agriculture

Special Issue Reprint

Rational Use of Feed to Promote Animal Healthy Feeding

Edited by
Tatiana Dumitra Panaite and Mihaela Hãbeanu

mdpi.com/journal/agriculture

MDPI

Rational Use of Feed to Promote Animal Healthy Feeding

Rational Use of Feed to Promote Animal Healthy Feeding

Guest Editors

Tatiana Dumitra Panaite
Mihaela Hãbeanu

MDPI

Basel • Beijing • Wuhan • Barcelona • Belgrade • Novi Sad • Cluj • Manchester

Guest Editors
Tatiana Dumitra Panaite
Nutrition Physiology Department
National Research and Development Institute for Biology and Animal Nutrition
Balotesti
Romania

Mihaela Hăbeanu
Mulberry Laboratory
Research Station for Sericulture Baneasa Bucharest
Bucharest
Romania

Editorial Office
MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

This is a reprint of the Special Issue, published open access by the journal *Agriculture* (ISSN 2077-0472), freely accessible at: https://www.mdpi.com/journal/agriculture/special_issues/O5JA83R715.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-3777-9 (Hbk)
ISBN 978-3-7258-3778-6 (PDF)
https://doi.org/10.3390/books978-3-7258-3778-6

Contents

About the Editors

Tatiana Dumitra Panaite

Tatiana Dumitra Panaite is a senior researcher who holds a PhD in Animal Science. Since 2009, she has served as the manager of the Nutritional Physiology Laboratory at the National Research and Development Institute for Animal Biology and Nutrition in Balotesti (INCDBNA-Balotesti). She specializes in the field of technical and scientific monogastric animal nutrition, including digestibility trials. Her current research interests include the optimization and development of nutritional solutions to limit pollution risks; the quality and safety of animal products and feed by developing nutritional strategies to obtain functional foods (eggs and meat); the optimization of new feed formulations to reduce the effects of thermal stress in monogastrics, including natural phytoadditives with antioxidant action; and the development of innovative nutritional solutions to reduce dependence on classic imported protein feeds and decrease the carbon footprint. She has experience in patent development, national and international project management (as a manager and collaborator), and has built strong relationships with industry partners and farmers. Tatiana is a member of both the WPSA Romanian branch and the EAAP. She has published numerous scientific papers, brochures, and books that significantly advance knowledge in the field.

Mihaela Hãbeanu

Mihaela Hãbeanu, a senior researcher in the field of animal nutrition, has over 29 years of rich scientific experience. From a managerial point of view, in 2010–2020, PhD Hãbeanu became head of the Animal Nutrition Laboratory. From 2014 to 2019, Hãbeanu was the Coordinator of Biotechnology Laboratory activities. Finally, from May 2020 to October 2022, she led the Animal Nutrition and Biotechnology Laboratory. She currently works at the Research Station for Sericulture Baneasa Bucharest in the sericulture field. In her area of interest, an important focus is the interrelation between nutritional factors and insect/animal metabolic responses, health status implications, dietary manipulation, product quality, digestibility, and animal performance, as well as fatty acid metabolism. She has also studied using silkworm by-products in animal feeding and valorization methods with a focus on pupae. Her scientific activity is reflected in > 80 published articles, of which 24 are ISI and 53 are BDI; 4 patents and 6 pending patent applications; 6 books (4 as the main author and 2 chapters in international books); guides/brochures; and communications at national/international conferences. Her project activity encompasses 38 projects: 9 as the project director, 1 as the scientific lead, 1 as the technical lead, and 1 as the economic lead. She has extensive experience in international projects, including Climate Neutral Farms and Life Green Sheep, as well as Erasmus and FP7 projects, bilateral projects with Vietnam, and others.

Preface

Our Special Issue on the "Rational Use of Feed to Promote Animal Healthy Feeding" promotes the utilization and reintegration of agricultural by-products in the animal feeding sector as strategic nutritional solutions to maintain economic efficiency and contribute to food security by presenting the complete valorization of feed resources within the context of a circular bioeconomy.

The studies within the reprint present the results and benefits of using fermented feed, probiotics, natural pigment supplementation, and alternative protein sources in animal farm feeding. Agricultural by-products were used in poultry feeding to either mitigate heat stress while maintaining productive performance or improve milk/cheese/meat quality in goats' and lambs' diets, focusing on analyzing their nutrient content and bioactive compounds. The topic of this reprint addresses farmers, feed manufacturers, the value-added products industry, and researchers.

We would like to express our gratitude to all the participants and colleagues for their research work, support, and contributions to this reprint.

Tatiana Dumitra Panaite and Mihaela Hăbeanu
Guest Editors

agriculture

MDPI

Editorial

Rational Use of Feed to Promote Animal Healthy Feeding

Tatiana Dumitra Panaite [1,*] and Mihaela Hăbeanu [2]

[1] Nutrition Physiology Department, National Research and Development Institute for Biology and Animal Nutrition, 077015 Balotesti, Romania
[2] Sericultural Research-Station Baneasa-Bucuresti, 69 București-Ploiești Avenue, 013685 Bucharest, Romania; mihaela.habeanu@scsbaneasa.ro
* Correspondence: tatiana.panaite@ibna.ro

Nowadays, ensuring global food production depends on various factors like climate change, resource scarcity, and a continuously increasing population, which creates tremendous pressure on traditional agricultural practices. Therefore, there is an urgent need to reimagine how to overcome limited natural resources to sustain food production, minimize environmental impact, and support animal and human nutrition. A well-defined strategic direction can be applied, focusing on a more in-depth exploration of underutilized feed resources and the valorization of agri-food by-products. While not a novel approach, this strategy enhances animal nutrition while promoting a circular bioeconomy.

Feed formulation based on conventional ingredients such as soybean meals and corn, often associated with deforestation, excessive water utilization, and significant greenhouse gas emissions, can be partially/ manipulated by including local, available, and accessible agri-food by-products to obtain high-quality animal feed, also reducing agricultural waste and farm environmental footprint. Valorizing agri-food by-products is not merely a technical solution; it represents a fundamental change in utilizing natural resources in agriculture. This path—*producing more or enough (re)using less*—aligns with present agricultural practices. Feed valorization methods such as fermentation can enhance these materials' digestibility and nutritional profile. To address the multifaceted challenges of global food production, adopting an integrated strategy that brings together technological innovation, supportive and adequate policies, and sustainable economic models is essential.

This Editorial provides an overview of recent studies exploring various feed interventions—including fermentation processes, probiotic supplementation, natural additives, agri-food by-products, and alternative protein sources—to enhance productivity and product quality in poultry and small ruminants and also provide nutritional solutions for thermal stress mitigation. In this Special Issue "Rational Use of Feed to Promote Animal Healthy Feeding", 10 papers were published, of which 9 research papers and 1 review paper were published. This Special Issue highlighted key research studies that explored nutritional interventions in broilers, laying hens, dairy goats, and lambs, emphasizing improvements in production performances, animal product quality, and gut health. Therefore, integrating these strategies into a circular bioeconomy framework is essential for achieving sustainable animal farming and ensuring food security.

Predescu et al. [1] reviewed the numerous effects of fermented feed utilization in broilers' feed, emphasizing the benefits of fermented feed in reducing the by-products antinutrients and enhancing nutrient availability, reducing pathogenic microorganisms, and promoting a healthier gut microbiome. Beneficial microorganisms, particularly lactic acid bacteria, enhance gut health and immune response. Performance parameters like feed conversion ratio (FCR), final body weight (FBW), and feed intake (FI) are positively influenced by fermented feeds.

Citation: Panaite, T.D.; Hăbeanu, M. Rational Use of Feed to Promote Animal Healthy Feeding. *Agriculture* **2025**, *15*, 644. https://doi.org/10.3390/agriculture15060644

Received: 25 February 2025
Accepted: 7 March 2025
Published: 18 March 2025

Citation: Panaite, T.D.; Hăbeanu, M. Rational Use of Feed to Promote Animal Healthy Feeding. *Agriculture* **2025**, *15*, 644. https://doi.org/10.3390/agriculture15060644

Complementing this, Ren et al. [2] investigated the effects of xylooligosaccharide (XOS) on the growth performance and intestinal health of broiler chickens challenged by avian pathogenic Escherichia coli (APEC). An improvement in FBW and average daily gain (ADG) was observed in APEC-challenged broilers, while FCR was also improved during the initial days of the experiment. The negative effects registered in APEC-challenged broilers led to decreased FBW and ADG and increased FCR, indicating that growth retardation was primarily due to intestinal disruption rather than reduced appetite. The XOS supplementation increased the count and density of jejunal goblet cells (crucial for maintaining the intestinal barrier). The jejunal villus height (VH) and crypt depth (CD) were significantly improved in XOS-supplemented broilers, indicating better intestinal morphology. The cecal microbiota was positively influenced by increasing the counts of Lactobacillus and enhancing the production of short-chain fatty acids (SCFAs) like acetate, butyrate, and valerate. The inflammatory cytokine expression in the jejunum was diminished in XOS-supplemented groups, suggesting a reduction in intestinal inflammation due to the protective effects of XOS against APEC. Additionally, XOS supplementation blocked the increases in the expression of virulent genes associated with *E. coli*, further contributing to maintaining intestinal health in challenged broilers.

Dumitru et al. [3] assessed the probiotic potential of Bacillus licheniformis (BL) in broilers fed cowpea-based diets; BL significantly improved BWG overall during the growing and finisher rearing phases. Improved tibia iron (Fe) and phosphorus (P) mineralization were observed while also reducing the calcium-phosphorus (Ca:P) ratio. Microbial analysis revealed that BL inclusion decreased coliform counts in the CWP diet and reduced *E. coli* in the ileum. Additionally, it lowered *Clostridium* spp. and *Enterococcus* spp. in the cecum of broilers on soybean meal (SBM) diets. The presence of *Staphylococcus* spp. in broiler feces was also reduced in CWP groups.

Matache et al. [4] investigated natural pigment supplementation from marigold and paprika extracts in laying hens' diets. Their findings revealed that these natural additives utilize enhanced yolk color and improved egg quality during storage, serving as an effective alternative to synthetic colorants. Cornescu et al. [5] evaluated the effects of white grape pomace supplementation (6%) in laying hens' diets under normal, low, and high-temperature conditions. While the benefits were most visible under low thermal stress conditions, improvements in parameters such as egg weight, production, and yolk quality indicate that such by-products dietary inclusion can be effectively used to support poultry performances under varying environmental conditions.

Other authors, such as Antunovic et al. [6], tested grape seed cake (5% and 10%) in lactating dairy goats, concluding that a 10% inclusion rate significantly enhances milk antioxidative activity. This improvement is attributed to the high polyphenol content of the grape seed cake, which helps alleviate lactation-induced stress and improve milk quality. A significant reduction in somatic cell count (SCC) was observed in the milk from goats in the grape seed cake group (10%) compared to the control and grape seed cake group (5%) groups, suggesting beneficial effects on udder health. The activity of superoxide dismutase (SOD) and glutathione reductase (GR) was significantly higher in the milk from the grape seed cake group (10%), indicating an enhanced milk antioxidative status. Cismileanu et al. [7] experimented with the substitution of conventional protein sources (sunflower meal 11.5% DM basis) with dietary linseed or hempseed in the diets of late lactation Murciano-Granadina dairy goats. Including these oilseeds significantly improved the fatty acid profiles of both milk and cheese. Notably, the enhanced levels of omega-3, omega-6 polyunsaturated fatty acids, and conjugated linoleic acid (CLA) contributed to lower atherogenic and thrombogenic indices, thereby elevating the nutritional quality of dairy products. Salavardic et al. [8] investigated the effects of extruded flaxseed (9%)

and pumpkin seed cake (16%) as alternative protein sources in diets for growing Alpine goat kids. Although these dietary modifications did not alter the average daily weight gain, significant results in hematological and biochemical parameters indicated potential benefits in immune modulation. Costa et al. [9] examined the impact of incorporating sunflower cake (150, 300, and 450 g/kg DM) of high-oleic sunflower cakes derived from high-oleic seeds on finishing lambs, evaluating performance, carcass characteristics, meat quality, and intramuscular fatty acid composition. While sunflower cake inclusion did not significantly influence weight gain, dry matter intake, or metabolizable energy intake, an increase in neutral detergent fiber (NDF) and ether extract (EE) intake was noticed. Including sunflower cakes reduced hot and cold carcass yields and intramuscular fat content; oleic acid, rumenic acid, and EPA fatty acids linearly increased with high-oleic sunflower cake. Including high-oleic sunflower cake reduced saturated fatty acids, except stearic acid. Filip et al. [10] investigated samples of golden apples, red apples, carrots, celery, beetroots, and red potato peel waste as animal feed, focusing on their nutrient content and bioactive compounds. Various analyses highlighted the value of waste in providing essential nutrients in animal diets. Several analyses were performed collectively (chemical composition, X-ray diffraction analysis, thermogravimetric and differential thermal analyses, antioxidant activity assessment: DPPH and ABTS, and total phenolic content analysis), providing a comprehensive understanding of the structural, thermal, chemical, and bioactive properties of fruit and vegetable waste, emphasizing their potential as alternative animal feed sources.

The papers published within this Special Issue presented evidence of the benefits of different feed interventions in animal farm nutrition. From fermented feeds that mitigate antinutritional factors to the use of agri-food by-products and natural additives that enhance product quality, these studies highlight diverse strategies to promote farm animal health and sustainability, especially in poultry and small ruminants.

