides hydrolysis stage is important in protein absorption because, although these are smaller in size compared to proteins, they are still too large to be absorbed by the small intestine mucosa [60]. Our results show that trypsin activities from duodenum and jejunum were insignificantly decreased and below the control level in all experimental groups E1 and E2, probably due to covalent attachment of the phenolic compounds from feed additives to reactive nucleophilic sites of the enzyme, affecting its

three-dimensional conformation and the active site. As a result, the rate of the catalyzed reaction decreased significantly [61].

Maltase and invertase are key enzymes of carbohydrate digestion; therefore, the relationship between cecum microbiota and small intestine enzyme activity could be due to glucose and fructose presence in the gastrointestinal tract, which could favor the *Lactobacilli* strains' presence as previously has been proved [62,63].

It has been shown that partial digests of protein in the culture medium was essential for growth of some lactic bacteria [64]. Also, peptides resulting in the small intestine due to trypsin activity could favor the relative abundance of *Firmicutes* [65].

An interesting observation is that, in hens from the E1 and E2 groups, duodenal invertase activity increased compared to control, whereas in the case of jejunum, this activity was higher for E1 and decreased for E2. Invertase is an enzyme with two active centers [66], one that catalyzes the hydrolysis of sucrose and the other involved in the hydrolysis of both maltose and other alpha-glucosides.

Probably due to the increased activity of invertase in the E2 group in duodenum, a lower amount of sucrose was present in jejunum, and this could explain the decreased invertase activity (Table 4) and the increased maltose hydrolysis (Table 4).

For *Aves* species, the digestion starts at the oral cavity and ends at the cloacal level, crossing several "stations" represented by a series of organs involved in the process [67]. The cecum is presented in the form of two extensions at the intersection of the small intestine with the large intestine [68] and is specialized in fiber digestion, nitrogen recycling through urine and osmotic regulation, and water resorption [69]. At the same time, certain types of B vitamins are released (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folic acid, and vitamin B_{12}). Dietary fiber components are not digested by endogenous digestive enzymes and consequently are substrates for bacterial fermentation in the distal part of the gut [70].

Our results indicate that group E2 (commercial recipe + 1% walnut leaves) has the highest probiotic potential, with this type of diet leading to a 5-fold increase in the level of lactobacilli and a significant decrease in *Enterobacteriaceae* compared to the control group. Moreover, probably higher concentrations of short chain fatty acids produced in the small intestine decreased the *Enterobacteriaceae* population [71], which is negatively correlated with the *lactobacilli* population (Table 5). Similarly, Leusnik et al. [72] showed that dietary supplementation with bilberry extract (80 mg/kg of feed) significantly decreased the size of *Enterococcus* spp. populations in broilers on day 28 of the experiment.

5. Conclusions

Our results indicate that basal diets enriched with bilberry and walnut leaves powder might change positively the microbiota of hens by modulating several digestive enzymes that favor the development of lactobacilli and decrease Enterobacteriaceae. As a result, we could conclude that supplementation of basal feed with herbal additives might increase the health status of poultry.

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Effects of Inclusion of Different Doses of *Persicaria odorata* Leaf Meal (POLM) in Broiler Chicken Feed on Biochemical and Haematological Blood Indicators and Liver Histomorphological Changes

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Simple Summary: The frequent use of antimicrobial growth promoters (AGPs) in poultry feed leads to antimicrobial resistance, resulting in a ban on their subtherapeutic use in food-producing animals. In this context, there is a dire need to find safe and potential alternatives. Recently, phytobiotics, especially herbs, have gained attention and have been studied extensively for their possible use as alternative poultry feed additives. *Persicaria odorata* is a herb (phytobiotic) that is reported to possess antioxidant, antimicrobial, immunomodulatory, and hepatoprotective properties. This study is the first of its kind to assess the effects of different doses of supplementation of *Persicaria odorata* leaf meal (POLM) on haematological blood indicators, serum biochemistry, organ parameters, and histomorphology of the liver in broiler chickens. The results revealed that the dietary supplementation of POLM enhanced the growth performance, positively improved the haematological indices and serum biochemistry profile with no deleterious effects on internal organs, and ameliorated the histomorphology of the liver, even at dietary supplementation of 8 g/kg. Thus, POLM would be safe at an inclusion rate of 8 g/kg as an alternative phytogenic feed additive in broiler chickens.

Abstract: This research was conducted to estimate the effects of *Persicaria odorata* leaf meal (POLM) on haematological indices, serum biochemical attributes, and internal organs parameters, including histomorphological features of the liver, in broiler chickens. A total of 120 one-day-old male broiler chicks (Cobb-500) were randomly allocated into four experimental groups. The dietary treatments were basal diet (BD), which served as the control (C), along with BD + 2 g/kg POLM (Po2), BD + 4 g/kg POLM (Po4), BD + 8 g/kg POLM (Po8), which were the supplemented groups. The body weight gain (BWG) showed a linear increase and feed conversion ratio (FCR) showed a linear decrease with increasing POLM dosage at day 42 ($p < 0.05$) and for the overall growth performance period

($p < 0.01$). On day 21 and day 42, the values of red blood cells (RBCs), white blood cells (WBCs), haemoglobin (Hb), and packed cell volume (PCV) showed linear increases ($p < 0.05$) as the dosage of POLM increased in the diet. On day 21, dietary supplementation of POLM linearly decreased ($p < 0.05$) the serum activity of alkaline phosphatase (ALP), aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), and serum levels of urea and creatinine. On the other hand, serum levels of total protein (TP), albumin, and globulin showed a linear increase ($p < 0.05$) as the POLM dosage increased. On day 42, the serum activity of AST and ALT and serum levels of glucose, cholesterol, triglycerides, urea, and creatinine showed linear decreases ($p < 0.05$) with increased levels of POLM in the diet. However, POLM supplementation linearly increased ($p < 0.05$) the serum levels of TP and globulin. Dietary inclusion of POLM did not influence the organ parameters and showed no adverse effects on the liver histomorphology. In conclusion, supplementation of POLM increased the growth performance, improving haematological indices and serum biochemistry profiles of broiler chickens without any deleterious effects on the liver histomorphology. The results of the present study provide evidence that POLM can be safely used at a dose rate of 8 g/kg of feed as an alternative to conventional antimicrobial growth promoters (AGPs).

Keywords: broiler chicken; feed additive; blood haematology; phytobiotics; serum biochemistry

1. Introduction

The ban against in-feed inclusion of antimicrobial growth promoters (AGPs) has increased the momentum in research to find potential alternatives [1,2]. An increasing interest has been seen in the study of phytobiotics as alternatives to AGPs. Among phytobiotics, herbs are of particular significance because of their secondary bioactive metabolites, such as flavonoids, which are potent antioxidants, thus helping to prevent oxidative stress and reduce the risk of developing chronic diseases [3,4]. Additionally, they are anti-inflammatory, immunomodulatory [5,6], antimicrobial, anthelmintic, [3,4,7], detoxifying, and digestion-stimulating substances [8].