Author Contributions: Conceptualization, T.D.P. and M.H.; writing—original draft preparation, T.D.P.; writing—review and editing, T.D.P. and M.H. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: The Guest Editors would like to thank all research collaborators and participants for their support and contribution to this Special Issue.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Predescu, N.C.; Stefan, G.; Rosu, M.P.; Papuc, C. Fermented Feed in Broiler Diets Reduces the Antinutritional Factors, Improves Productive Performances and Modulates Gut Microbiome—A Review. *Agriculture* **2024**, *14*, 1752. [CrossRef]
2. Ren, L.; Cao, Q.; Ye, H.; Dong, Z.; Zhang, C.; Feng, D.; Zuo, J.; Wang, W. Supplemental Xylooligosaccharide Attenuates Growth Retardation and Intestinal Damage in Broiler Chickens Challenged by Avian Pathogenic *Escherichia coli*. *Agriculture* **2024**, *14*, 1684. [CrossRef]
3. Dumitru, M.; Lefter, N.A.; Ciurescu, G.; Draghici, R. Effect of Bacillus licheniformis on Growth, Bone Mineralization, and Intestinal Microbiota in Broilers Fed Cowpea Diets. *Agriculture* **2024**, *14*, 2013. [CrossRef]
4. Matache, C.-C.; Cornescu, G.M.; Drăgotoiu, D.; Cișmileanu, A.E.; Untea, A.E.; Sărăcilă, M.; Panaite, T.D. Effects of Marigold and Paprika Extracts as Natural Pigments on Laying Hen Productive Performances, Egg Quality and Oxidative Stability. *Agriculture* **2024**, *14*, 1464. [CrossRef]
5. Cornescu, G.M.; Panaite, T.D.; Cișmileanu, A.E.; Sărăcilă, M.; Untea, A.E.; Varzaru, I. White Grape Pomace Effect on Laying Hens' Productivity, Egg Quality Traits, and Antioxidant Capacity Under Normal, Heat, and Cold Thermal Conditions. *Agriculture* **2024**, *14*, 2209. [CrossRef]
6. Antunović, Z.; Novoselec, J.; Klir Šalavardić, Ž.; Steiner, Z.; Drenjančević, M.; Pavić, V.; Đidara, M.; Ronta, M.; Jakobek Barron, L.; Mioč, B. The Effect of Grape Seed Cake as a Dietary Supplement Rich in Polyphenols on the Quantity and Quality of Milk, Metabolic Profile of Blood, and Antioxidative Status of Lactating Dairy Goats. *Agriculture* **2024**, *14*, 479. [CrossRef]

7. Cismileanu, A.E.; Toma, S.M.; Ropota, M.; Dragomir, C.P.; Cornescu, G.M.; Dragomir, C. Obtaining Goats' Dairy Products Enriched in Healthy Fatty Acids by Valuing Linseed or Hempseed as Dietary Ingredients. *Agriculture* **2024**, *14*, 1498. [CrossRef]
8. Klir Šalavardić, Ž.; Novoselec, J.; Đidara, M.; Antunović, Z. Blood Parameter Response in Growing Alpine Goat Kids Fed Diets Containing Extruded Flaxseed or Pumpkin Seed Cake. *Agriculture* **2024**, *14*, 1667. [CrossRef]
9. Costa, D.M.; Alvarenga, T.I.R.C.; dos Santos, I.J.; Dias Junior, P.C.G.; Alvarenga, F.A.P.; Alves, N.G.; Furusho-Garcia, I.F. Performance, Carcass Traits and Meat Quality of Lambs Fed with Increasing Levels of High-Oleic Sunflower Cake. *Agriculture* **2025**, *15*, 191. [CrossRef]
10. Filip, M.; Vlassa, M.; Petean, I.; Țăranu, I.; Marin, D.; Perhaiță, I.; Prodan, D.; Borodi, G.; Dragomir, C. Structural Characterization and Bioactive Compound Evaluation of Fruit and Vegetable Waste for Potential Animal Feed Applications. *Agriculture* **2024**, *14*, 2038. [CrossRef]

agriculture

MDPI

Review

Fermented Feed in Broiler Diets Reduces the Antinutritional Factors, Improves Productive Performances and Modulates Gut Microbiome—A Review

Nicoleta Corina Predescu [1,*], Georgeta Stefan [2], Mihaela Petronela Rosu [1] and Camelia Papuc [3]

[1] Preclinical Sciences Department, Faculty of Veterinary Medicine of Bucharest, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 105 Splaiul Independentei, District 5, 050097 Bucharest, Romania; petronela.rosu@fmvb.usamv.ro
[2] Clinical Sciences 1 Department, Faculty of Veterinary Medicine of Bucharest, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 105 Splaiul Independentei, District 5, 050097 Bucharest, Romania; georgeta.stefan@fmvb.usamv.ro
[3] Academy of Romanian Scientists (AOSR), 54 Splaiul Independentei, District 5, 050094 Bucharest, Romania; cami_papuc@yahoo.com
* Correspondence: corina.predescu@fmvb.usamv.ro

Abstract: The aim of this review is to highlight the most beneficial effects of dietary fermented feed in correlation with decreasing the antinutrient concentration in vegetal matrices usually used for broiler nutrition. Rational feed formulation is critical for animals because it improves animal performance, and provides the animal with the necessary nutrients to develop strong bones, muscles and tissues, and a properly functioning immune system. Fermentation of animal feed is useful as compounds with high molecular mass are converted into energy and compounds with lower molecular mass in the presence of enzymes produced mainly by bacteria and yeasts. Fermentation products contain probiotic compounds with beneficial effects on the health of the animal microbiome. Feed fermentation has other roles such as converting antinutrients into beneficial substances for animal organisms, and some studies have shown that fermentation of feed decreases the risk of antinutrient components presence. For the bibliographic research, different platforms were used (PubMed, Science Direct, MDPI resources), and numerous words or combinations of terms were used to find the latest information. Fermented feed utilization has been shown to enhance growth performance while promoting a healthier gut microbiome in animals.

Keywords: fermented feed; broiler; antinutrient components; gut microbiome; biofilm; bioavailability; goblet cells

Citation: Predescu, N.C.; Stefan, G.; Rosu, M.P.; Papuc, C. Fermented Feed in Broiler Diets Reduces the Antinutritional Factors, Improves Productive Performances and Modulates Gut Microbiome—A Review. *Agriculture* **2024**, *14*, 1752. https://doi.org/10.3390/agriculture14101752

Academic Editor: Lin Zhang

Received: 19 August 2024
Revised: 23 September 2024
Accepted: 3 October 2024
Published: 4 October 2024

1. Introduction

Sustainable and physiological growth [1], improving meat quality and feed conversion efficiency of farm broilers represent the main directions of this sector [2,3]. Increased oxidative stress has a tremendous impact on the health status and well-being of the birds [4]. In fact, stress management presented a big concern for researchers all over the world, for the last ten years. In order to reduce the unintended side effects, many solutions were proposed and tested [2]. In European Union until 2006, for both therapeutic and non-therapeutic applications antibiotics were used in animal production [5], posing a threat to product safety due to antimicrobial residues and increasing the risk of microbial resistance development and spread in the poultry environment [3].

To mitigate this risk, alternative strategies are being explored to reduce antibiotic use in chicken farms [5,6]. Research has focused on finding natural agents that can serve as growth promoters while maintaining low mortality rates, high animal yield, and protecting the environment and consumer health [7].

Research has simultaneously explored the use of plant-derived antioxidant compounds [8], bacteriophage therapy [9], micronutrient supplementation, and fermented feed [10] in animal feed as strategies to alleviate various negative impacts on animal health, human health, and environmental sustainability.

In general, animal agricultural sectors and especially, poultry farms have grown more interested in using plants and their derivatives as feed additives throughout the past ten years to preserve or enhance their health and productivity [11]. Secondary plant metabolites, polyphenols, are responsible for most host health benefits [12]. Polyphenols are well known for their antioxidant [13], immunomodulatory [14], anti-mutagenic [15], and anti-inflammatory effects [16]. Despite these benefits, polyphenols have been shown to have poor gastrointestinal absorption as well as reduced quantities in target cells, limiting their effectiveness as antioxidants. Some polyphenols' poor bioavailability necessitates additional research to fully realize their potential in poultry livestock rearing [17]. For example, tannins are plant polyphenols, known for their properties like binding to proteins to form large-molecular compounds, and complex metal ions, decreasing their availability for animals [18].

So, researchers are looking for solutions to increase the accessibility and bioavailability of proteins, metal ions, tannins, and other molecules [19]. A brief search on the main platforms for scientific journals (MDPI, PubMed, and Science Direct) revealed an increased number of articles related to the use of fermented feed for broilers reared in the farm system, to improve the bioavailability of the healthy molecules.

Indeed, feeds are the most important instruments used to achieve the mentioned goals (rapid growth, enhanced meat quality, and increased feed conversion efficiency in farms raising broiler chickens).

The use of fermented feeds in broiler nutrition has gained attention due to its potential impact on growth performance [20]. Also, many studies proved that fermented feed has a salutary effect on the broiler gut microbiome [21]. In general, the fermentation process results in the breakdown of complex compounds in the presence of microorganisms, leading to the production of smaller and more beneficial metabolites [21], that have a positive impact on the health and antioxidant status of the broiler [20]. Also, fermentation reduces the antinutrient components (ANC) in the feed, conducting to increase of food-beneficial nutrient bioavailability [19–21].

Healthy fermented feed alternatives include probiotics, prebiotics, enzymes, organic acids, immunostimulants, bacteriocins, bacteriophages [9], or feed additives [8]. Probiotics used in fermentation become the main microorganisms delivered by feed; the probiotic's metabolites lower the pH of the feed and help reduce harmful microorganisms [22]. Probiotics like *Lactobacillus*, *Bacillus subtilis*, and yeast can improve immunological, antioxidant, and production function as well as remodel gut microbiota and alleviate intestinal dysbiosis and inflammation [23]. Probiotics are added to a feed substrate and fermented under controlled conditions to make fermented feed. Probiotics take over in the feed during fermentation, generating metabolites that raise crude protein content, lower pH, limit mycotoxin synthesis, reduce dangerous microbes, and remove allergens and antinutritional factors. Some studies recommend fermented feed for better tastes thanks to the production of acids, alcohols, ketones, and esters during the process [24,25].

The aim of the present review is to emphasise and discuss the most beneficial effects of using fermented feed for broilers in the context of achieving the expected growth performance in connection with gut microbiome health. Notably, for the present review, the most beneficial effects of feed fermentation are presented in correlation with decreasing the concentration of ANC in vegetal matrices usually used for broiler nutrition.

Feed of vegetal origin, that undergone microbial fermentation, usually with the aid of lactic acid bacteria (LAB), is referred as fermented feed. Through this method, the feed's nutritional profile changes, its digestibility is increased, and the broilers' gastrointestinal health is improved [20]. Probably the most important fact is that fermented feed contains live probiotics, organic acid (like lactic acid), lowering the pH, which can inhibit harmful pathogens, naturally preserves the feed, and reducing the risk of spoilage [25].

2. Fermentation Used in Feed Processing

Fermented feeds have been gaining attraction in poultry farming as a potential way to improve both growth performance and gut health in broilers [24,26]. It is known that fermentation is a complex process, and lately, it gained more and more application from the food industry to the feed industry. The fermentation process is based on the breaking down of complex compounds in feedstuffs, making nutrients more digestible for animals. This can lead to improved growth performance and feed efficiency. Fermentation can preserve feedstuffs and extend their shelf life by reducing spoilage caused by moulds and bacteria [27]. Moreover, fermentation allows for the utilization of non-conventional feed sources, such as industrial waste products, which can help reduce reliance on traditional feed ingredients. The use of probiotics (live microorganisms) and prebiotics (fibres that promote beneficial gut bacteria) in conjunction with fermentation can further enhance the benefits for animal health [24].

There are various techniques for making fermented feeds from vegetal matrices, such as liquid fermentation, solid fermentation, and ensiling, which are the most used over the last couple of decades [20]. *Fermented liquid feed* provides not only water and digestible nutrients, but also organic acids and beneficial microorganisms such as lactic acid bacteria and yeasts. *Solid-state fermentation*, as opposed to liquid fermentation, has advantages such as reduced wastewater generation, increased product stability, reduced energy consumption and easier transportation [22]. In general, the fermentation substrate used for *ensiling* consists of fresh, water-rich plant material that supports a high number of active microorganisms [28]. For instance, mechanical chopping during harvesting may lead to an increase in lactic acid bacteria (LAB) counts, followed by *Enterobacteriaceae* and yeasts. Whatever type of fermentation is used, most of the research studies suggest a concentration of live microorganisms in the biomass between 10^5 and 10^9 CFU/g [28].

Spontaneous fermentation, although possible in all types of fermentation, should be avoided because of the risk of growth of harmful microorganisms and toxic metabolites. The development of harmful microorganisms is reduced under normal production conditions and can be effectively controlled by applying good quality control practices as well as by optimizing fermentation parameters [21,22]. These parameters include composition of raw materials used, starter culture formulation, fermentation conditions, and post-fermentation processes. For instance, the selection of enzymes and microorganisms depends on the characteristics of the substrate, and pretreatments (mechanical, thermal, chemical) can facilitate fermentation. The choice between enzymatic treatments and fermentation varies depending on the properties of the feed and enzymes used. Finally, nutritional assessments are essential to determine both feed improvement and nutritional value [24–26,28].

All the three fermentation processes described above, can help reduce ANC in feedstuffs, such as tannins and phytates, which can interfere with nutrient absorption [29]. In Figure 1 are presented the most basic ingredients of the fermentation process of milled vegetal material with the addition of water (chlorine-free water is preferred to avoid killing fermentation sensitive microorganisms) and substances to stimulate bacteria and yeast for multiplication and fermentation of matrices. Reduced ANC, increased feed shelf life, alternative feed source utilization and enhanced probiotic and probiotic delivery are probably some of the most important reasons to use fermented feed for broiler chickens' nutrition.

Legumes and cereals are part of the base diet for all farm animals including broilers. Because they provide significant amounts of carbs, protein, vitamins, and dietary fibre, these grains are vital to the broiler chicken diet [24]. To promote growth and maintain health, these nutrient sources are necessary for daily nourishment. However, a lot of research regarding the plant composition in proteins, lipids, carbohydrates, vitamins, and minerals provide evidence and debate the issue regarding the presence of ANC, which reduce the bioavailability of good nutrients [30].

In Table 1 are presented a few of the most used vegetal matrices of basal diet of broiler corelated with ANC and ways to mitigate their effects.

Table 1. Antinutrient components found in plants, side effects and biotechnological solutions to mitigate them.

Vegetal Matrices	Antinutrient Components (ANC)	Side Effects of Antinutrient Components	Biotechnological Solutions for Antinutrients Mitigation	References
Soybean	Trypsin inhibitors, phytic acid, saponins and lectins	Reduce the digestibility and absorption of the nutrients in the animal organism	Fermentation in the presence of *Lactobacillus plantarum* reduced the anti-nutritional factors in the milled soybeans	[31–33]
Corn	Phytate, tannins and polyphenols	Bind dietary minerals in the gastrointestinal tract, decreasing their bio-accessibility and bioavailability	Fermentation with *L. plantarum* A6, *L. brevis* G11, *L. brevis* G25, *L. fermentum* N33, *L. fermentum* N25, *L. buchneri* M11, and *L. cellobiosus* M41, reduced the antinutritional factors.	[29,34–36]
Sorghum	Tannin (phenolic compounds), and dhurrin	Affect the growth, feed intake, digestion of protein, egg production, and deformities of the legs of broiler chickens	The two-step procedure of sinking in a NaOH solution and fermentation (using *L. bulgaricuss*, *L. casei*, and *L. brevis*) lowers the ANC and enhances the nutritional composition.	[37–41]
Barley	β-glucan	Decrease digestibility, form gels in aqueous media and excretion of sticky droppings	Fermentation of barley with *Rhizopus oligosporus*, and supplementation with β-glucanase reduced ANC	[42,43]
Wheat	Phytate, protease inhibitors, tannins, lectins, alkaloids, and oxalate	Reduces the bioavailability of essential micronutrients such as iron and zinc	Fermentation (*Bacillus* sp. TMF–2) as well as genetic engineering led to the reduction of ANC	[44,45]
Oats	Phytate, tannin, and oxalate	Disrupt the stomach's normal functioning and prevent nutrients from being absorbed.	*Saccharomyces cerevisiae* fermentation, germination, de-branning, autoclaving, soaking reduced ANC	[46,47]
Rye	Water-soluble pentosans	Clogged digestive tract with a viscous pentosan solution that prevents food nutrients being absorbed; esophageal and gastric blockages	Fermentation with 1k2079 *L. plantarum*, 1k2103 *Pediococcus pentosaceus*, and 1k2082 *L. lactis* enzymes seem to reduce ANC	[48–50]
Alfalfa	Saponins	Growth rate retardation	Fermentation with LAB reduced ANC	[51–53]

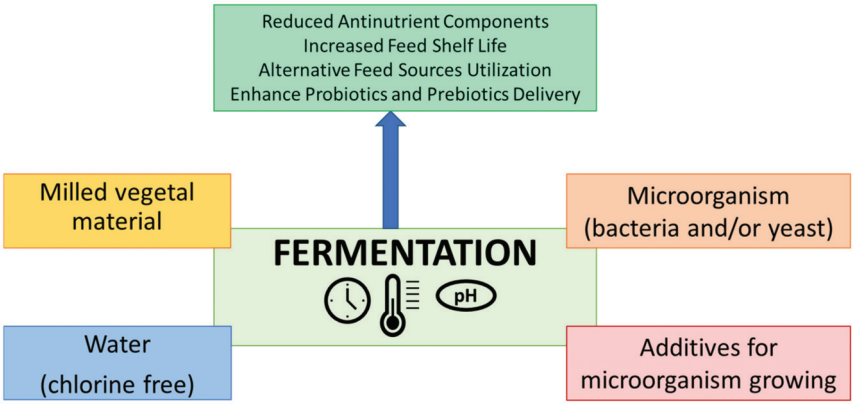

Figure 1. The ingredients involved in the fermentation process (wet milled vegetal material in the presence of microorganisms acting in specific pH and temperature conditions) are in correlation with four of the most important arguments for using fermentation to improve feed safety and quality for broiler nutrition.

Worldwide, soybean (*Glycine max*) is the primary source of protein and amino acids utilized in animal feed formulations, especially for monogastric animals. High inclusion rates of high-quality vegetable protein, particularly in diets for chicken, can be found in soybeans, which have a less changeable chemical makeup than other protein sources. In addition to their high protein content, soybeans also include a variety of variable amounts of substances that are generally regarded as antinutritional. Phytic acid, saponins, isoflavones, and trypsin inhibitors are a few examples [31]. Nowadays, it is believed that eating controlled concentration of these substances can have positive biological effects on blood cholesterol levels and cancer prevention [33]. Most of the scientific results addressed the variations in the concentrations of phytic acid, saponins, isoflavones, and trypsin inhibitors. Soybean processing alters the levels of these minor elements in a variety of ways. During the processing of soybeans by fermentation is created conventional protein components, flours, isolates, and concentrates, without ANC [32].

Corn or maize (*Zea mays*) provides an affordable and easy-to-access source of protein and energy, being utilized as an important ingredient in commercial chicken feed. The high carbohydrate content, and amino acid profile of corn protein give chickens the energy they need to thrive and regulate their body temperature. Also, corn contains higher levels of ANC, phytate and polyphenols, that form complexes with metal ions [34,35]. With the help of the enzymes produced during fermentation, ANC are reduced, making fermentation an effective technique for enhancing the macro, trace, and total minerals levels of corn genotypes. Tannase and phytase, two enzymes produced during fermentation by microorganisms, are thought to be responsible for reducing antinutrients (polyphenols and phytate) in corn genotypes [36]. This likely explains the increase in trace minerals after corn fermentation. One possible mechanism for releasing minerals from phytate could be dephosphorylation, whereby the removal of phosphate groups from the inositol ring reduces phytate's ability to bind minerals, increasing the bioavailability of vital dietary minerals. Fermenting grains flour for 14 days resulted in significant increases in total and extractable zinc, manganese, copper, and cobalt levels [29].

Both ruminant and nonruminant farming systems can use sorghum (*Sorghum bicolor* L.) as a source of protein and energy. Sorghum can be a good source of grain in an animal's diet if it is processed properly and combined with other feed ingredients in a balanced manner [38]. Further research is required to fully comprehend important antinutritive characteristics of sorghum, such as tannin (phenolic compounds), phytic acid, and dhurrin [37]. Apart from its application in the food animal industry, the problem lies in the high tannin content of sorghum, a water-soluble polyphenolic metabolite that inhibits

poultry growth. Increased tannin concentrations slow down protein digestion and form chelate, which makes them ANC [39]. Tannins negatively affect the growth, feed intake, digestion of protein, egg production, and deformities of the legs of broiler chickens. Dhurrin, a cyanogenic glucoside, by a specific enzyme pathway generate hydrogen cyanide (HCN), toxic for organisms [40]. It decreases the broilers' apparent nutritional digestibility, promotes cytochrome oxidase system inactivation causing anoxia of the central nervous system, which can lead to death in a matter of seconds. To inhibit the antinutritive effects, processing in the presence of microorganism like lactic acid bacteria (LAB) is used [37,41].

Research has shown that the inclusion of barley (*Hordeum vulgare* L.) with β-glucan in the diets of young chicks can hinder growth and lead to problems such as sticky droppings. However, these negative effects can be mitigated either by fermenting the barley or by adding β-glucanase producing microorganisms to barley-rich diets [42]. The adverse effects appear to be associated with nutrient digestion and bowel transit problems, but there is no conclusive evidence that treatments aimed at removing β-glucan can improve nutrient digestibility in barley-based diets. Although there is a substantial amount of literature on this topic, very few studies have made significant advances for a certain answer [42]. Some recent research has looked at the antinutritive effects of pentosans present in other cereals, but there have been no direct tests on β-glucan isolated from barley in poultry diets, nor examinations of its impact on nutrient uptake by poultry. Probably, further research on the biochemistry and molecular biology of fermentation microorganisms with glucan-degrading enzymes could provide a deeper insight into their role in poultry nutrition [43].

Antinutritional factors are harmful compounds in wheat (*Triticum aestivum*) that interfere with the absorption and bioavailability of nutrients in monogastric animals like pigs and poultry, so impact its nutritional quality. Wheat, a key source for energy and protein, contains several ANC like phytate, protease inhibitors, tannins, lectins, alkaloids, and oxalate. Phytate, for instance, reduces the bioavailability of essential micronutrients such as iron and zinc [44]. Various strategies, including fermentation as well as genetic engineering, aim to mitigate these effects. Enzyme degradation of tannins—proteins complex in seed provide an efficient solution (this network being responsible for trapping metal ions). Rather than germination, processing by fermentation increases the content of iron and zinc, with the highest iron content observed in fermented samples (5.52 mg/100 g) [45].

Oats (*Avena sativa* L.) stand out among cereals because of their high dietary fiber content and nutritional value, which includes proteins, balanced amino acids, minerals, unsaturated fatty acids, vitamins, antioxidants, and phenolic compounds [46]. Their high soluble and insoluble fiber level is especially sought because it has been associated with several health advantages. Also, important concentrations of ANC are present in oats, it can disrupt the stomach's normal functioning and prevent nutrients from being absorbed [47]. Also, rough texture, tough hull removal, low digestibility, and antinutrient elements, limit the use of oats in animal diet. To lower ANC, several processing methods, including fermenting, grinding and boiling, can help address these problems by increasing the availability of minerals, protein, and carbohydrates [47].

The rye grain (*Secale cereale*) contains several nutritionally beneficial dietary components. Poultry should not receive more than 5–7% of their diet, according to zootechnical requirements. The primary cause of rye grain's restricted use as animal feed is its high level of antinutrient ingredients, like water-soluble pentosans [48]. Water-soluble pentosans' capacity to absorb water and create a very dense solution gives their negative feeding characteristics. Animals' digestive tracts are clogged with a viscous pentosan solution that prevents food nutrients from being absorbed. High rye grain consumption causes indigestion, weakens farm animals, and slows down the forage's passage through the digestive system [49]. Poultry may experience oesophageal and gastric blockages. The immune systems of the birds will be impacted by the addition of rye to their diet. To prepare rye grain for feeding animals, there are several methods. Winter rye grain is currently subjected to enzymatic treatments to counteract its antinutritional qualities and improve its

nutritional digestibility. Studies indicate that applying enzyme preparations to the aqueous grain extract of winter rye can considerably lower its viscosity [50].

Because it has a high protein content and a high dry matter yield, alfalfa (*Medicago sativa* L.) is an essential livestock feed crop in both developed and developing countries. This plant's primary ANC are saponins, and because of their detrimental effects on animal performance, its high protein content has not been able to be used to its full potential as animal feed [51]. Saponins are glycosides that have a polycyclic aglycone moiety connected to a carbohydrate. These moieties can be either of the C_{27} steroid or the C_{30} triterpenoid, which are together referred to as sapogenins [52]. The flavor of saponins is unpleasant, and they have foaming qualities. For non-ruminant animals (pigs and chicks), the most important negative effect is growth rate retardation, which is mainly caused by a decrease in feed intake [6]. To fully detoxify the seeds of this antinutrient, the dry (raw) bean requires a long cooking time. Alternatively, fermentation with LAB microorganism, dramatically eliminates the majority of these antinutrients with a significantly lower energy cost than cooking [53].

Fermentation increases feed digestibility because fermentation break down lipids, complex carbohydrates and proteins into their simple biochemical units (e.g., fatty acids, monosaccharides or amino acids), making them easier for broilers to digest and absorb nutrients [20]. According to another study, the enhanced digestibility of fermented feeds may be responsible for the beneficial effects observed in the gastrointestinal environment/condition (e.g., the reduction of gastric pH and pathogenic microbial activity as well as the increase in the production of short chain fatty acids [SCFA]), which in turn enhances the growth performance of chickens [54].

Fermentation of fresh vegetal nutrients reduces the size of the complex molecules, with anti-nutritional characteristics, to easily absorbable structures in the digestive tract. Some of these molecules (Figure 2A), like protein-tannin complex, metal—starch—phytate complex or inhibitor–enzyme complex. After fermentation process (Figure 2B) of vegetal feed, the complex molecules are degraded, reducing the concentration of ANC, increases the concentration of beneficial compounds (antimicrobial peptides, metal ions, antioxidants, vitamins, short chain fatty acids, and amino acids) released from the complex networks and acting as prebiotics in the broiler gastrointestinal tract (GIT). Additionally, fermentation brings beneficial microorganisms, probiotics, to the digestive tract of the broiler improving the health status of the birds (Figure 2C). Broilers ingest fermented feed with decreased concentration of antinutritional compounds and enhanced prebiotics and probiotics levels, elevate the meat nutritional value. The human consumer, as the final link in the chain, is the beneficiary of the positive consequences of feeding fermented feed to broilers (Figure 2D).

Tannins have been shown to bind proteins via hydrogen bonding and hydrophobic interactions, resulting in protein-tannin complexes [55]. They impact the digestibility of proteins and carbohydrates, adding to the diet's lower calorie value. Tannins interact with digestive enzymes such as trypsin and amylase, leaving them unavailable for digestion reactions. Tannins can form a complex compound with vitamin B, making it inaccessible for body needs [31,56].

Phytic acid is present in many plants and is also called inositol hexa-phosphate or IP6. It forms salts of the mono or/and divalent cations K^+, Ca^{2+}, and Mg^{2+}. In this way, phytates serve as reservoirs of cations, with high-energy phosphoryl groups [56]. Because they can act as chelators of free iron are considered efficient natural antioxidant. Phytates are powerful anions in a broad pH range, impacting the bioavailability of divalent and trivalent mineral ions such as Ca^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, or Fe^{3+} in diet [57]. The effect of increasing phytate intake on mineral deficiency is dependent on what else is consumed. It has been claimed that the interaction between phytate and polysaccharides like starch, lowers carbohydrate bioavailability and breakdown, and influences starch metabolism as the development of the phytate-carbohydrate complex [31].

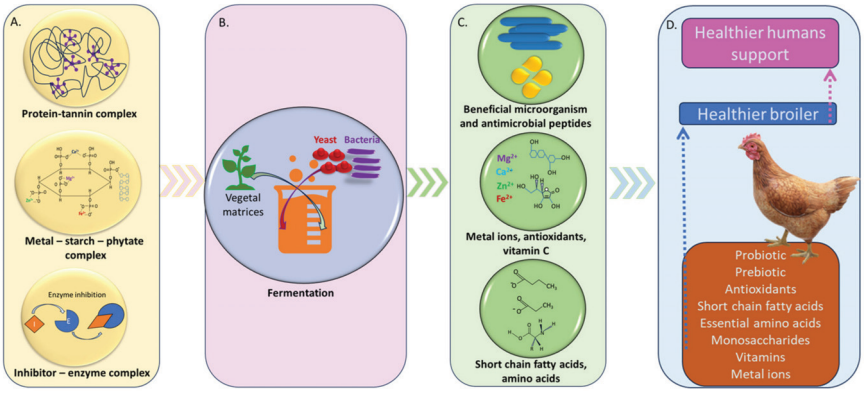

Figure 2. The influence of fermenting plant material rich in anti-nutritional compounds on feed quality and implications for broiler health and, consequently, human health. (**A**) Most often found antinutritional complex in feed protein-tannin complex, metal—starch—phytate complex, inhibitor—enzyme complex; (**B**) Fermentation process enhancing feed quality and safety through microbial degradation of the complex compounds into smaller and easily absorbable compounds; (**C**) Highlighted products found in fermented feed like beneficial microorganism and antimicrobial peptides, metal ions, antioxidants, vitamins, short chain fatty acids, amino acids; (**D**) consequences of administration of fermented feed to broiler and humans as final receiver.

Protease inhibitors are found in many plants, including legume and cereal seeds used for broiler nutrition. Protease inhibitors function as competitive inhibitors, binding to the enzyme's active site and forming a complex with a low dissociation constant at neutral pH [57]. The inhibitor imitates the substrate, resulting in an inhibitor-enzyme complex that cannot be removed using the usual process [55]. This blocks the enzyme's active site and effectively silences its activity. For instance, amylase inhibitors are thought to have two functions: they protect the seeds from microbial diseases and pests while also inhibiting endogenous amylase [31,56].

Nutrient digestibility is a measurement of feed nutritional processes related to capacity and intestinal function. Beneficial microorganisms in fermentation produce metabolites like lactic acid, bacteriocin, antibacterial substances, and alcohols, which can lower digestive tract pH, inhibit harmful bacteria like *Escherichia coli*, and improve intestine digestion and absorption [55]. Addition of fermented material, broilers' apparent calcium metabolic rates increased. Adding 20% fermented feed enhanced the apparent metabolic rate of Calcium. This is because the lactic acid produced by fermentation lower the feed pH, creating a favourable acidic environment in digestive system for calcium absorption [58].