Herbs have shown positive effects on the performance and biological health of broiler chickens [9,10], can improve haematological blood indicators and serum biochemical attributes [11–13], and have also been reported to regulate the kidney and liver functions [14,15]. Among such herbs, *Persicaria odorata*, of the family Polygonaceae, is of important significance. *P. odorata* is a perennial herb that can grow up to 1.0 m in lowlands and 1.5 m in hilly areas. This plant possesses long and lanceolate leaves measuring 0.5–2.0 cm in width and 5–7 cm in length [16,17], and is used traditionally and regularly in Southeast Asian cuisine. *P. odorata* has many common names, such as Vietnamese cilantro and Vietnamese mint. In Malaysia, Indonesia, Singapore, and Brunei, it is called "*daun laksa*" or "*daun kesum*" [16]. The dried grounded leaves of *P. odorata* contain 3.5% crude protein, 0.83% crude fat, 10.66% crude fiber, and 1.83% ash [18]. It is a powerful antioxidant [17] that contains essential oils [19] and flavonoids [20]. Among these flavonoids, myricetin, quercetin, gallic acid, and coumaric acid are essential bioactive compounds [21]. Its high polyphenolic content, quercetin and myricetin, has been suggested to be responsible for its antioxidant activity [22,23], which can inhibit lipid peroxidation [24]. Moreover, previous studies have shown that *P. odorata* was non-toxic in murine model [25,26].

Phytobiotics are assumed to be natural, safe, and residue-free substances, which may have mildly toxic effects compared to commonly used synthetic AGPs [27]. Literature is scarce about the safe use of herbal plants and their optimal dosage; thus, there is a dire need to estimate the appropriate dosage and the possible side effects of natural feed additives, which might be used as safe alternative growth promoters in poultry production. The present study aims to estimate the effects of different doses of supplementation of POLM on the growth performance, haematological blood indicators, serum biochemical attributes, histomorphology of the liver, and internal organ parameters in broiler chickens.

2. Materials and Methods

2.1. Source and Preparation Method for Diets

Fresh samples of *P. odorata* were obtained from the Universiti Putra Malaysia herbal farm. The obtained samples were authenticated from the Biodiversity Unit, Institute of Biosciences, Universiti Putra Malaysia, and deposited with voucher no. SK3296/18 for future reference. For sample preparation, fresh leaves of *P. odorata* were dried using an oven set to 50 °C for 72 h and milled to a fine powder. The obtained sample (fine powder) was stored at 4 °C until further use.

2.2. Experimental Birds and Diets

The experimental procedures and animal handling were approved by the institutional animal care and use committee, Universiti Putra Malaysia (UPM/IACUC/AUP-R033/2018). A total of 120 one-day-old male broiler chicks (Cobb500) were procured from a local hatchery. Upon arrival, the birds were wing-tagged and allocated randomly into 4 treatment groups with 5 replicates of 6 birds each. The birds were raised in cages with wire meshed floor (length 120 cm × width 120 cm × height 45 cm), which were placed in a conventional open-sided shed. The birds in all replicates were reared under the same environmental and management conditions. The cyclic temperature in the house ranged between a maximum of 34 °C and a minimum of 24 °C, while the humidity ranged between a maximum of 91% and a minimum of 65%. Commencing from day one, birds had free access to water and feed, while the lighting was continuous. The basal diet without any premixing of anticoccidial, antimicrobial, and antioxidant drugs or feed enzymes was procured from the feed supplier and processed into experimental diets at a feed mixing facility at the Universiti Putra Malaysia. The current study consisted of four dietary treatments, which were given for 42 days (starter period = 1 to 21days, finisher period = 22 to 42 days). The dietary treatments were the basal diet (BD), which served as the control (C); and BD + 2 g/kg POLM (Po2), BD + 4 g/kg POLM (Po4), and BD + 8 g/kg POLM (Po8), which were the treatment groups. The experimental diets were formulated in the composition that meets or exceed NRC [28] recommendations (Table 1). The experimental broiler chickens were vaccinated with Newcastle disease (ND) and infectious bronchitis (IB) vaccines at day 4 and day 21, respectively; and vaccinated with the infectious bursal disease (IBD) vaccine on day 7 via an intra-ocular route.

Table 1. Ingredients (% as feed) and nutritional analysis of the basal diet.

Ingredients %	Starter Period	Finisher Period
Corn	54.40	58.95
Soybean Meal (SBM) (44%)	33.90	28.00
Fish Meal	5.33	5.74
Palm Oil	2.64	4.14
Salt	0.38	0.28
Limestone	1.06	0.86
Dicalcium Phosphate	1.09	0.84
Mineral Mix ††	0.28	0.28
Vitamin Mix †	0.29	0.29
DL-Methionine	0.18	0.18
Choline chloride	0.10	0.10
L-Lysine	0.35	0.34
Calculated analysis (%) *		
Crude Protein, %	22.00	20.00
Metabolise Energy (ME) MJ/kg	13.10	13.40
Crude Fat, %	5.21	7.01
Available P, %	0.43	0.35
Calcium, %	0.99	0.90

† Premixed administered vitamins per (kg) of dietary feed: Vitamin K (menadione) 1.33 (mg); Vitamin A (retinol), 1950 (µg); Vitamin D_3 30 (µg); Vitamin E, 0.02 (mg); riboflavin, 2.0 (mg); Biotin, 0.03 (mg); Vitamin B_{12}, 0.03 (mg); VitaminB_1, 0.83 (mg); Vitamin B_3 24 (mg); Vitamin B_6, 1.37 (mg); Folic acid, 0.33 (mg); Calcium D-Panthothenate, 3.69 (mg). †† Premixed administered minerals per kg of dietary feed: Zinc, 100.01 (mg); iron, 120.0 (mg); Mg, 16.0 (mg); I, 0.8 (mg); Co, 0.6 (mg); Cu, 19.99 (mg). Diet C = Control (0 g/kg medicinal herb; *P. odorata*); Diet Po2 = 2 g/kg *P. odorata*; Diet Po4 = 4 g/kg *P. odorata*; Diet Po8 = 8 g/kg *P. odorata*; * Calculated according to NRC [28].

2.3. Growth Performance Measurement

The initial body weights of the birds were recorded on day 1, followed by weekly recording of live body weight (BW) and feed intake (FI) throughout the entire experiment. The body weight gain (BWG) and feed conversion ratio (FCR) were calculated. Mortality was recorded whenever it occurred.

2.4. Sample Collection

On day 21 and day 42, two broiler chickens were randomly selected per replicate (n = 10) per experimental group. From each bird, the blood samples were collected via brachial (wing) vein to check haematological blood indicators and serum biochemical indices.

2.5. Analysis of Haematological Blood Indicators

For haematological blood indicator analyses, the blood samples were collected in the K_3 EDTA tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). Blood haematology parameters, including red blood cell (RBCs) and white blood cell (WBCs) counts, haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentrations (MCHC) were measured within 2 h post blood collection using a haematology analyser (ABC Vet®, ABX Diagnostics, Montpellier, France).

2.6. Serum Biochemical Indices

For serum biochemical analyses, blood samples were collected in a plain tube (BD Vacutainer®, Franklin Lakes, NJ, USA) and subjected to centrifuge at 3000×g for 15 min to obtain serum, which was stored at −20 °C until further analyses. The serum biochemical indices, including aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP), urea, creatinine, triglycerides, cholesterol, glucose, total protein (TP), albumin, and globulin; and serum electrolytes, including sodium (Na), chloride (Cl), and potassium (K), were measured with commercial kits (Roche "Basal" Diagnostica, Rotkreuz, Switzerland) using an autochemistry analyser (Bio Lis 24i Chemistry Analyser, Tokyo, Japan).

2.7. Determination of Relative Internal Organ Weights

On day 42, two birds per replicate (n = 10) from each experimental group were selected randomly and slaughtered according to the procedure designated in Malaysian Standard (MS) 1500: 2009 [29]. The following equation was used to calculate the dressing percentage (DP):

$$\text{DP} = (\text{eviscerated carcass weight/live weight}) \times 100 \quad (1)$$

The weights of the gizzard, heart, liver, kidney, spleen, pancreas, and bursa of Fabricius were taken and expressed as percentages (%) of the live body weight.

2.8. Liver Histomorphology Assessment

The liver samples were collected from birds slaughtered to determine internal organ parameters. For histomorphological studies, the liver tissues were kept for 48 h in 10% buffered formalin and subjected to a series of dehydration cycles in an automated tissue processor (Leica ASP 3000, Wetzlar, Germany). The liver tissues were embedded using a paraffin embedding system (Leica RM 2155, Wetzlar, Germany). Tissue sections up to 4–5 μm in size were obtained using a microtome (Leica Jung Multicut 2045, Wetzlar, Germany) and stained using haematoxylin and eosin staining. For the histomorphology, the tissues were examined under a light microscope (Leica DM LB2, Wetzlar, Germany).

2.9. Data Analyses

The data analyses were carried out with SAS 9.4 [30] (SAS Institute Inc., Cary, NC, USA) by one-way ANOVA using the general linear model procedure. Group differences were compared by Duncan's multiple range test. The effects of POLM supplementation at different doses were measured using an orthogonal polynomial contrast test for linear and quadratic effects. The differences were considered as significant at $p < 0.05$.

3. Results

3.1. Growth Performance

The growth performance of broiler chickens is shown in Table 2. On day 21, compared to the control BWG was significantly increased ($p < 0.05$) in the Po8 group; however, FI and FCR were not affected ($p > 0.05$) by dietary supplementation of POLM. On day 42, compared to the control, BWG was significantly increased ($p < 0.05$) in POLM-supplemented groups (Po2, Po4, and Po8). Furthermore, FCR was decreased ($p < 0.05$) in POLM-supplemented diets compared to the control group. Additionally, FI was not affected ($p > 0.05$) by dietary supplementation of POLM. Regarding the overall growth performance of broiler chickens (days 1–42), the maximal increase ($p < 0.05$) for the BWG and the lowest ($p < 0.05$) FCR were seen in dietary group Po8 compared to the control group. In addition, the BWG showed a linear increase ($p < 0.05$) and FCR showed a linear decrease ($p < 0.05$) with increasing POLM dosage on day 42 ($p < 0.05$) and for the overall growth performance period.

Table 2. Effects of different doses of supplementation of *Persicaria odorata* leaf meal (POLM) on the growth performance of broiler chickens.

Parameters	Treatment					*p*-Value	*p*-Value of Contrast	
	C	PO2	PO4	PO8	SEM	ANOVA	Linear	Quadratic
Initial BWT (g/bird)	40.31	40.30	40.32	40.30	0.031	0.994	0.9901	0.956
FI (g/d/bird)								
1–21 d	48.69	49.30	49.11	49.47	0.361	0.942	0.975	0.924
22–42 d	142.95	144.10	143.97	143.51	0.412	0.788	0.802	0.836
1–42 d	95.82	96.70	96.54	96.49	0.287	0.749	0.943	0.995
BWG (g/d/bird)								
1–21 d	30.04 ^{b}	31.38 ^{a,b}	31.94 ^{a,b}	32.97 ^{a}	0.372	0.048	0.164	0.974
22–42 d	78.06 ^{b}	80.50 ^{a}	80.68 ^{a}	82.53 ^{a}	0.499	0.005	0.047	0.725
1–42 d	54.23 ^{c}	55.94 ^{b}	56.31 ^{b}	57.65 ^{a}	0.340	0.0003	0.003	0.430
FCR								
1–21 d	1.60	1.57	1.54	1.51	0.021	0.215	0.307	0.999
22–42 d	1.83 ^{a}	1.79 ^{a,b}	1.78 ^{a,b}	1.74 ^{b}	0.012	0.048	0.047	0.908
1–42 d	1.77 ^{a}	1.73 ^{a}	1.72 ^{ab}	1.67 ^{b}	0.011	0.012	0.004	0.549
Mortality rate (%) 1–42 d								
	6.66	0.00	3.66	0.00	-	-	-	-

$^{a–c}$ indicate that values in the same row with different superscripts are significantly different ($p < 0.05$). FI: feed intake; BWG: body weight gain; FCR: feed conversion ratio; C: control; basal diet alone; Po2: basal diet+ POLM 2 g/kg; Po4: basal diet+ POLM 4 g/kg; Po8: basal diet+ POLM 8 g/kg. SEM: standard error of mean.

3.2. Haematological Blood Indicators

Data in Tables 3 and 4 illustrate the impacts of different doses of POLM on the haematological blood indicators of broilers on day 21 and day 42, respectively. On day 21, compared to the control, RBC counts were improved ($p < 0.05$) in the Po8 group. On the other hand, PCV was significantly increased in the POLM-supplemented groups (Po2, Po4, and Po8) compared with the control group. The Hb concentration was higher ($p < 0.05$) in the Po4 group and the maximum value ($p < 0.05$) was recorded in the Po8 group compared to the control group. Furthermore, the WBC counts were increased ($p < 0.05$) in dietary group Po8 relative to the control group. However, MCV, MCH, and MCHC were not affected

($p > 0.05$) by the dietary supplementation of POLM. In addition, POLM supplementation linearly increased RBC ($p = 0.048$), Hb ($p = 0.048$), and WBC counts ($p = 0.0001$) with increasing POLM dosage.