In the same time, research suggests that fermented feeds can be a promising strategy for enhancing growth performance and gut health in broilers. However, the specific effects can vary depending on factors like the type of fermentation, the ingredients used, and the inclusion level in the diet. Furthermore, improved growth in birds is attributed to elevated activity of digestive enzymes such lipases, amylases, trypsin, and proteases in broilers fed fermented diets [59].

Enhanced nutrient availability by previously subject to fermentation of row vegetal matrices or plant byproducts. Beneficial microbes in fermented feeds can produce enzymes and organic acids that further improve nutrient utilization. While there is evidence linking fermentation to enhanced palatability and nutrient digestibility, fermentation resulted in a decrease in the amount of feed consumed by broilers throughout the starter and grower phases [60]. The hens' growth rates were slowed down as a result during these stages, but surprisingly not during the finishing phase.

The ability of animals to convert feed into muscle tissues is shown by their slaughter performance [61]. The two main indicators used to assess carcass yield and meat quality

in broilers are muscle and visceral (abdominal) fat. The addition of FTLM to the broiler diets raised the weights of the thigh and breast muscles [62]. Also, Yang et al. (2008) noted a rise in the weights of the cut parts (thighs and breast) of broilers given fermented herbal items [63].

3. Effects of Fermented Feeds on Broilers Growth Performance and Gut Microbiome

3.1. Improved Growth Performance

Resources like cereals, different vegetal beans, and agricultural by-products are insufficient to improve growth performance in broiler? It was shown previously that ANC in the feed, reduce digestibility and have an impact on survival rates. To reduce ANC, fermentation is the most used method [24]. Fermentation feed can stimulate growth, take the place of antibiotics, recycle waste (use byproducts to cut waste), and alleviate feed scarcity [22].

To evaluate the growth performance of an animal, in this case, broiler, considered parameters are feed conversion ratio (FCR), body weights gaining (BW) and feed intake (FI) [24]. Those tree parameters are linked by a mathematical relationship. FCR represents the ratio between FI and BW. The research indicate that the microorganisms used for feed fermentation influence the balance [64].

It's crucial to increase broiler feed conversion rate, and for broilers feed with fermented feed, FCR is between 1.35–1.70 g feed/g gain, depending on the concentration of fermented feed added to the basal ratio and the age of the broilers [24]. The most recommended solution by the researchers is represented by feed fermentation. Indeed, probiotic fermentation of different materials produces fermented feed, which increases the beneficial nutrients and decreases the ANC [65].

According to the study [21], corn, soy-bean, and wheat, 6:2:2 (*w*:*w*:*w*), fermented with *Lactobacillus casei* (*L. casei*) was used as fermented feed additive. Four groups of chickens were investigated as follows NC (negative control received basal diet), PC (positive control received basal diet +antibiotic 15 ppm), FFL (fermented feed low received basal diet + 0.3 kg/t FFA), and FFH (fermented feed additive high received 3 kg/t FFA). The results of the study [21] indicated that FFL and PC diets had a higher FCR than the NC from 0–42 days, and the FFH and FFL groups gained greater BW (1–21 days). Also, microorganisms with beneficial properties, such as *Lactobacillus aviarus* genus, *Lactobacillus* spp., and *Lactobacillus phylum Delsulfobacterota* and class *Desulfovibriona*, were also tended to increase in the FFH and FFL fermented feed groups compared to the PC and NC groups. Pathogenic microorganisms were also significantly reduced in the group treated with FFH and PC [21].

Irawan et al., [62] investigated the effect of fermented soybean meal (FSBM) in the presence of *Lactic acid bacteria* and yeast on chickens' performance. Comparing broiler trials fed with and without FSBM revealed that replacing SBM with FSBM had a substantial favorable effect on the BW of broiler chickens [66]. Except for the feed fermentation in the presence of *Aspergillus niger*, the majority of microbial fermentation (*Lactic acid bacteria* and yeast) resulted in a considerable rise of broiler BW. In the same research, positive correlation was found between broiler average daily gain and the diet supplementation with FSBM fermented in the presence of *Aspergillus oryzae*, mixed probiotics + bromelain, *Bacillus subtilis*, and *Lactobacillus microorganisms*. Also, fermented feed seems to be responsible for increasing the FI [66].

The most important microorganisms used to obtain fermented feed are belong to *Ruminococcaceae*, *Lactobacillaceae*, and unclassified *Clostridiales*. The growth performance parameters obtained after feeding broilers with fermented feed in the presence of these bacteria are shown in Table 2.

Table 2. Examples of microorganisms used for preparation of fermented feed corelated with some growth performance parameters.

Fermented Feed Microorganisms	Vegetal Matrices	Effects of Fermented Feed Groups Compared to the Control Group		References
		Growth Performance Parameters	Other Parameters	
Lactobacillus casei (C37M41)	Corn, soybean, and wheat, 6:2:2 (*w*:*w*:*w*)	- ↗ BW during the first 21 days days for broiler treat with fermented feed in concentration of 3 kg · t^{-1} ↗ FCR during 0–42 days for broiler treat with fermented feed in concentration of 0.3 kg · $tone^{-1}$	- ↙ pathogenic microorganisms (phylum *Delsulfobacterota* and class *Desulfovibriona* and *Negativicutes*) - ↗ beneficial microorganisms like *Lactobacillaceae* family	[21]
Bacillus amyloliquefaciens	Rice bran	- ↗ fermented feed consumption in the first three weeks, - ↗ FCR at the first three weeks of the feeding trial and BW	Zero mortality recorded during the experiment	[60]
Lactobacillus and *Bacillus subtilis*	Corn, soybean, cottonseed, and rapeseed (different ratio)	- ↗ growth performance after addition of 10% fermented feed, in the first 21 days - ↗ growth performance after addition of 5% fermented feed in 0–42 days of broiler life	- ↗ breast fat - ↙ cholesterol content in broiler meat - ↗ immune factors like IgM, IgG, and interferon-γ	[26]
Streptococcus alactolyticus, *Lactobacillus acidophilus*, *Lactobacillus reuteri*	*Astragalus* powder	- ↗ Final BW and ADG and ↙ F/G ratio for fermentation feed group compared to control and unfermented feed group	- ↗ serum IgA and IgG, serum albumin, serum total antioxidant capacity - ↙ serum and liver tissue malondialdehyde on day 28 and day 42.	[27]
Bacillus licheniformis	Corn–soybean (different ratio)	↗ growth and digestibility for fermented feed group compared with other tested broiler groups	- ↗ duodenal and ileal villi heights - crypt depths weren't not altered	[67]
Aspergillus oryzae 3.042	Soybean	- ↗ Fermented feed broiler presented growth and feed conversion promotion compared to another tested group	- ↗ activities of trypsin, lipase, and protease in the intestinal content	[59]
Bacillus spp., *Lactobacillus* spp., Saccharomyces cerevisiae	Corn, soybean and wheat bran, 6:2:2 (*w*:*w*:*w*)	- ↗ nutrient absorption; - ↙ the abdominal fat rate and the muscle fat	- ↙ feed pH value and anti-nutritional factor concentrations - ↗ good bacteria like *Parasutterella*, *Butyricicoccus* and *Erysipelotrichaceae*	[68]

Average daily gain—ADG; feed to gain ratio—F/G; ↗ means increased; ↙ means decreased

In the last years, besides corn, soybean, cotton-seed, and rapeseed, other vegetal sources are used as feed for broiler. Leaf meals (LM) are leaves and twigs dried, pulverized, and used as a feed for livestock. They are an important management tool during the dry months when fresh fodder is in short supply [68]. For instance, tropical leaf meals (TLMs) can be used in poultry production due to their health and nutritional benefits. However, their large-scale use is limited due to their high crude fiber content and moderate anti-nutritional factors. Fermentation, particularly microbial fermentation, has been shown to reduce these factors, increase nutritional benefits, and improve growth performance in broiler production. Ogbuewu et al. [61] find out that the growth performance of broilers fed with fermented tropical leaf meals (FTLM) may be linked to decreased ANC levels and the breakdown of complex biomass by fermentation microorganisms. This may also improve intestinal health and function, as the intestine is the primary site for immunity, nutrient digestion, and uptake [61].

Fermented feeds have been found to promote growth in broilers by increasing intestinal length indices, maintaining normal gut microbial ecosystems, and improving intestinal morphology, such as villus height. These feeds also improve digestion and absorption, leading to improved production performance [20]. This can be sustained by the study of Saleh et al. [67], where *Bacillus* spp. appear to be a viable alternative for antibacterial growth promoters that improve animal health and performance. *Bacillus licheniformis* can sporulate, rendering them stable under thermal processing of feed and resistant to enzymatic digestion along the gastrointestinal tract (GIT). *Bacillus licheniformis* addition in broiler drinking water or diets boosted growth performance by increasing the body weight and feed conversion ratio of broilers [67].

3.2. Gut Microbiota Modulation

The microbiota is the entire microbial population (including viruses, bacteria, fungi, protists, and archaea) that lives in complex multicellular organisms such as plants, animals, and humans [9]. The microbiota in broiler chickens' gastrointestinal tract (GIT) plays a crucial role in their health, immune system, and productivity. It reduces colonization by enteric pathogens and prevents inflammation, leaky gut, and other gut-related disorders [69]. Broiler microbiota composition is influenced by factors like age, diet, genetics, and antibiotic use. Broad-spectrum antibiotics cause collateral damage, leading to dysbiosis and antibiotic resistance. Poultry production systems use antibiotics for growth promotion, but indiscriminate use reduces microbiota stability and *Lactobacillus* population in broilers. Long-term antibiotic usage has disrupted the gut microbiome of the animals in general, and impaired immune function [56]. Furthermore, increasing treatment resistance among pathogenic strains has emerged as one of the world's most severe issues. The observations described above has given rise to a growing interest in the management of infections caused by antibiotic resistant pathogens by selectively targeting the disease-causing bacteria, without disturbing the commensal microbiota of the GIT [70].

Due to their immature immunological and digestive systems, chickens have poor nutrition utilization and low condition resistance. 44 bacterial strains have been licensed by the Food and Drug Administration (FDA) and Association of American Feed Control Officials (AAFCO) for use in fermented feed, which has improved animal husbandry [71]. By secreting chemical signals known as auto-inducers, which alter bacterial behaviour, bacteria are able to interact with one another across cells. Bacteria also utilize the quorum sensing mechanism of bacterial communication to communicate with their host. Probiotics may impact pathogenic bacteria's ability to sense quorum, which could change how harmful they are [72]. For instance, fermentation products from *L. acidophilus La-5* significantly reduced the extracellular secretion of a chemical signal (autoinducer-2) by human enterohaemorrhagic *E. coli* serotype O157:H7. This led to the in vitro suppression of the expression of the virulence gene (LEE, locus of enterocyte effacement). This interferes with quorum sensing and stops *E. Coli* serotype O157:H7 from colonizing the GIT [73].

In order to mitigate the concentration of ANC, cereals and vegetables commonly used in broiler feed are most often subjected to fermentation and then fed to the animals [73]. Fermented mixed feed had lower pH, phytic acid, trypsin inhibitor, and β-glucan concentrations, compared to unfermented feed. Fermentation increased crude protein content, but unfermented mixed feed contained higher molecular mass proteins (60–120 kDa) [68].

Fermentation of feed introduces beneficial bacteria like *Lactobacillus* and *Bifidobacterium* to the gut. These microbes compete with harmful bacteria for space and resources, promoting a healthy gut balance. The analysis reveals positive correlations between intestinal morphology and certain bacteria related to gut health, while negative correlations exist with bad microorganism. Fermentation improves feed safety by preventing the growth of pathogenic bacteria [74].

The diverse habitats that make up the gastrointestinal lumen's various segments' distinct functions are reflected by them. Additionally, the gut lumen has a variety of habitats that could add to the microbiota's spatial variability [68]. The most listed chemical gradients (like, pH, bile concentrations, etc.), nutritive molecules availability, and immunological interactions are some of the possible causes of this heterogeneity [75]. The upper section of the gut is thought to have a bacterial density of 10–10^3 bacteria per gram of stomach and duodenal contents, in the ileum and jejunum, it rises to 10^4–10^7 bacteria/gram of content [76].

The broiler intestinal health is very broad and relies on an understanding of nutrition, intestinal morphology, and gut microbiota [77]. All of these components interact with one another to ensure the appropriate functioning and dynamic balance of the GIT lumen (Figure 3). The intestinal histological structure has a crucial impact in the GIT's ability to move nutrients from the lumen into the systemic circulation. As shown in Figure 3A, the gut mucosa functions as both a physical and immunological defensive barrier. The barrier is primarily composed of the mucus layer, biofilm, microorganism metabolites, and secretory immunoglobulin A (sIgA) molecules, which are linked to the specialized epithelial central single cell layer with the epithelium's tight junction proteins (ETJP) and the inner lamina propria, which is made up of immune cells, loose connective tissue, blood vessels, and lymphatics [78,79]. Goblet cells secrete high molecular weight glycoproteins, known as intestinal mucous layer [80]. Enterocytes, goblet cells, and other specialized cells form a continuous and polarized monolayer. It separates the lumen of the intestine and lamina propria. In the absence of specialized transporters, cell membranes are impermeable to hydrophilic solutes, limiting their transit through ETJP. Diffusion and endocytosis are the primary mechanisms for lipophilic or large-molecule absorption [79]. Junctional complexes govern the transport of molecules between ETJP. Generally speaking, there are two ways that nutritional compounds can go from the intestinal lumen to the subepithelial space: transcellular and paracellular [81]. Large antigenic molecules, lipophilic substances, and nutrients will prefer the transcellular pathway. It assumed binding to certain transporters, endocytosis, or passive diffusion to move molecules across the ETJP. Ions, particularly cations, and tiny hydrophilic molecules (<600 Da) will leave the lumen by paracellular transport pathway. These substances will diffuse via the intercellular gaps between neighbouring IECs, with the TJs acting as the rate-limiting step for epithelial permeability [79,82]. Fermented feed contains small molecules with molecular weight less than 600 Daltons, like amino acids, monosaccharides, antioxidants like vitamin C or polyphenols, SCFA. These molecules are easily absorbed by villus enterocyte which is the absorptive surface of the intestines. Also, the study prove that increased dimensions of intestinal villi are connected with an increase in the absorbent surface of the intestines as well as the absorbing rate of the intestine [80]. Good microbiome is preserved by competition with the pathologic microorganism (Figure 3A).