Table 3. Haematological blood indicators of broilers fed with different supplementation doses of POLM at day 21.

Parameters	Treatment					*p*-Value	*p*-Value of Contrast	
	C	PO2	PO4	PO8	SEM	ANOVA	Linear	Quadratic
RBCs ($mm^3 \times 10^6$)	2.39 ^{b}	2.70 ^{a,b}	2.78 ^{a,b}	2.92 ^{a}	0.08	0.036	0.048	0.545
PCV (%)	30.38 ^{b}	32.50 ^{a}	33.13 ^{a}	32.86 ^{a}	0.37	0.006	0.218	0.950
Hb (g/dL)	9.35 ^{b}	10.55 ^{a,b}	10.84 ^{a}	10.94 ^{a}	0.25	0.035	0.048	0.999
MCV (fL)	129.72	122.77	126.43	115.12	2.76	0.219	0.088	0.234
MCH (pg)	39.73	39.26	39.40	38.04	0.56	0.586	0.813	0.158
MCHC (%)	30.85	32.39	34.26	34.79	0.57	0.535	0.151	0.974
WBCs ($mm^3 \times 10^6$)	22.49 ^{b}	22.66 ^{b}	23.84 ^{a,b}	24.23 ^{a}	0.27	0.032	0.0001	0.430

^{a,b} indicate that values in the same row with different superscripts are significantly different ($p < 0.05$). RBCs: red blood cells; PCV: packed cell volume; Hb: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBCs: white blood cells; C: control; basal diet alone; Po2: basal diet+ POLM 2 g/kg; Po4: basal diet+ POLM 4 g/kg; Po8: basal diet+ POLM 8 g/kg.

Table 4. Haematological blood indicators of broilers fed on different supplementation doses of POLM at day 42.

Parameters	Treatment					*p*-Value	*p*-Value of Contrast	
	C	PO2	PO4	PO8	SEM	ANOVA	Linear	Quadratic
RBCs ($mm^3 \times 10^6$)	2.54 ^{b}	2.85 ^{a,b}	2.89 ^{a,b}	3.00 ^{a}	0.07	0.069	0.046	0.800
PCV (%)	31.14 ^{b}	33.34 ^{a,b}	33.40 ^{a,b}	33.89 ^{a}	0.40	0.039	0.231	0.559
Hb (g/dL)	9.62 ^{b}	10.50 ^{a,b}	10.82 ^{a,b}	11.13 ^{a}	0.22	0.047	0.047	0.980
MCV (fL)	123.43	120.03	117.21	113.76	2.05	0.215	0.016	0.990
MCH (pg)	38.02	37.66	37.75	37.15	0.46	0.671	0.205	0.641
MCHC (%)	30.86	31.59	32.38	32.75	0.35	0.202	0.039	0.710
WBCs ($mm^3 \times 10^6$)	22.67 ^{b}	22.86 ^{b}	23.98 ^{a,b}	25.04 ^{a}	0.33	0.027	0.0001	0.998

^{a,b} indicate that values in the same row with different superscripts are significantly different ($p < 0.05$). RBCs: red blood cells; PCV: packed cell volume; Hb: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBCs: white blood cells; C: control; basal diet alone; Po2: basal diet+ POLM 2 g/kg; Po4: basal diet+ POLM 4 g/kg; Po8: basal diet+ POLM 8 g/kg.

On day 42, compared with the control group, RBC, WBC, PVC, and Hb counts increased ($p < 0.05$) in the Po8 group. On the other hand, POLM supplementation did not influence ($p > 0.05$) MCV, MCH, or MCHC values in experimental broiler chickens. In addition, POLM supplementation linearly increased RBC ($p = 0.046$), Hb ($p = 0.047$), MCV ($p = 0.016$), MCHC ($p = 0.039$), and WBC counts ($p = 0.0001$) with increasing POLM dosage.

3.3. Serum Biochemistry

Data in Tables 5 and 6 show the influences of different doses of POLM on the serum biochemistry of broilers on day 21 and day 42, respectively. On day 21, compared to the control group, the AST activity in the serum was significantly decreased ($p < 0.05$) with increasing levels of POLM supplementation. However, the serum activity of ALT was decreased in Po8 group compared with the control group. On the other hand, the activity of ALP in the serum was not affected ($p > 0.05$) by dietary supplementation of POLM. The serum TP level was increased ($p < 0.05$) in dietary group Po8 compared to the control group. Furthermore, the serum levels of albumin and globulin (except Po2) were increased ($p < 0.05$) in POLM-supplemented groups compared to the control group. In contrast with the control group, except for Po2, the serum levels of urea and creatinine were decreased ($p < 0.05$) in POLM-supplemented groups. On the other hand, supplementation of POLM did not influence ($p > 0.05$) serum levels of glucose, cholesterol, triglycerides, K, or Cl in experimental broiler chickens. In addition, there was a linear decrease in the serum activity levels of ALP ($p = 0.048$) and AST ($p = 0.000$) and the serum levels

of urea ($p = 0.044$) and creatinine ($p = 0.000$) as the POLM dosage increased. However, a linear and quadratic decrease in the serum activity of ALT ($p = 0.000$) was observed with increasing POLM dosage. The POLM supplementation linearly increased the serum levels of TP ($p = 0.000$), albumin ($p = 0.022$), and globulin ($p = 0.000$) with increasing supplementation levels in the diet.

Table 5. Biochemical indicators of chicken blood from birds fed different supplementation doses of POLM at day 21.