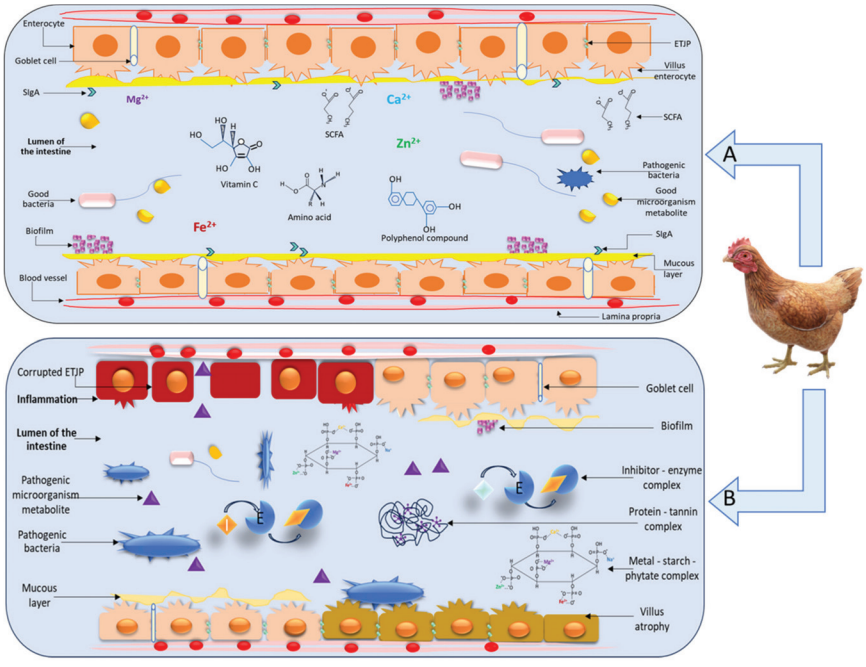

Figure 3. Intestinal cytoprotective effect of fermented feed (**A**) and unfermented feed (**B**). Probiotic and prebiotic condition and stabilizes the gut structure; reduce pathogenic bacteria, increase small molecules content (amino acids, monosaccharides, antioxidants like vitamin C, polyphenols, SCFA) and free ions (Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}); increases the number of beneficial bacterial metabolites like bacteriocins; stabilize mucus layer; (**A**); unfermented feed promote longer period for digestion of protein—tannin complex, and metal—phytate complex, increased of inhibitor—enzyme complex, enterocyte villus atrophy, inflammation via pathogenic bacteria metabolites, development of pathogen microorganism on account of the fall in good bacteria number, goblet cells perturbation with the result of diminishing of mucus layer secretion (**B**).

So, intestinal morphology (like, villus height, crypt depth) changes in response to exogenous agents, such as the presence or lack of good nutrient feed or pathological circumstances. Based on the previous presented fact, fermented food is quickly absorbed from the lumen of the intestine, also stimulate the peristalsis of the intestine. Also, unfermented feed stays longer time in the intestine and the presence of ANC conduct to modification and pathology development like inflammation, villus atrophy, corrupted epithelium's tight junction proteins (ETJP), and goblet cells perturbation and decreasing in mucous layer secretion represent an unwanted scenario having dramatic consequences on broiler health (Figure 3B). Mucous layer is protecting the enterocyte and, in its absence, the perturbance of microbiome appear and together with pathological microorganism metabolites can pass from the intestine lumen complicated even more the broiler health. The decreased number of healthy microbes allow the development of the rest of the unwanted bacteria and yeast. The important concentration of ANC (like, protein-tannin complex, metal—starch—phytate complex, inhibitor—enzyme complex) overlap existing problems (Figure 3B).

The gastrointestinal tract (GIT) microflora is primarily composed of bacteria, with minor populations of fungi and protozoa. Because different bacterial species have distinct growth requirements and substrate preferences, the chemical content of diet might affect the composition of microflora in GIT. Fermented feeds promote wellness by lowering feed viscosity [82]. Fermented barley, wheat, oats, and rye diets substantially raised caecal butyrate and propionate levels. Nutrient degradation and solubilization enhance

accessible substrates for microbial fermentation in the intestine, including oligosaccharides and monosaccharides. Increasing of short-chain fatty acids (SCFA) from digestion due to fermented substrates promote the growing of the healthier microflora (for example, Lactic Acid Bacteria (LAB)) [83]. Other study showed that certain beneficial bacteria can synthesize essential vitamins and SCFAs, further contributing to broiler health and growth. The number of LAB adhering to the gut mucosa forming biofilm is more developed when the number of benefic bacteria increased and significantly dropped in the case of pathologic case. The lately situation conduct the formation of pathogenic microorganism biofilm [84]. Similarly, in the presence of prebiotic compounds, which pass undigested, the growth of beneficial bacteria is promoted [85].

Many research indicate that fermented feed may improve immune function [23]. A balanced gut microbiome can strengthen the immune system, making broilers less susceptible to diseases. The study conducted by [68] found that fermented feed, particularly 6% and 8%, significantly improved jejunum secretory immunoglobulin A concentration (sIgA is an important component of the immune shield) and jejunum morphology in laying hens, indicating its positive effects on gut mucosa barrier function and reducing adverse effects on gut health [24].

Figure 4 emphasizes the most important achievements of the present paper. Fermented feed decreased the concentration of ANC and enhanced the concentration of beneficial probiotics, enzymes and metabolites. Beside this, the fermentation process has acidic pH and molecules with lower molecular mass. Those factors stimulate gut microbiota by growing the number of good microorganism and regulation of the immune response. Healthy broiler chicken will improve growth performance (feed conversion ratio (FCR), body weights gaining (BW) and feed intake (FI)).

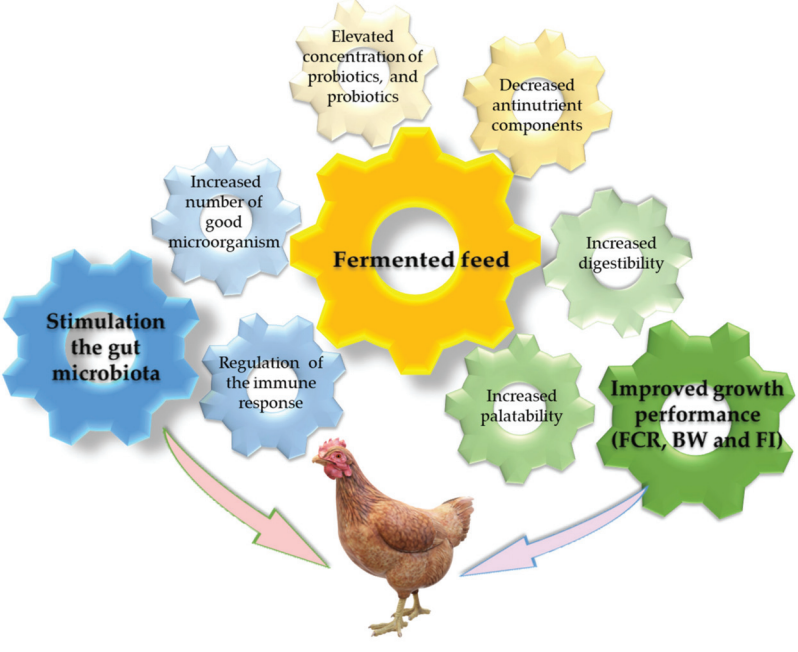

Figure 4. Intercorrelation between gut microbiota and growth performance under the influence of fermented feed in broiler chicken.

4. Conclusions

Proper control of fermentation parameters and the use of good quality practices are essential whatever fermentation techniques—liquid, solid-state, and ensiling—offering

distinct benefits for enhancing vegetal-based feeds, including decreased concentration of antinutrient components. Tailoring the fermentation process to the specific substrate characteristics, selection of fermentation microorganism and conducting regular nutritional assessments ensure improved feed quality and safety.

Using fermented feeds in broiler nutrition has shown promising effects on growth performance and gut health. However, it's essential to consider various factors, including feed composition, fermentation conditions, and the specific needs of the broiler production system. Fermented feeds often contain higher levels of beneficial microorganisms, most lactic acid bacteria. These bacteria can contribute to a healthier gut environment by stimulating and supporting a balanced immune response the host or exhibiting anti-inflammatory properties.

Studies suggest that fermented feeds can positively influence intestinal morphology, including increased villus height and crypt depth, which is indicative of better nutrient absorption. Fermented feeds may stimulate the production of mucin, a protective layer in the gut. This can contribute to a healthier gut lining and protection against pathogens.

Certain fermented feed components, such as prebiotics and short chain fatty acids, can influence the relative abundance of specific microbial populations, promoting a balanced microbiota. Fermented feeds may enhance the palatability of the diet, leading to increased feed intake and subsequently supporting growth performance.

Research in this field continues to explore optimal strategies for incorporating fermented feeds into broiler diets. The type and composition of the feed used in fermentation, as well as the specific fermentation microorganisms employed, can impact the outcomes.

Author Contributions: Conceptualization, N.C.P. writing—original draft preparation, N.C.P., M.P.R., G.S. and C.P.; writing—review and editing, N.C.P. and C.P.; validation, N.C.P., M.P.R., G.S. and C.P.; resources, NCP.; visualization, N.C.P., M.P.R., G.S. and C.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: This work was supported by the Romanian UEFISCDI project PN-III-P2-2.1-PED2021-2001, no. 631PED/2022.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Maharjan, P.; Martinez, D.A.; Weil, J.; Suesuttajit, N.; Umberson, C.; Mullenix, G.; Hilton, K.M.; Beitia, A.; Coon, C.N. Review: Physiological Growth Trend of Current Meat Broilers and Dietary Protein and Energy Management Approaches for Sustainable Broiler Production. *Animal* **2021**, *15*, 100284. [CrossRef]
2. Zampiga, M.; Calini, F.; Sirri, F. Importance of feed efficiency for sustainable intensification of chicken meat production: Implications and role for amino acids, feed enzymes and organic trace minerals. *J. World's Poult. Sci.* **2021**, *77*, 639–659. [CrossRef]
3. Siegel, P.B. Evolution of the Modern Broiler and Feed Efficiency. *Annu. Rev. Anim. Biosci.* **2014**, *2*, 375–385. [CrossRef] [PubMed]
4. Desbruslais, A.; Wealleans, A.L. Oxidation in Poultry Feed: Impact on the Bird and the Efficacy of Dietary Antioxidant Mitigation Strategies. *Poultry* **2022**, *1*, 246–277. [CrossRef]
5. Krysiak, K.; Konkol, D.; Korczyński, M. Overview of the Use of Probiotics in Poultry Production. *Animals* **2021**, *11*, 1620. [CrossRef] [PubMed]
6. Xu, C.; Kong, L.; Gao, H.; Cheng, X.; Wang, X. A Review of Current Bacterial Resistance to Antibiotics in Food Animals. *Front. Microbiol.* **2022**, *13*, 822689. [CrossRef]
7. Selaledi, L.A.; Hassan, Z.M.; Manyelo, T.G.; Mabelebele, M. The Current Status of the Alternative Use to Antibiotics in Poultry Production: An African Perspective. *Antibiotics* **2020**, *9*, 594. [CrossRef]
8. Panaite, T.D.; Saracila, M.; Papuc, C.P.; Predescu, C.N.; Soica, C. Influence of Dietary Supplementation of *Salix alba* Bark on Performance, Oxidative Stress Parameters in Liver and Gut Microflora of Broilers. *Animals* **2020**, *10*, 958. [CrossRef]
9. Clavijo, V.; Morales, T.; Vives-Flores, M.J.; Reyes Muñoz, A. The gut microbiota of chickens in a commercial farm treated with a Salmonella phage cocktail. *Sci. Rep.* **2022**, *12*, 991. [CrossRef]
10. Mangan, M.; Siwek, M. Strategies to combat heat stress in poultry production-A review. *J. Anim. Physiol. Anim. Nutr.* **2024**, *108*, 576–595. [CrossRef]
11. Ivanova, S.; Sukhikh, S.; Popov, A.; Shishko, O.; Nikonov, I.; Kapitonova, E.; Krol, O.; Larina, V.; Noskova, S.; Babich, O. Medicinal Plants: A Source of Phytobiotics for the Feed Additives. *J. Agric. Food Res.* **2024**, *16*, 101172. [CrossRef]