Parameters	Treatment					*p*-Value	*p*-Value of Contrast	
	C	PO2	PO4	PO8	SEM	ANNOVA	Linear	Quadratic
ALP (U/L)	1794.1	1801.00	1819.20	1863.10	16.94	0.487	0.048	0.828
AST (U/L)	216.8 ^{a}	196.9 ^{b}	190.1 ^{c}	183.7 ^{d}	2.18	<0.0001	0.000	0.985
ALT (U/L)	6.84 ^{a}	7.02 ^{a}	6.97 ^{a}	6.36 ^{b}	0.06	<0.0001	0.000	0.000
Total protein (g/dL)	2.311 ^{c}	2.430 ^{b,c}	2.540 ^{a,b}	2.650 ^{a}	0.03	0.001	0.000	1.000
Albumin (g/dL)	1.112 ^{b}	1.212 ^{a}	1.229 ^{a}	1.287 ^{a}	0.02	0.015	0.022	0.662
Globulin (g/dL)	1.198 ^{b}	1.218 ^{ab}	1.311 ^{a}	1.364 ^{a}	0.02	0.040	0.000	0.769
Glucose (mmol/L)	14.90	14.30	13.70	13.10	0.58	0.744	0.380	1.000
Cholesterol (mmol/L)	3.00	2.98	2.97	2.82	0.04	0.477	0.056	0.473
Triglycerides (mmol/L)	0.93	0.92	0.93	0.91	0.01	0.898	0.727	0.636
Na (mmol/L)	128.5 ^{b}	132.90 ^{a,b}	136 ^{a,b}	137.9 ^{a}	1.48	0.049	0.060	0.943
K (mmol/L)	5.23	4.35	4.18	4.09	0.19	0.145	0.627	0.985
Cl (mmol/L)	106.4	108.66	108.9	109.6	1.55	0.904	0.918	0.993
Urea (mmol/L)	0.44 ^{a}	0.40 ^{b}	0.38 ^{c}	0.37 ^{c}	0.01	<0.0001	0.044	0.924
Creatinine (mmol/L)	28.28 ^{a}	28.65 ^{a}	24.48 ^{b}	23.45 ^{b}	0.68	0.005	0.000	0.112

$^{a–d}$ indicate that values in the same row with different superscripts are significantly different ($p < 0.05$). ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; C: control; basal diet alone; Po2: basal diet+ POLM 2 g/kg; Po4: basal diet+ POLM 4 g/kg; Po8: basal diet+ POLM 8 g/kg.

Table 6. Biochemical indicators of chicken blood from birds fed different supplementation doses of POLM at day 42.

Parameters	Treatment					*p*-Value	*p*-Value of Contrast	
	C	PO2	PO4	PO8	SEM	ANNOVA	Linear	Quadratic
ALP (U/L)	1650.19	1659.70	1652.80	1671.80	13.27	0.943	0.825	0.722
AST (U/L)	224.50 ^{a}	203.70 ^{b}	200.00 ^{b}	195.80 ^{b}	2.27	<0.0001	0.003	0.991
ALT (U/L)	8.34 ^{a}	6.98 ^{b}	6.95 ^{b}	5.75 ^{c}	0.19	<0.0001	0.000	0.004
Total protein (g/dL)	2.418 ^{c}	2.582 ^{b}	2.668 ^{b}	2.744 ^{a}	0.03	0.0008	0.001	0.988
Albumin (g/dL)	1.180 ^{b}	1.269 ^{a,b}	1.251 ^{a,b}	1.311 ^{a}	0.02	0.049	0.313	0.279
Globulin (g/dL)	1.238 ^{b}	1.313 ^{a,b}	1.417 ^{a}	1.434 ^{a}	0.03	0.027	0.006	0.374
Glucose (mmol/L)	15.90	15.70	14.20	13.30	0.63	0.414	0.021	0.874
Cholesterol (mmol/L)	3.23 ^{a}	3.03 ^{b}	2.95 ^{b}	2.77 ^{c}	0.04	<0.0001	0.000	0.451
Triglycerides (mmol/L)	1.08 ^{a}	0.94 ^{b}	0.80 ^{c}	0.75 ^{c}	0.03	<0.0001	0.000	0.355
Na (mmol/L)	136.1	136.60	140.10	141.6	1.42	0.461	0.059	0.850
K (mmol/L)	5.55 ^{a}	4.53 ^{b}	4.24 ^{b}	4.03 ^{b}	0.19	0.020	0.149	0.985
Cl (mmol/L)	112.3	109.93	110	109.3	1.68	0.933	0.968	0.984
Urea (mmol/L)	0.48 ^{a}	0.42 ^{b}	0.36 ^{c}	0.35 ^{c}	0.01	<0.0001	0.000	0.031
Creatinine (mmol/L)	31.11 ^{a}	30.07 ^{a}	23.73 ^{b}	21.14 ^{b}	0.89	<0.0001	0.000	0.022

$^{a–c}$ indicate that values in the same row with different superscripts are significantly different ($p < 0.05$). ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; C: control; basal diet alone; Po2: basal diet+ POLM 2 g/kg; Po4: basal diet+ POLM 4 g/kg; Po8: basal diet+ POLM 8 g/kg.

On day 42, the activity of AST and ALT in the serum was decreased ($p < 0.05$) with increasing POLM supplementation. However, the serum activity of ALP was not influenced by ($p > 0.05$) POLM supplementation in experimental broilers. The serum level of TP was increased ($p < 0.05$) in POLM supplementation groups compared to the control group. The serum levels of albumin in the Po8 group and globulin in Po4 and Po8 groups were significantly increased ($p < 0.05$) compared with the

control group. Serum levels of cholesterol, triglycerides, and urea were decreased ($p < 0.05$) with increasing POLM supplementation levels, in comparison with the control group. Birds in Po8 and Po4 groups showed decreased serum creatinine levels compared with the Po2 and control groups. However, POLM supplementation did not vary ($p > 0.05$) the serum level of Na, Cl, or glucose in the experimental birds. In addition, there was a linear decrease in serum levels of AST ($p = 0.003$), glucose ($p = 0.002$), cholesterol ($p = 0.000$), and triglycerides ($p = 0.000$) as the POLM supplementation increased. Furthermore, linear and quadratic decreases in serum levels of ALT ($p = 0.000$; 0.004), urea ($p = 0.000$; 0.03), and creatinine (0.000; 0.022) were observed as the POLM supplementation levels increased. Moreover, the serum levels of TP ($p = 0.001$) and globulin ($p = 0.006$) showed linear increases with increased supplementation of POLM.

3.4. Dressing Percentage and Relative Internal Organs Weights

The dietary supplementation of POLM did not influence ($p > 0.05$) the dressing percentages or relative internal organs weights across experimental broiler chickens (Table 7). Moreover, there were linear increases in the dressing percentage ($p = 0.040$) and relative spleen weight ($p = 0.000$) with increasing levels of POLM.

Table 7. Dressing percentages and relative internal organs weights of broilers fed different supplementation doses of POLM at day 42.