12. Abdel-Moneim, A.E.; Shehata, A.M.; Alzahrani, S.O.; Shafi, M.E.; Mesalam, N.M.; Taha, A.E.; Swelum, A.A.; Arif, M.; Fayyaz, M.; El-Hack, M.E.A. The Role of Polyphenols in Poultry Nutrition. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 1851–1866. [CrossRef] [PubMed]
13. Papuc, C.; Goran, G.V.; Predescu, C.N.; Nicorescu, V.; Stefan, G. Plant Polyphenols as Antioxidant and Antibacterial Agents for Shelf-Life Extension of Meat and Meat Products: Classification, Structures, Sources, and Action Mechanisms. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 1243–1268. [CrossRef]
14. Mamun, M.A.A.; Rakib, A.; Mandal, M.; Kumar, S.; Singla, B.; Singh, U.P. Polyphenols: Role in Modulating Immune Function and Obesity. *Biomolecules* **2024**, *14*, 221. [CrossRef] [PubMed]
15. Masiala, A.; Vingadassalon, A.; Aurore, G. Polyphenols in Edible Plant Leaves: An Overview of Their Occurrence and Health Properties. *Food Funct.* **2024**, *15*, 6847–6882. [CrossRef]
16. Bié, J.; Sepodes, B.; Fernandes, P.C.B.; Ribeiro, M.H.L. Polyphenols in Health and Disease: Gut Microbiota, Bioaccessibility, and Bioavailability. *Compounds* **2023**, *3*, 40–72. [CrossRef]
17. Bešlo, D.; Golubić, N.; Rastija, V.; Agić, D.; Karnaš, M.; Šubarić, D.; Lučić, B. Antioxidant Activity, Metabolism, and Bioavailability of Polyphenols in the Diet of Animals. *Antioxidants* **2023**, *12*, 1141. [CrossRef]
18. Zhang, L.; Guan, Q.; Jiang, J.; Khan, M.S. Tannin Complexation with Metal Ions and Its Implication on Human Health, Environment and Industry: An Overview. *Int. J. Biol. Macromol.* **2023**, *253*, 127485. [CrossRef]
19. Wu, H.; Oliveira, G.; Lila, M.A. Protein-binding Approaches for Improving Bioaccessibility and Bioavailability of Anthocyanins. *Compr. Rev. Food Sci. Food Saf.* **2022**, *22*, 333–354. [CrossRef]
20. Sugiharto, S.; Ranjitkar, S. Recent advances in fermented feeds towards improved broiler chicken performance, gastrointestinal tract microecology and immune responses: A review. *Anim. Nutr.* **2019**, *5*, 1–10. [CrossRef]
21. Peng, W.; Talpur, M.Z.; Zeng, Y.; Xie, P.; Li, J.; Wang, S.; Wang, L.; Zhu, X.; Gao, P.; Jiang, Q.; et al. Influence of fermented feed additive on gut morphology, immune status, and microbiota in broilers. *BMC Vet. Res.* **2022**, *18*, 218. [CrossRef] [PubMed]
22. Xu, F.; Wu, H.; Xie, J.; Zeng, T.; Hao, L.; Xu, W.; Lu, L. The Effects of Fermented Feed on the Growth Performance, Antioxidant Activity, Immune Function, Intestinal Digestive Enzyme Activity, Morphology, and Microflora of Yellow-Feather Chickens. *Animals* **2023**, *13*, 3545. [CrossRef] [PubMed]
23. Zhang, M.; Yang, Z.; Wu, G.; Xu, F.; Zhang, J.; Luo, X.; Ma, Y.; Pang, H.; Duan, Y.; Chen, J.; et al. Effects of Probiotic-Fermented Feed on the Growth Profile, Immune Functions, and Intestinal Microbiota of Bamei Piglets. *Animals* **2024**, *14*, 647. [CrossRef] [PubMed]
24. Zhu, X.; Tao, L.; Liu, H.; Yang, G. Effects of fermented feed on growth performance, immune organ indices, serum biochemical parameters, cecal odorous compound production, and the microbiota community in broilers. *Poult. Sci.* **2023**, *102*, 102629. [CrossRef]
25. Leeuwendaal, N.K.; Stanton, C.; O'Toole, P.W.; Beresford, T.P. Fermented Foods, Health and the Gut Microbiome. *Nutrients* **2022**, *14*, 1527. [CrossRef]
26. Sun, H.; Chen, D.; Cai, H.; Chang, W.; Wang, Z.; Liu, G.; Deng, X.; Chen, Z. Effects of Fermenting the Plant Fraction of a Complete Feed on the Growth Performance, Nutrient Utilization, Antioxidant Functions, Meat Quality, and Intestinal Microbiota of Broilers. *Animals* **2022**, *12*, 2870. [CrossRef] [PubMed]
27. Han, S.; Xu, G.; Zhang, K.; Ahmad, S.; Wang, L.; Chen, F.; Liu, J.; Gu, X.; Li, J.; Zhang, J. Fermented Astragalus Powder, a New Potential Feed Additive for Broilers to Improve the Growth Performance and Health. *Animals* **2024**, *14*, 1628. [CrossRef]
28. Dai, Z.; Cui, L.; Li, J.; Wang, B.; Guo, L.; Wu, Z.; Zhu, W.; Wu, G. *Fermentation Techniques in Feed Production*; Elsevier eBooks: Amsterdam, The Netherlands, 2020; pp. 407–429.
29. Sokrab, A.M.; Mohamed Ahmed, I.A.; Babiker, E.E. Effect of fermentation on antinutrients, and total and extractable minerals of high and low phytate corn genotypes. *J. Food Sci. Technol.* **2014**, *51*, 2608–2615. [CrossRef]
30. Samtiya, M.; Aluko, R.E.; Dhewa, T. Plant food anti-nutritional factors and their reduction strategies: An overview. *Food Prod. Process. Nutr.* **2020**, *2*, 6. [CrossRef]
31. Salim, R.; Nehvi, I.B.; Mir, R.A.; Tyagi, A.; Ali, S.; Bhat, O.M. A review on anti-nutritional factors: Unraveling the natural gateways to human health. *Front. Nutr.* **2023**, *10*, 1215873. [CrossRef]
32. Anderson, R.L.; Wolf, W.J. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *J. Nutr.* **1995**, *125*, 581S–588S. [CrossRef]
33. Adeyemo, S.M.; Onilude, A.A. Enzymatic Reduction of Anti-Nutritional Factors in Fermenting Soybeans by *Lactobacillus plantarum* Isolates from Fermenting Cereals. *Niger. Food J.* **2013**, *31*, 84–90. [CrossRef]
34. Gogoi, P.; Sharma, P.; Mahajan, A.; Goudar, G.; Chandragiri, A.K.; Sreedhar, M.; Longvah, T. Exploring the nutritional potential, anti-nutritional components and carbohydrate fractions of Indian pigmented maize. *Food Chem. Adv.* **2023**, *2*, 100176. [CrossRef]
35. Șonea, C.; Toader, M.; Năstase, I.P. The quality of maize grains in organic farming system. *Rom Biotechnol. Lett.* **2020**, *25*, 1781–1789. [CrossRef]
36. Roger, T.; Léopold, T.N.; Funtong, M.C.M. Nutritional Properties and Antinutritional Factors of Corn Paste (Kutukutu) Fermented by Different Strains of Lactic Acid Bacteria. *Int. J. Food Sci.* **2015**, *2015*, 1–13. [CrossRef] [PubMed]
37. Emkani, M.; Oliete, B.; Saurel, R. Effect of Lactic Acid Fermentation on Legume Protein Properties, a Review. *Fermentation* **2022**, *8*, 244. [CrossRef]

38. Etuk, E.B.; Ifeduba, A.V.; Okata, U.E.; Chiaka, I.; Charles, O.I.; Okeudo, N.J.; Esonu, B.O.; Udedibie, A.B.I.; Moreki, J.C. Nutrient Composition and Feeding Value of Sorghum for Livestock and Poultry: A Review. *Anim. Sci. Adv.* **2012**, *2*, 510–524.
39. Aladeen, A.M.A.; Omer Elbashier, M. The Effect of Replacement of Sorghum with Millet on Broilers Performance. *Int. J. Sci. Res.* **2013**, *6*, 2319–7064.
40. McCuistion, K.C.; Selle, P.H.; Liu, S.Y.; Goodband, R.D. Sorghum as a feed grain for animal production. In *Sorghum and Millets. Chemistry, Technology, and Nutritional Attributes*, 2nd ed.; Taylor, J., Duodu, K., Eds.; Elsevier Inc. in cooperation with AACC International: Amsterdam, The Netherlands, 2019; pp. 355–391.
41. Gunawan, S.; Dwitasari, I.; Rahmawati, N.; Darmawan, R.; Wirawasista Aparamarta, H.; Widjaja, T. Effect of Process Production on Antinutritional, Nutrition, and Physicochemical Properties of Modified Sorghum Flour. *Arab. J. Chem.* **2022**, *15*, 104134. [CrossRef]
42. Karunaratne, N.D.; Newkirk, R.W.; Ames, N.P.; Van Kessel, A.G.; Bedford, M.R.; Classen, H.L. Effects of exogenous β-glucanase on ileal digesta soluble β-glucan molecular weight, digestive tract characteristics, and performance of coccidiosis vaccinated broiler chickens fed hulless barley-based diets with and without medication. *PLoS ONE* **2021**, *16*, e0236231. [CrossRef]
43. McNab, J.M.; Smithard, R.R. Barley β-Glucan: An Antinutritional Factor in Poultry Feeding. *Nutr. Res. Rev.* **1992**, *5*, 45–60. [CrossRef] [PubMed]
44. Ram, S.N.; Narwal, S.; Gupta, O.P.; Pandey, V.; Singh, G. 4—Anti-nutritional factors and bioavailability: Approaches, challenges, and opportunities. In *Wheat and Barley Grain Biofortification*; Woodhead Publishing Series in Food Science, Technology and Nutrition; Gupta, O.P., Pandey, V., Narwal, S., Sharma, P., Ram, S., Singh, G.P., Eds.; Woodhead Publishing: Sawston, UK, 2020; pp. 101–128. [CrossRef]
45. Tanasković, S.J.; Šekuljica, N.; Jovanović, J.; Gazikalović, I.; Grbavčić, S.; Đorđević, N.; Sekulić, M.V.; Hao, J.; Luković, N.; Knežević-Jugović, Z. Upgrading of Valuable Food Component Contents and Anti-Nutritional Factors Depletion by Solid-State Fermentation: A Way to Valorize Wheat Bran for Nutrition. *J. Cereal Sci.* **2021**, *99*, 103159. [CrossRef]
46. Alemayehu, G.F.; Forsido, S.F.; Tola, Y.B.; Teshager, M.A.; Assegie, A.A.; Amare, E. Proximate, mineral and anti-nutrient compositions of oat grains (*Avena sativa*) cultivated in Ethiopia: Implications for nutrition and mineral bioavailability. *Heliyon* **2021**, *7*, e07722. [CrossRef] [PubMed]
47. Mehta, B.; Jood, S. Anti-Nutritional Factors and Mineral Content of Different Oat (*Avena sativa* L.) Varieties. *Food Sci. Res. J.* **2018**, *9*, 117–120. [CrossRef]
48. Jasinska-Kuligowska, I.; Kuligowski, M.; Kolodziejczyk, P.; Michniewicz, J. Effect of fermentationextrusion and baking processes on content of fructans in rye products. *Zywnosc-Nauka Technol. Jakosc* **2013**, *20*, 129–141.
49. Ismagilov, R.; Ayupov, D.; Nurlygayanov, R.; Ahiyarova, L.; Abdulloev, V. Ways to reduce anti-nutritional substances in winter rye grain. *Physiol. Mol. Biol. Plants* **2020**, *26*, 1067–1073. [CrossRef]
50. Osman, A.; Hartung, C.B.; Lingens, J.B.; Rohn, K.; Schreiner, T.; Ahmed, M.F.E.; Hankel, J.; Abd El-Wahab, A.; Visscher, C. Fermentation Characteristics of Rye and Sorghum Depending on Water:Feed Ratio. *Fermentation* **2022**, *8*, 155. [CrossRef]
51. Kumar, R. Anti-nutritional factors, the potential risks of toxicity and methods to alleviate them in Legume Trees and Other Fodder Trees as Protein Sources for Livestock. In Proceedings of the FAO Expert Consultation, Kuala Lumpur, Malaysia, 14–18 October 1991; FAO Animal and Health paper. Speedy, A., Pugliese, P., Eds.; FAO: Rome, Italy, 1991; p. 102.
52. Shqueir, A.A.; Brown, D.L.; Taylor, S.J.; Rivkin, I.; Klasing, K.C. Effects of solvent extraction, heat treatments and added cholesterol on *Sesbania sesban* toxicity in growing chicks. *Anim. Feed Sci. Technol.* **1989**, *27*, 127–135. [CrossRef]
53. Raharjo, Y.C.; Cheeke, P.R.; Patton, N.M. Effect of cecotrophy on the nutrient digestibility of alfalfa and black locust leaves. *J. Appl. Rabbit Res.* **1990**, *613*, 56–61.
54. Sharma, R.; Garg, P.; Kumar, P.; Bhatia, S.K.; Kulshrestha, S. Microbial Fermentation and Its Role in Quality Improvement of Fermented Foods. *Fermentation* **2020**, *6*, 106. [CrossRef]
55. Ramireddy, L.; Radhakrishnan, M. *Cold Plasma Applications on Pulse Processing*; Elsevier eBooks: Amsterdam, The Netherlands, 2021; pp. 295–307.
56. Dittoe, D.K.; Olson, E.G.; Ricke, S.C. Impact of the Gastrointestinal Microbiome and Fermentation Metabolites on Broiler Performance. *Poult. Sci.* **2022**, *101*, 101786. [CrossRef] [PubMed]
57. Saadi, S.; Saari, N.; Ghazali, H.M.; Abdulkarim, M.S. Mitigation of Antinutritional Factors and Protease Inhibitors of Defatted Winged Bean-Seed Proteins Using Thermal and Hydrothermal Treatments: Denaturation/Unfolding Coupled Hydrolysis Mechanism. *Curr. Res. Food Sci.* **2022**, *5*, 207–221. [CrossRef] [PubMed]
58. Li, J.; Tao, L.; Zhang, R.; Yang, G. Effects of Fermented Feed on Growth Performance, Nutrient Metabolism and Cecal Micro-flora of Broilers. *Anim. Biosci.* **2022**, *35*, 596–604. [CrossRef]
59. Feng, J.; Liu, X.; Xu, Z.R.; Wang, Y.Z.; Liu, J.X. Effects of Fermented Soybean Meal on Digestive Enzyme Activities and Intestinal Morphology in Broilers. *Poult. Sci.* **2007**, *86*, 1149–1154. [CrossRef]
60. Haryati Supriyati, T.; Susanti, T.; Susana, I.W.R. Nutritional value of rice bran fermented by *Bacillus amyloliquefaciens* and humic substances and its utilization as a feed ingredient for broiler chickens. *Asian Aust. J. Anim. Sci.* **2015**, *28*, 231–238. [CrossRef] [PubMed]
61. Ogbuewu, I.P.; Mabelebele, M.; Mbajiorgu, C.A. Determination of performance response of broilers to fermented tropical leaf meal supplementation using meta-analytical method. *Trop. Anim. Health Prod.* **2024**, *56*, 98. [CrossRef]