Parameters	Treatment					*p*-Value	*p*-Value of Contrast	
	C	PO2	PO4	PO8	SEM	ANNOVA	Linear	Quadratic
Dressing %	71.16	71.85	73.09	74.35	0.60	0.367	0.040	1.000
Gizzard	1.88	2.18	2.2	2.28	0.09	0.218	0.897	0.987
Liver	2.31	2.27	2.25	2.31	0.09	0.978	0.888	0.863
Heart	0.63	0.61	0.6	0.61	0.04	0.973	0.990	0.964
Kidney	0.61	0.59	0.63	0.62	0.03	0.950	0.683	0.737
Spleen	0.13	0.13	0.14	0.15	0.01	0.146	0.000	0.440
Pancreas	0.21	0.23	0.23	0.23	0.01	0.894	0.920	0.979
Bursa of Fabricius	0.2	0.22	0.21	0.22	0.01	0.675	0.949	0.222

C: control; basal diet alone; Po2: basal diet+ POLM 2 g/kg; Po4: basal diet+ POLM 4 g/kg; Po8: basal diet+ POLM 8 g/kg.

3.5. Morphological Analyses of Liver

Figure 1a–d show the histomorphologies of the liver sections of the experimental birds. The histomorphological examination of the liver section of the control group showed congestion of the central vein (CV), with loosening of the endothelium and vacuolar degeneration of the hepatocytes (Figure 1a). The liver lobule sections of the POLM-supplemented groups (Po2, Po4, and Po8) showed normal architecture of the hepatic lobules and central veins with intact endothelia and hepatic sinusoids. Furthermore, multifocal areas of the RBCs were seen in sinusoidal capillaries, without any infiltrative evidence of inflammatory cells within the liver parenchyma (Figure 1b–d). A gradual histomorphological improvement in the normalcy level of the hepatocytes was noticed with increasing POLM supplementation levels. The hepatocyte architecture was clearer and showed no vacuolation or degenerative changes in the Po8 group compared to Po2 and Po4, where low levels of vacuolation and fatty changes were noticed within hepatocytes in a centrally magnified area of the hepatic lobule sections.

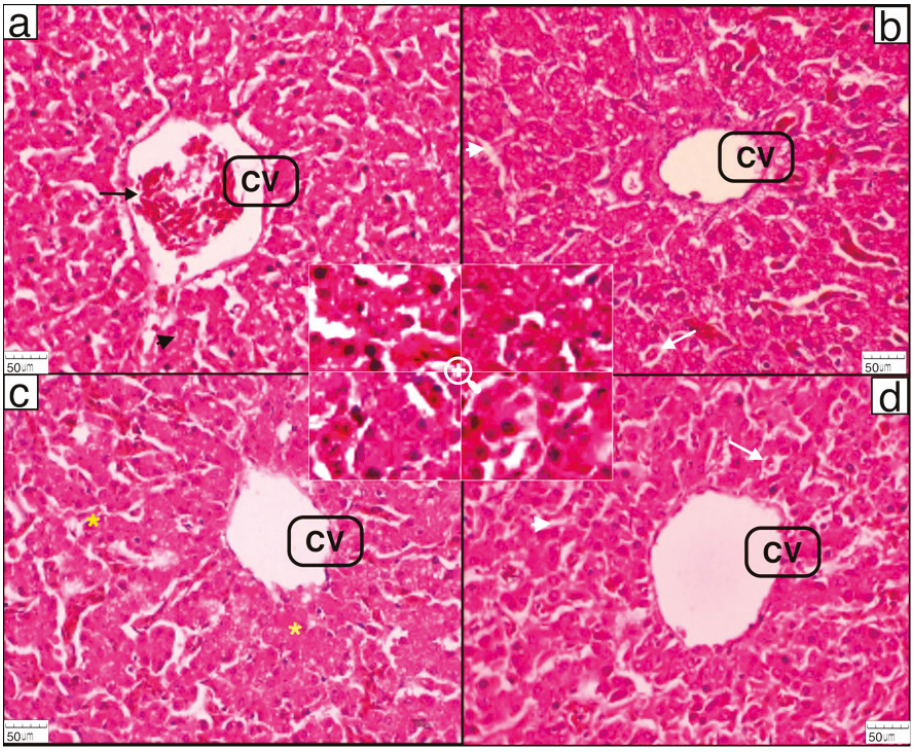

Figure 1. Photomicrograph image. (**a**) Liver lobule section of the control group, showing partial congestion (black arrow) in the central vein (CV) and vacuolar degeneration of the hepatocytes (black arrowhead). (**b–d**) Liver lobule sections of the Po2, Po4, and Po8 groups, showing central veins with intact endothelia, RBCs within sinusoids (white arrow), and radiating sinusoidal spaces (white arrowhead); there is no evidence of the infiltration of inflammatory cells in the liver parenchyma. (**c**) Hepatocytes showing normal architecture (asterisk). The magnified area in the center of the image shows histomorphological features of hepatocytes, where the hepatocytes in the Po8 group (**d**) have the clearest and healthiest architecture compared to the other groups (H&E:Haematoxylin and Eosin; 400X).

4. Discussion

4.1. Growth Performance

Supplementation of POLM enhanced the growth performance of broiler chickens. In this study, compared to the control group, BWG was significantly increased ($p < 0.05$) in the Po8 group on day 21; however, FI and FCR were not affected ($p > 0.05$) by dietary supplementation of POLM. Aroche et al. [31] reported that the dietary inclusion of phytobiotics in the form of 0.5 % mixed powder of *M. citrifolia*, *P. guajava*, and *A. occidentale* improved the feed efficiency, which resulted in increased BWG. The *P. guajava and M. citrifolia* contain flavonoids and possess antioxidant and antimicrobial properties [32]. The flavonoid contents of these phytobiotics are assumed to increase the growth performance in supplemented broiler chickens [33,34]. The secondary metabolites of herbs such as alkaloids, tannins, and flavonoids positively influence the birds' health, as they possess antimicrobial, anti-inflammatory, and antioxidant properties [35]. Thus, the inclusion of phytobiotics as a dietary supplement help to improve the growth in chickens [36–38].

On day 42, compared to the control group, BWG was increased ($p < 0.05$) and FCR was significantly decreased in POLM-supplemented groups (Po2, Po4, and Po8). Additionally, The BWG showed a linear increase and FCR showed a linear decrease ($p < 0.05$) with increasing POLM dosage. Supplementation of POLM resulted in enhanced BWG in broiler chickens, which may have been due to the flavonoids and the secondary bioactive compound quercetin, which has a primary role and was successfully quantified from POLM. Quercetin is a flavone that should improve growth in birds by upregulating growth hormone, triggering the hepatic growth hormone receptor; this stimulus increases the concentration of insulin-like growth factor-1 [39]. Kim et al. [40] reported increased broiler growth by supplementing quercetin in broilers. Quercetin can limit the effects of oxidative stress [41] and pro-inflammatory cytokines such as TNF-α, interleukin, and cyclooxygenase-2 [42], thus modulating the gut environment to better utilise the nutrients, improving the growth in birds.