62. Chen, B.; Li, D.; Leng, D.; Kui, H.; Bai, X.; Wang, T. Gut microbiota and meat quality. *Front. Microbiol.* **2022**, *13*, 951726. [CrossRef] [PubMed]
63. Yang, X.Y.; Li, Y.X.; Li, Y. Effect of Ginkgo biloba extract on growth performance, slaughter performance and immune index in broilers. *J. Fujian Agric. For. Univ.* **2008**, *3*, 295–298.
64. Agboola, T.O.; Balogun, O.L.; Rahji, M.A.Y. Stock Size and Relative Efficiency in Broiler Production in SouthWestern Nigeria: A Normalized Profit Function Approach. *IOSR J. Agric. Vet. Sci.* **2014**, *7*, 43–50. [CrossRef]
65. Castellone, V.; Bancalari, E.; Rubert, J.; Gatti, M.; Neviani, E.; Bottari, B. Eating Fermented: Health Benefits of LAB-Fermented Foods. *Foods* **2021**, *10*, 2639. [CrossRef]
66. Irawan, A.; Ratriyanto, A.; Respati, A.N.; Ningsih, N.; Fitriastuti, R.; Suprayogi, W.P.S.; Hadi, R.F.; Setyono, W.; Akhirini, N.; Jayanegara, A. Effect of feeding fermented soybean meal on broiler chickens' performance: A meta-analysis. *Anim. Biosci.* **2022**, *35*, 1881–1891. [CrossRef] [PubMed]
67. Saleh, A.A.; Shukry, M.; Farrag, F.; Soliman, M.M.; Abdel-Moneim, A.E. Effect of Feeding Wet Feed or Wet Feed Fermented by Bacillus licheniformis on Growth Performance, Histopathology and Growth and Lipid Metabolism Marker Genes in Broiler Chickens. *Animals* **2021**, *11*, 83. [CrossRef]
68. Liu, Y.; Feng, J.; Wang, Y.; Lv, J.; Li, J.; Guo, L.; Min, Y. Fermented Corn-Soybean Meal Mixed Feed Modulates Intestinal Morphology, Barrier Functions and Cecal Microbiota in Laying Hens. *Animals* **2021**, *11*, 3059. [CrossRef] [PubMed]
69. Bajagai, Y.S.; Klieve, A.V.; Dart, P.J.; Bryden, W.L. Probiotics in animal nutrition—Production, impact and regulation. In *FAO Animal Production and Health Paper*; Makkar, H.P.S., Ed.; FAO: Rome, Italy, 2016; Volume 179.
70. Fathima, S.; Shanmugasundaram, R.; Adams, D.; Selvaraj, R.K. Gastrointestinal Microbiota and Their Manipulation for Improved Growth and Performance in Chickens. *Foods* **2022**, *11*, 1401. [CrossRef]
71. Pendleton, B. The regulatory environment. In *Direct-Fed Microbial, Enzyme and Forage Additive Compendium*; The Miller Publishing Company: Minnetonka, MN, USA, 1998; Volume 4, pp. 47–52.
72. Medellin-Peña, M.J.; Wang, H.; Johnson, R.; Anand, S.; Griffiths, M.W. Probiotics affect virulence-related gene expression in Escherichia coli O157: H7. *Appl. Environ. Microbiol.* **2007**, *73*, 4259–4267. [CrossRef]
73. Meng, Q.; Yan, L.; Ao, X.; Zhou, T.; Wang, J.S.; Garrido-Galand, A.; Asensio-Grau, J.; Calvo-Lerma, A.; Heredia, A. The potential of fermentation on nutritional and technological improvement of cereal and legume flours: A review. *Int. Food Res.* **2021**, *145*, 110398. [CrossRef]
74. Xiang, H.; Sun-Waterhouse, D.; Waterhouse, G.I.N.; Cui, C.; Ruan, Z. Fermentation-enabled wellness foods: A fresh perspective. *Food Sci. Hum. Wellness* **2019**, *8*, 203–243. [CrossRef]
75. Tropini, C.; Earle, K.A.; Huang, K.C.; Sonnenburg, J.L. The Gut microbiome: Connecting spatial organization to function. *Cell Host Microbe* **2017**, *21*, 433–442. [CrossRef]
76. Farré, R.; Fiorani, M.; Abdu Rahiman, S.; Matteoli, G. Intestinal Permeability, Inflammation and the Role of Nutrients. *Nutrients* **2020**, *12*, 1185. [CrossRef]
77. Izadi, H.; Arshami, J.; Golian, A.; Raji, M.R. Effects of Chicory Root Powder on Growth Performance and Histomorphometry of Jejunum in Broiler Chicks. *Dir. Open Access J.* **2013**, *4*, 169–174.
78. Raza, A.; Bashir, S.; Tabassum, R. An update on carbohydrases: Growth performance and intestinal health of poultry. *Heliyon* **2019**, *5*, e01437. [CrossRef] [PubMed]
79. Vancamelbeke, M.; Vermeire, S. The Intestinal Barrier: A Fundamental Role in Health and Disease. *Expert Rev. Gastroenterol. Hepatol.* **2017**, *11*, 821–834. [CrossRef] [PubMed]
80. Dao, D.P.D.; Le, P.H. Histology, Goblet Cells. In *StatPearls [Internet]*; StatPearls Publishing: Treasure Island, FL, USA, 2024. Available online: https://www.ncbi.nlm.nih.gov/books/NBK553208/ (accessed on 23 September 2024).
81. Horowitz, A.; Chanez-Paredes, S.D.; Haest, X.; Turner, J.R. Paracellular Permeability and Tight Junction Regulation in Gut Health and Disease. *Nat. Rev. Gastroenterol. Hepatol.* **2023**, *20*, 417–432. [CrossRef] [PubMed]
82. Ménard, S.; Cerf-Bensussan, N.; Heyman, M. Multiple facets of intestinal permeability and epithelial handling of dietary antigens. *Mucosal Immunol.* **2010**, *3*, 247–259. [CrossRef] [PubMed]
83. Annunziata, G.; Arnone, A.; Ciampaglia, R.; Tenore, G.C.; Novellino, E. Fermentation of Foods and Beverages as a Tool for Increasing Availability of Bioactive Compounds. Focus on Short-Chain Fatty Acids. *Foods* **2020**, *9*, 999. [CrossRef] [PubMed]
84. Siddique, A.; Azim, S.; Ali, A.; Adnan, F.; Arif, M.; Imran, M.; Ganda, E.; Rahman, A. *Lactobacillus reuteri* and *Enterococcus faecium* from Poultry Gut Reduce Mucin Adhesion and Biofilm Formation of Cephalosporin and Fluoroquinolone-Resistant *Salmonella enterica*. *Animals* **2021**, *11*, 3435. [CrossRef]
85. Iqbal, Y.; Cottrell, J.J.; Suleria, H.A.R.; Dunshea, F.R. Gut Microbiota-Polyphenol Interactions in Chicken: A Review. *Animals* **2020**, *10*, 1391. [CrossRef]

agriculture

MDPI

Article

Structural Characterization and Bioactive Compound Evaluation of Fruit and Vegetable Waste for Potential Animal Feed Applications

Miuța Filip [1], Mihaela Vlassa [1,*], Ioan Petean [2], Ionelia Țăranu [3], Daniela Marin [3], Ioana Perhaiță [1], Doina Prodan [1], Gheorghe Borodi [4] and Cătălin Dragomir [3]

[1] Raluca Ripan Institute for Research in Chemistry, Babeș-Bolyai University, 30 Fântânele Street, 400294 Cluj-Napoca, Romania; miuta.filip@ubbcluj.ro (M.F.); ioana.perhaita@ubbcluj.ro (I.P.); doina.prodan@ubbcluj.ro (D.P.)
[2] Faculty of Chemistry and Chemical Engineering, Babeș-Bolyai University, 11 Arany Janos Street, 400028 Cluj-Napoca, Romania; ioan.petean@ubbcluj.ro
[3] National Research and Development Institute for Animal Biology and Nutrition-IBNA Balotesti, 1 Calea București Street, 077015 Balotești, Romania; ionelia.taranu@ibna.ro (I.Ț.); daniela.marin@ibna.ro (D.M.); catalin.dragomir@ibna.ro (C.D.)
[4] National Institute for Research and Development of Isotopic and Molecular Technologies, 65-103 Donath Street, 400293 Cluj-Napoca, Romania; gheorghe.borodi@itim-cj.ro
* Correspondence: mihaela.vlassa@ubbcluj.ro

Abstract: Agricultural waste from the fruit and vegetable industry is used as an alternative source of animal feed, but detailed investigations are required. The aim of this work was to conduct a physico-chemical characterization, through analytical techniques, of fruit and vegetable wastes such as those of golden apples, red apples, carrots, celery, beetroots, and red potato peels. The bioactive compounds in the samples indicated a high carbohydrate content of 50.38 g/100 g in golden apples and 59.38 mg/100 g of organic acids in celery. In addition, the total phenolic content (TPC, mg gallic acid equivalent/g dry weight) varied between 3.72 in celery and 15.51 in beetroots. The antioxidant capacity values were significant. A thermal analysis showed thermal stability and weight loss, underscoring the composition of the solid samples. An infrared spectroscopy (FTIR) analysis showed C-H, O-H, C=O, and N-H functional groups in non-starchy carbohydrates, organic acids, and proteins. Microscopic techniques revealed the microstructure, particle size, and semicrystalline profile of the samples. The ultrastructure (determined via atomic force microscopy (AFM)) of celery consisted of a smooth and uniform surface with a lignin and cellulose texture. These results highlight the importance of fruit and vegetable waste as an alternative source of essential nutrients and bioactive compounds for animal feed.

Keywords: fruit and vegetable waste; physico-chemical methods; structural characterization; bioactive compounds

Citation: Filip, M.; Vlassa, M.; Petean, I.; Țăranu, I.; Marin, D.; Perhaiță, I.; Prodan, D.; Borodi, G.; Dragomir, C. Structural Characterization and Bioactive Compound Evaluation of Fruit and Vegetable Waste for Potential Animal Feed Applications. *Agriculture* **2024**, *14*, 2038. https://doi.org/10.3390/agriculture14112038

Academic Editor: Antonio Natalello

Received: 12 September 2024
Revised: 16 October 2024
Accepted: 26 October 2024
Published: 12 November 2024

1. Introduction

The waste of fruits and vegetables, obtained after processing in order to obtain food products, contains compounds that are identified in the scientific literature as materials intended for human consumption that are subsequently evacuated, lost, degraded, or contaminated [1]. Agricultural wastes are regarded as a loss of valuable biomass and nutrients, since these wastes have the potential to become useful products or even raw materials for other industries. It is well known that fruits and vegetables are good sources of valuable organic and mineral compounds, along with having high amounts of dietary fiber [2]. In particular, the by-products of the fruit and vegetable industry are of interest, since they are inexpensive and available in large quantities [3]. Agricultural waste (pomace, seeds, peels, stems, pulp, etc.) represents a high percentage of processed fruits and vegetables,

at 20–35% [4]. This fact led to the accumulation of biodegradable matter rich in organic compounds, which can contribute to major environmental pollution through decomposing waste in landfills and the release of harmful gases with greenhouse effects [4,5]. Moreover, the costs to dispose of these agricultural wastes are becoming higher and higher [5]. At present, according to the current EU legislation, a small amount comprising 5–10% of the food waste generated can be used in animal feed [6]. Recent studies considered the characterization of different agricultural by-products in order to use them in the future as animal feed, with the aim of achieving positive effects on animal health and performance, minimizing waste management costs, and avoiding environmental pollution [7]. Moreover, due to the recycling of waste, various food by-products have been included in the catalog of feed materials [8], updated in 2017, through the European Commission regulations [9]. Thus, fruit and vegetable waste could be used as a substitute in animal nutrition and as part of cereal grains and plant protein sources, which would diminish the food competition between humans and animals [10]. In this respect, various fruit and vegetable by-products, which are abundant in bioactive compounds, are used by humans and animals for their nutritional purposes [11].

Carrots (*Daucus carota*) may be processed into juice, concentrates, or dried food [12]. After juicing, approximately 30–50% of the initial mass becomes a by-product of the beverage industry as pulp [13], which provides an inexpensive, sustainable, and renewable source of cellulose, fibers, vitamins, phenolic compounds, and flavonoids [14,15]. Using up to 5% dried carrot processing waste in broiler diets enhances productive performance and economic efficiency [16]. The processing of beetroots (*Beta vulgaris*) results in large amounts of waste materials, including the flesh, crown, and peel [17]. These waste products are being increasingly recognized as a natural source of bioactive compounds with a rich nutritional and commercial value, such as pigments and fibers [18]. The strong antioxidant activity of beetroots is attributed to its content of polyphenolic compounds, and they can be used to stabilize free radicals, preventing the oxidation of biological molecules [19]. In addition, the dietary addition of beetroot waste and carrot waste has been shown to influence the total carotenoid content in the skin and muscle tissues of goldfish (*Carassius auratus*) [20].

Apple (*Malus domestica*) pomace (the pulpy residue remaining after the fruit has been crushed) accounts for ~25% of apples; thus, the remains from the apple juice and cider industry can generate massive amounts of waste. These fruit waste products are a good source of carbohydrates and functionally important bioactive molecules such as proteins, vitamins, minerals, and natural antioxidants [21]. Potatoes (*Solanum tuberosum*) are among the most important agricultural crops that, in order to be consumed, are usually peeled during processing, forming potato peel waste that can vary between 15 and 40% of the initial mass, depending on the peeling method [22]. Potato peels are a by-product rich in starch, non-starchy polysaccharides, lignin, polyphenols, proteins, and small amounts of lipids [23]. In addition, this waste represents a rich material for the extraction of biologically valuable compounds, such as natural antioxidants, dietary fibers, biopolymers, etc. [23]. This waste can be used as an alternative to animal feed due to its natural sources of energy and fiber and its low protein levels [24]. Celery (*Apium graveolens* L.) is rich in vitamins, carotene, protein, cellulose, and other nutrients and is a good source of flavonoids, volatile oil, and antioxidants [25].

For the valorization of agri-food waste, celery root peels were studied by Uzel Aşkın in a case study of raw material [26]. In addition to being a very good source of carbohydrates, celery root peels are a good source of phenolic compounds and pectin, since most of the phenolic components in celery are in the peel [26].

There is growing interest in investigating the potential of by-products as substitutes for conventional feed. These wastes are obtained in significant quantities during the manufacture of agricultural products and by-products that are processed annually in many agricultural countries [6,10].

The aim of the present study was to investigate the physico-chemical and structural properties of some vegetable and fruit wastes to highlight the valuable amounts of essential nutrients and beneficial bioactive compounds contained in these products, which can be exploited as additives in animal feed for the support of animal health.

2. Materials and Methods

2.1. Sample Preparation

The analytical experiments were carried out in 2023 and 2024. In the current study, fruit waste (golden apples and red apples) and vegetable waste (celery, carrots, beetroots, and red potato peels) were provided by a local food and canning manufacturer from Vâlcea County, Romania (45.04412/24.31295). After the manufacturer obtained juice extractions, the waste of the fruit and vegetable samples (approximately 500 g/sample) was collected in order to be lyophilized for further chemical analyses.

The lyophilization process consisted of two operations: freezing the samples in successive stages and subliming the ice with the help of a higher vacuum. A LyoQuest freeze-dryer (Azbil Telstar, S.L.U., Terrassa, Spain) was used to dry the samples. The residues were placed on lyophilizer shelves. Lyophilization was conducted for 45.5 h in four steps at −40 °C and −0.5 mbar of pressure. The temperature of the lyophilizer shelves and the sample temperature were measured using temperature sensors.

2.2. Chemical Composition

The crude chemical composition was determined according to the WEENDE scheme for the determination of dry matter (DM) (103 °C), crude protein (CP), crude fat (CF), crude cellulose (CC), crude ash (CA), and organic substances. The CC was assessed according to Taranu et al. [27]. The CP in the residues was determined with the semi-automatic classic Kjeldahl method using a Kjeltek auto 1030—Tecator (Tecator AB, Hőganäs, Sweden) [27]. The ether extract was analyzed using an improved version of the classical continuous solvent extraction method with a Soxhlet extractor, followed by a fat measurement after solvent removal [27]. The DM and CA were determined in accordance with a method from the literature [27]. Neutral detergent fibers (NDFs) were quantified using the Van Soest method reported in [28], and acid detergent fibers (ADFs) were quantified using the Van Soest method reported in [29].

The residue samples were analyzed for their concentration of macro-elements; calcium (Ca) was analyzed according to Anzano et al. [30], and sodium (Na), potassium (K), and magnesium (Mg) were analyzed using atomic absorption spectrometry (AAS) on a Thermo Electron atomic absorption spectrophotometer (Thermo Fisher Scientific Inc., Göteborg, Sweden) [31]. The concentrations of the micro-minerals copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn) were determined using AAS after microwave digestion and mineralization with nitric acid [32]. The working parameters were as follows: wavelength (nm): 324.8 (Cu), 279.5 (Mn), and 213.9 (Zn); bandpass (nm): 0.5 (Cu), 0.2 (Mn), and 0.5 (Zn); lamp current (mA): 5 (Cu), 12 (Mn), and 10 (Zn). The samples were analyzed in triplicate.