The overall growth performance (1–42 days) of broiler chickens showed that compared to the control group, BWG was maximumly increased ($p < 0.05$) and FCR was significantly decreased ($p < 0.05$) in the Po8 dietary group. In addition, the BWG showed a linear increase ($p < 0.01$) and FCR showed a linear decrease ($p < 0.01$) with increasing POLM dosage. Salami et al. [43] identified that the incorporation of medicinal herbs as feed additives in the broiler chickens' diets improved the FCR in the last growth phase of the birds. Other studies also suggested the roles of flavonoids in the growth performance of broiler chickens [34,44]. The present study results are in agreement with Mpofu et al. [45], where inclusion of *L. javanica* at the rate of 5 g/kg in broiler chickens' diets had a positive impact on overall growth. In another study, Paraskeuas et al. [36] reported that inclusion of phytobiotics such as eugenol, menthol, and anethol at the rate of 100 to 150 mg/kg in feed improved the nutrient digestibility, thus enhancing the growth measures in broiler chickens.

Conclusively, the supplementation of POLM in broiler chickens showed a positive effect on growth performance, hence effectively increasing the BWG and feed efficiency with decreased FCR. These findings are in agreement with the positive results of the previous studies, where in-feed phytobiotics were tested in broiler chickens [31,45,46]. Thus, the present results show that POLM effectively enhanced the growth performance of broiler chickens, even at the supplementation rate of 8 g/kg.

4.2. Haematological Blood Indicators

Haematology blood tests of experimental animals are very significant when evaluating the toxic effects of a supplemented compound or plant extract. Haematology blood tests are also tools that can be used to determine the physiological and pathological statuses of the organisms [47]. The haematological blood indicators in this study were found to be within normal ranges [48]. The normal blood haematology values in this study indicated the adequacy of nutrients and better immune status of the broiler chickens supplemented with POLM.

The current study findings indicated significant increases in RBC and WBC counts, and in Hb and PCV values. These outcomes are comparable with the results of Reis et al. [49], who indicated that inclusion of phytobiotics such as cinnamic aldehyde, thymol, and carvacrol in broiler chickens significantly increased erythrocyte counts and haemoglobin in comparison with the control. Similar findings in another study were reported by Krauze et al. [50], who studied the dietary effects of probiotic *Bacillus subtilis* (0.25 g/L) *Enterococcus faecium* (0.25 g/L), and phytobiotics containing cinnamon oil (0.25 mL/L) in broiler chickens and found improvements in the immune system and parameters such as RBCs and Hb. In another experiment, Gilani et al. [11] examined the efficacy of organic acids and phytobiotics (possessing flavonoids) in poultry feed as alternatives to AGPs, observing significant increases in RBC and WBC counts, as well as an increase in PCV in broiler chickens. Similarly, broiler chickens fed Garden cress (*Lepidium satvium*) seed powder [51], cayenne pepper (*Capsicum frutescens*) and turmeric (*Curcuma longa*) powders [52], and pawpaw leaf and seed meal [53] showed increased values of Hb, PCV, and RBCs.

The present study results were not significant ($p > 0.05$) for MCV, MCH, or MCHC in experimental broiler chickens. These results affirm the findings of Oghenebrorhie and Oghenesuvwe [54],

who reported no significant results for MCV, MCH, or MCHC among broilers supplemented with *Moringa oleifera* leaf meal (MOLM).

In conclusion, the dietary supplementation of POLM improved the RBCs, WBCs, PCV, and Hb, suggesting better utilisation of the dietary nutrients.

4.3. Serum Biochemistry

Serum biochemical parameters show the metabolism of nutrients in the body and highlight the possible changes resulting from intrinsic and extrinsic factors [55,56]. The liver is one of the largest and most vital organs of living organisms, and it has a pivotal role in detoxification, metabolism, and elimination of endogenous and exogenous substances [57]. The activity levels of ALP, AST, and ALT are considered as diagnostic tools that may be used to evaluate hepatotoxicity [58]. Any pathological manifestation or toxicity results in enhanced activity levels of AST and ALT [59]. Moreover, their activity levels are considered as specific indicators of liver injury or impairment [60]. The current study results showed decreased ($p < 0.05$) serum activity of AST and ALT by increasing the POLM supplementation dosage. However, the serum activity of ALP was not influenced by ($p > 0.05$) POLM supplementation in experimental broiler chickens. The decreased activity of ALT and AST indicated the hepatoprotective nature of the POLM. The POLM possesses a significant concentration of flavonoids and secondary metabolites, including quercetin, which is believed to be responsible for hepatoprotective activity [17,61]. Farag and El-Rayes [62] revealed the hepatoprotective effect of quercetin from bee pollen in broilers, which has an ability to restrict oxidative damage to the liver. Another study by Odetola et al. [12] showed that the graded supplementation of *Petiveria alliacea* root meal in broiler chickens significantly decreased the activity of AST. In a previous study, Oloruntola et al. [63] found that the dietary inclusion of pawpaw and bamboo leaf meal significantly decreased the activity of ALT in broiler chickens.

Serum proteins are primarily synthesised in the liver and their concentrations reflect the functional status of hepatocytes. Any decline in the levels of serum proteins (TP, albumin, and globulin) may be the result of hepatic insufficiency, malnutrition, and active inflammation, which may be due to the recurrent infections and immune deficiency [64]. Furthermore, the serum protein levels of birds are considered important indicators for the determination of their health status. The fattening period of broiler chickens is very short, and there is a rapid accumulation of building proteins in the body tissues, which may significantly influence the concentrations of proteins in the blood, as well as their composition [65]. This rapid growth trend requires intensive erythropoiesis and haemoglobin synthesis, which can result in increased globulin production, potentially affecting the concentrations of serum protein levels in growing chickens [66,67]. The current study results showed that the inclusion of POLM significantly increased the levels of TP, albumin, and globulin compared to the control group. In addition, TP, albumin, and globulin showed linear increases with increasing supplementation of POLM. The present study results are in accordance with the results of Goerge et al. [68], who noted higher serum TP levels in broilers fed a ginger-powder-supplemented diet at starting and finishing phases. Abudabos et al. [69] reported trends for serum TP and globulin for broilers fed anise and thyme essential oils that were in agreement with the current study.

In birds, the normal reference range of serum glucose is 200 to 500 mg/dL [48]. The present study showed that the serum glucose concentrations were not influenced by POLM in the experimental chickens; however, numerically high values were recorded in the control group compared to supplemented groups. The current findings are in line with the study by Abudabos et al. [69], where serum glucose did not differ significantly in experimental broilers supplemented with phytogenic feed additives.

The serum concentrations of cholesterol and triglycerides are considered to be indicators of lipid metabolism [70]. The current study findings showed that the serum levels of triglycerides and cholesterol were not affected by POLM supplementation at day 21, however significant decreases in the levels of triglyceride and cholesterol were noted in POLM-supplemented groups relative to control on

day 42. Furthermore, it was observed that the increasing dosages of POLM linearly decreased the serum levels of triglycerides and cholesterol. The current study results are endorsed by Vispute et al. [13], who reported that dill and hemp seed (possessing flavonoids) significantly decreased the serum levels of triglycerides in the final growth phase. Similarly, our results are in agreement with Zhang et al. [71], who specified that the supplementation of *Chinese bayberry* leaves in chickens' diets significantly decreased the serum concentrations of triglycerides and cholesterol. Our outcomes are affirmed by Zhou et al. [72] and Niu et al. [73], who reported that dietary supplementation of broilers with fermented *Ginkgo biloba* rations and fermented *Ginkgo biloba* leaves can significantly decrease the serum levels of triglycerides and cholesterol. Similar results were shared by Gilani et al. [11], who revealed that phytobiotics, organic acids, and their combinations resulted in significantly reduced serum levels of cholesterol and triglycerides in broiler chickens.

Electrolyte balance plays an important role in acid–base balance and ultimately modifies the performance of broiler birds. Any alteration in the acid–base balance results in malfunction of the biochemical and metabolic pathways, which results in an inability to maintain the physiological status of the birds. The minerals Na, K, and Cl are essential for acid–base and osmotic balance, as well as transport of substances across the cell membranes. Thus, they play vital roles in the metabolisms of living organisms. Any imbalance in these minerals can directly alter the acid–base balance, metabolic functions, and ultimately the performance of broiler chickens [74]. In the present study, the Na, K, and Cl values were within normal ranges. These results are in accordance with Malahubban and Ab Aziz, [75], who reported the graded supplementation of Misai Kucing (*Orthosiphon stamineus*) in broiler chicken.

The kidneys are considered the second target organs that may be injured due to metabolic dysfunctions. Kidney function plays a key role in measuring the possible toxicity of any compound. The status of kidney function can be measured via the increase or decrease in serum levels of urea and creatinine. Higher creatinine levels result from reduced glomerular filtration, which reflects kidney impairment [76], while an elevated serum urea level indicates cardiac and renal tissue injuries. The current study findings showed that serum levels of creatinine and urea were significantly decreased as the POLM dosage increased. These findings indicated that POLM had no deleterious effects on kidney function. Various studies using phytobiotics supplementation in broiler chickens have supported our present study results, including the work by Rubio et al. [77], Ahmad et al. [78], and Adegoke et al. [52].

In conclusion, our findings showed decreased activity of AST and ALT with reduced serum levels of urea and creatinine. These results highlighted that POLM supplementation was useful in terms of liver and kidney function, and was safe even at 8 g/kg in broiler chickens. Additionally, the increased blood protein levels (TP, albumin, and globulin) in this study might be due to the antioxidant and immunomodulatory properties of the POLM supplementation [5,6].

4.4. Relative Internal Organs Weights

The relative internal organs weights served as an indicator for the responses of animals towards any in-feed toxic substance that may result in an increase or decrease in internal organs weights [79]. In the current study, no macroscopic alterations, such as hypertrophy or atrophy, injury, and swelling, were noticed in any internal organ. Furthermore, dietary inclusion of POLM did not influence the relative organs weights in experimental broiler chickens. The outcomes of our present study are in agreement with Oloruntola [53], who observed that the relative internal organs weights of the broilers were not influenced by dietary inclusion of seed meal and pawpaw leaf meal. Similar observations were described in the work by Rubio et al. [77] and Vispute et al. [13], where dietary addition of phytobiotics did not influence the relative organs weights in broiler chickens.

In conclusion, the constant relative internal organs weights of the broilers across experimental groups suggested that, POLM supplementation had no adverse effect on internal organs of the broiler chickens.

4.5. Histomorphological Analysis of the Liver

The histomorphological study of the liver revealed that POLM supplementation did not show deleterious effects on liver tissues. The vacuolar degeneration was more frequent in the control group compared to the hepatic tissue samples of all the POLM-supplemented groups. The microscopic characteristics of the hepatic tissues showed positive impacts on the histomorphologies of the livers, as seen in Figure 1b–d compared to Figure 1a (control group). The histomorphological changes in the present study were comparable to the previous study by Quereshi et al. [80], in which the hepatoprotective effects of *fenugreek* seeds and *dandelion* leaves in broiler chickens resulted in normal architecture of the hepatic parenchyma. Additionally, the hepatoprotective effects of these phytobiotics were suggested to be due to the presence of flavonoids in *dandelion* leaves and *fenugreek* seeds. Klaric et al.'s [15] results also supported the results of the present study, where supplementation of phytobiotics such as propolis and bee pollen, which possess flavonoids, ameliorated the liver morphology compared to the control group. Additionally, normal hepatocytes without regressive lesions were noticed in the supplemented groups compared to the control. The control group showed extensive regressive lesions in liver tissue sections. In another study, Farag and El-Rayes [62] observed that the dietary supplementation of bee pollen in broiler chickens' diets ameliorated the hepatic parenchyma and reduced tissue injury. Furthermore, flavonoids such as quercetin might have a protective effect against oxidative damage of the liver.

Inflammatory responses and oxidative stress are key factors that can damage the liver. Any substance that can diminish the oxidative stress and inflammation can produce hepatoprotective effects and reduce hepatic injury. In the present study, the POLM supplementation produced protective effects on the hepatocytes. These hepatoprotective effects were primarily due to quercetin, which produce effects by limiting oxidative stress [41]; pro-inflammatory cytokines such as TNF-α, IL-6, and COX-2 [42]; and nuclear factor NF-κB, probably via interference of the signalling of the toll-like receptor TLR_4 [81]. Furthermore, quercetin increases the non-enzymatic and enzymatic antioxidants by stimulating the Nrf2–ARE signalling pathway in cells, which might positively influence the liver status and function [82]. Conclusively, increasing the POLM supplementation levels has a gradual ameliorating histomorphological effect on hepatocytes. A predominantly healthy architecture of the liver parenchyma was noticed in POLM-supplemented group Po8 compared to the Po2 and control groups.

5. Conclusions

The current study results showed that the dietary inclusion of POLM supplementation in broiler chickens enhanced the growth performance and positively improved haematological blood indicators and serum biochemistry attributes, with no deleterious effects on the internal organs. Additionally, broilers chicken fed a diet supplemented with POLM at a rate of 8 g/kg showed the most promising results in terms of growth performance, as well as for the tested blood and serum biochemistry parameters, and retained relatively normal hepatic parenchyma. Thus, POLM supplementation at 8 g/kg would be the appropriate dose as an alternative feed additive for broiler chickens.

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