2.3. HPLC Determination of Carbohydrates, Organic Acids, and Individual Polyphenols

Carbohydrates, organic acids, and phenolic compounds were analyzed using high-performance liquid chromatography (HPLC) on a Jasco chromatograph (Jasco Corporation, Tokyo, Japan) equipped with a UV/Vis detector, a refractive index detector, and an injection valve with a 20 µL sample loop (Rheodyne®, Thermo Fisher Scientific, Waltham, MA, USA). To collect and process the chromatographic data, the ChromPass software (version v1.7, Jasco International Co., Ltd., Tokyo, Japan) was used. The HPLC analyses of carbohydrates were carried out by adapting the method reported in [33]. The determination of organic acids and the analyses of individual phenolic compounds (flavonoids and phenolic acids) were performed according to the methods presented by Filip, M et al. [34,35].

2.4. Analysis of Total Phenolic Content (TPC)

The TPC was measured using the Folin–Ciocalteu colorimetric method with a Specord 205 spectrophotometer (Analytik Jena, GMbH, Berlin, Germany), which was used to detect the blue complex at 760 nm; gallic acid was used as a reference standard [36]. The TPC of each lyophilized compound was quantified as the mg gallic acid equivalent per 100 g of dry weight (mg GAE/100 g). All the determinations were performed in triplicate and the data are presented as the mean ± standard deviation (SD).

2.5. Antioxidant Activity

Two different chemical methods, namely DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), were used to evaluate the antioxidant activity of the studied samples.

DPPH● radical scavenging assay: A spectrophotometrically modified DPPH method was used to determine the antioxidant activity of the studied samples at 517 nm against methanol as the blank [37]. The free radical scavenging activity of the sample extracts was measured using the absorbance with standard solutions of methanolic Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The effective concentrations of DPPH were expressed in μmol Trolox/100 g dry weight. All the determinations were performed in triplicate and the data are presented as the mean ± standard deviation.

ABTS+● radical scavenging assay: The antioxidant activity of the samples, determined using the ABTS method, was based on the percentage inhibition of the peroxidation of this radical according to a previously described method [38], with modifications. The ABTS+● radical was generated during a chemical reaction between an ABTS aqueous solution and potassium persulfate [39]. The antioxidant capacity of the studied sample extracts was calculated using a standard curve drawn up for Trolox solutions at 734 nm and expressed as a μmol Trolox equivalent/100 g dry sample. All the determinations were performed in triplicate and the results are presented as the mean ± SD.

2.6. Thermogravimetric Analysis (TGA) and Differential Thermal Analysis (DTG)

The thermal behavior of all the samples was evaluated based on a TG-DTG analysis. The measurements were carried out in the temperature range of 25 to 1200 °C with a heating rate of 10 °C/min, under an inert N_2 atmosphere and at a flow rate of 60 mL/min on a Mettler-Toledo TGA/SDTA851 instrument (Mettler-Toledo, Schwerzenbach, Switzerland).

2.7. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

An analysis was performed using a Fourier-transform infrared spectrophotometer (FTIR) (Jasco FTIR-610) (Jasco® International Co., Ltd., Tokyo, Japan) equipped with an attenuated total reflectance (ATR) accessory with a horizontal ZnSe crystal (Jasco PRO400S). The samples were placed in direct contact with the ZnSe crystal and then the spectra were recorded at a resolution of 4 cm^{-1}. The scans were repeated 100 times.

2.8. X-Ray Diffraction (XRD)

The powder X-ray diffraction patterns were obtained with a Bruker D8 Advance powder diffractometer (Brucker Company, Karlsruhe, Germany) at 40 kV and 40 mA, equipped with an incident beam Ge 111 monochromator using CuKα1 radiation (λ = 1.540598 Å). The spectra were scanned at a diffraction angle (2θ) range of 5–80° and a step size of 0.05°/step and 2 s/step. The patterns were indexed using the Dicvol method [40].

2.9. Scanning Electron Microscopy (SEM) Analysis

The analysis was performed on an INSPECT S spanning electron microscope (FEI Company, Hillsboro, OR, USA). The experiment was conducted in a low vacuum at 80 torr, each powder sample was mounted on a stub using carbon tape, and field emissions were performed at 5 kV.

2.10. Atomic Force Microscopy (AFM) Analysis

The AFM investigation was effectuated with a JSPM 4210 scanning probe microscope produced by Jeol Company, Tokyo, Japan. The samples were probed in tapping mode using NSC 15 Hard cantilevers produced by MikroMasch Company, Sofia, Bulgaria; they had a resonant frequency of 330 kHz and a force constant of 40 N/m. The topographic images were obtained at a scanning rate of 1–2 Hz, depending on the surface complexity. The images were analyzed with WinSPM 2.0 Processing software, powered by Jeol Company, Tokyo, Japan. The fine microstructural details were observed for a scanned area of 20 μm × 20 μm and the nanostructural details were observed for an area of 2 μm × 2 μm. At least three different macroscopic areas were investigated for each sample, and the Ra and Rq surface roughness parameters were measured.

3. Results

3.1. Chemical Composition

As can be seen from Table 1, there was variation in the DM, with values ranging from 11.85 to 19.30% at a temperature of 65 °C, and between 89.96 and 95.72% at a temperature of 103 °C. The highest CP concentration of all the residues was found for beetroots (16.08%), followed by red potato peels (15.58%).

Table 1. The crude chemical composition and fiber content of fruit and vegetable waste.

Chemical Composition (%)	Golden Apples	Red Apples	Carrots	Celery	Beetroots	Red Potato Peels
DM (65 °C)	18.56 ± 0.17	19.30 ± 0.16	11.85 ± 0.11	12.10 ± 0.12	13.94 ± 0.14	15.67 ± 0.14
DM (103 °C)	95.04 ± 0.13	89.86 ± 0.19	93.19 ± 0.15	95.00 ± 0.11	94.18 ± 0.11	95.72 ± 0.11
CP	2.43 ± 0.09	2.45 ± 0.06	5.79 ± 0.06	6.97 ± 0.08	16.08 ± 0.07	15.58 ± 0.08
CF	0.76 ± 0.04	1.16 ± 0.03	0.14 ± 0.015	0.52 ± 0.03	0.29 ± 0.02	0.12 ± 0.01
CA	1.48 ± 0.07	1.75 ± 0.08	6.45 ± 0.08	10.38 ± 0.17	9.61 ± 0.07	5.79 ± 0.08
NDFs	22.42 ± 0.28	19.85 ± 0.10	14.19 ± 0.16	18.85 ± 0.12	33.15 ± 0.19	46.29 ± 0.16
ADFs	13.89 ± 0.16	14.90 ± 0.15	10.74 ± 0.18	14.96 ± 0.16	14.00 ± 0.26	10.72 ± 0.22
CC	14.49 ± 0.15	13.09 ± 0.13	8.83 ± 0.12	11.87 ± 0.23	13.07 ± 0.16	6.85 ± 0.24

The values are presented as the mean ± standard deviation (SD).

Celery presented the highest amount of CA (10.38%), followed by beetroots (9.61%), carrots (6.45%), and red potato peels (5.79%). Golden apples presented the lowest CA value (1.48%).

Measuring NDFs is the most common method of determining the quantity of fibers for a feed analysis, but they do not represent a unique class of chemical compounds. NDFs include most of the structural components in plant cells (lignin, hemicellulose, and cellulose), but not pectin. Hemicellulose, which is also a carbohydrate present in plant material, is taken into account only when calculating the amount of ADFs. The NDF content varied from the highest amounts, found in red potato peels (46.29%), beetroots, (33.15%), and golden apples (22.42%), to the lowest value, which was determined for carrots (14.19%). The samples of celery, red apples, beetroots, and golden apples presented the highest contents of ADFs (14.96, 14.90, 14.00, and 13.89%). With regard to the CC content, golden apples, red apples, and beetroots presented the highest values (14.49–13.97%), while carrots and red potato peels had the smallest values (8.83 and 6.85%).

Concerning the composition of micro- and macro-elements (see Table 2), celery represents a valuable source of Cu (10.480 ppm), Fe (116.400 ppm), Mn (21.190%), and Zn (40.250%), followed by beetroots, with 10.08 ppm of Cu and 33.050% of Zn. Still, red potato peels exhibited the highest concentration of Fe (249.400 ppm). Macro-elements were present in higher quantities in the celery, beetroot, and carrot samples. Thus, the values of K in beetroots (4.502%), celery (4.003%), and red potato peels (3.392%) were followed by the Na values in celery (0.602%), carrots (0.529%), and beetroots (0.367%). Higher values of Ca, P, and Mg were also found in the same vegetables.

Table 2. Composition of macro- and micro-minerals.

Waste	Ca	P	Mg	Na	K	Cu	Fe	Mn	Zn
	%	%	%	%	%	ppm	ppm	%	%
Golden apples	0.04 ± 0.002	0.11 ± 0.004	0.06 ± 0.002	0.008 ± 0.0004	0.75 ± 0.037	3.73 ± 0.186	26.09 ± 1.043	6.07 ± 0.303	3.46 ± 0.138
Red apples	0.38 ± 0.019	0.11 ± 0.004	0.105 ± 0.005	0.006 ± 0.0003	1.216 ± 0.061	5.660 ± 0.283	34.910 ± 1.746	5.06 ± 0.253	19.01 ± 0.951
Carrots	0.32 ± 0.013	0.31 ± 0.012	0.117 ± 0.005	0.529 ± 0.021	2.66 ± 0.106	4.16 ± 0.166	25.97 ± 1.039	10.18 ± 0.407	14.60 ± 0.584
Celery	0.48 ± 0.024	0.55 ± 0.028	0.122 ± 0.006	0.602 ± 0.030	4.003 ± 0.200	10.48 ± 0.419	116.40 ± 0.058	21.19 ± 0.848	40.25 ± 2.013
Beetroots	0.12 ± 0.006	0.38 ± 0.019	0.139±	0.367 ± 0.007	4.502 ± 0.225	10.08 ± 0.403	55.65 ± 2.226	16.59 ± 0.664	33.05 ± 1.322
Red potato peels	0.08 ± 0.005	0.33 ± 0.165	0.081 ± 0.004	0.027 ± 0.001	3.392 ± 0.169	8.79 ± 0.439	249.40 ± 9.976	13.66 ± 0.546	21.44 ± 0.858

The values are presented as the mean ± standard deviation (SD).

3.2. HPLC Determination of Carbohydrates, Organic Acids, and Individual Polyphenols

The total studied carbohydrates, presented in Figure 1, were found in the largest quantities (g/100 g) for the golden apple samples (50.38) and the carrot samples (46.89).

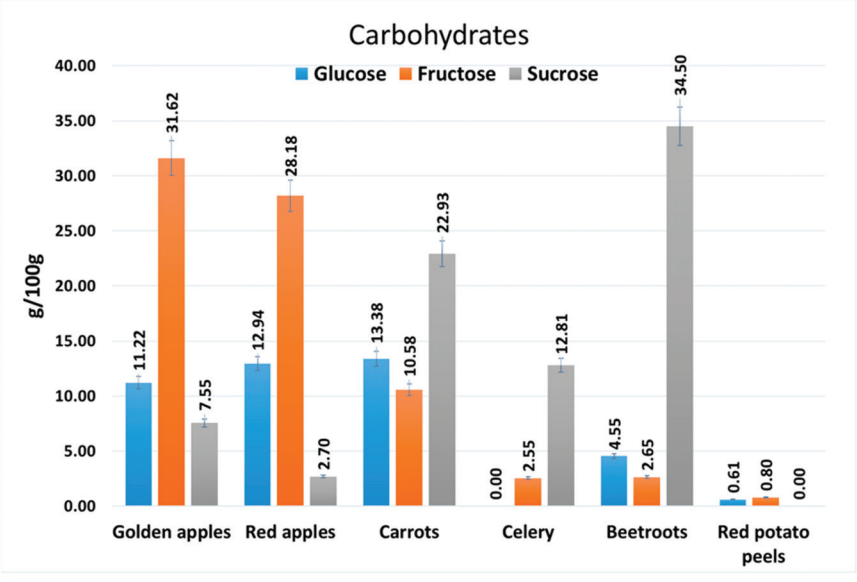

Figure 1. The carbohydrate content (mean ± SD) in fruit and vegetable waste.

On the other hand, the smallest value for the total studied carbohydrates was found for red potato peels at 1.41 g/100 g. The celery waste contained 15.35 g/100 g of the studied carbohydrates.

Regarding the organic acid content (Figure 2), the celery and carrot samples had the largest amounts, at 59.38 mg/100 g and 43.81 mg/100 g, respectively. Organic acids were found in relatively important quantities in the golden apple samples, at 35.63 mg/100 g, and the beetroot samples, at 29.96 mg/100 g.

The content of individual flavonoids and phenolic acids had values between 41.44 mg/100 g for the golden apple samples and 129.88 mg/100 g for the beetroot samples. We observed (Figure 3) that the vegetables samples, including those from beetroots, red potato peels, and celery, presented a larger quantity of flavonoids and phenolic acids than the fruit samples from golden and red apples.

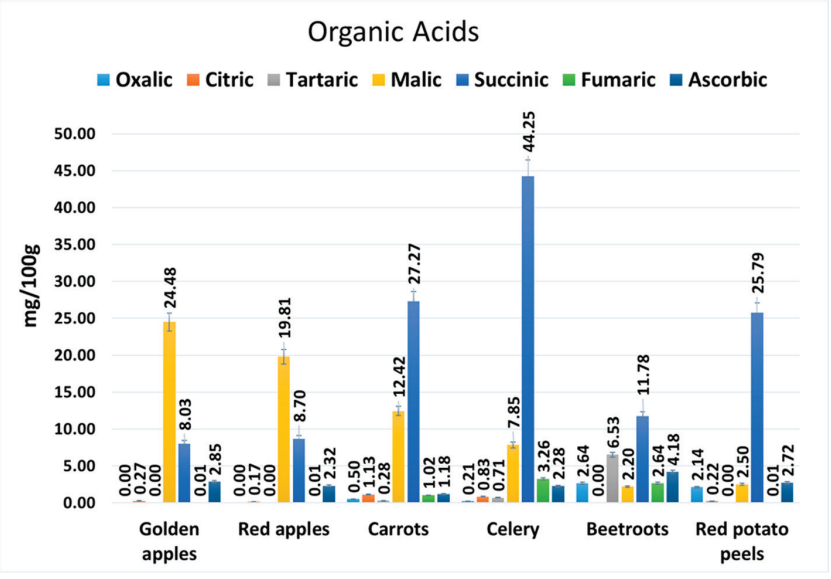

Figure 2. The organic acid content (mean ± SD) in fruit and vegetable waste.

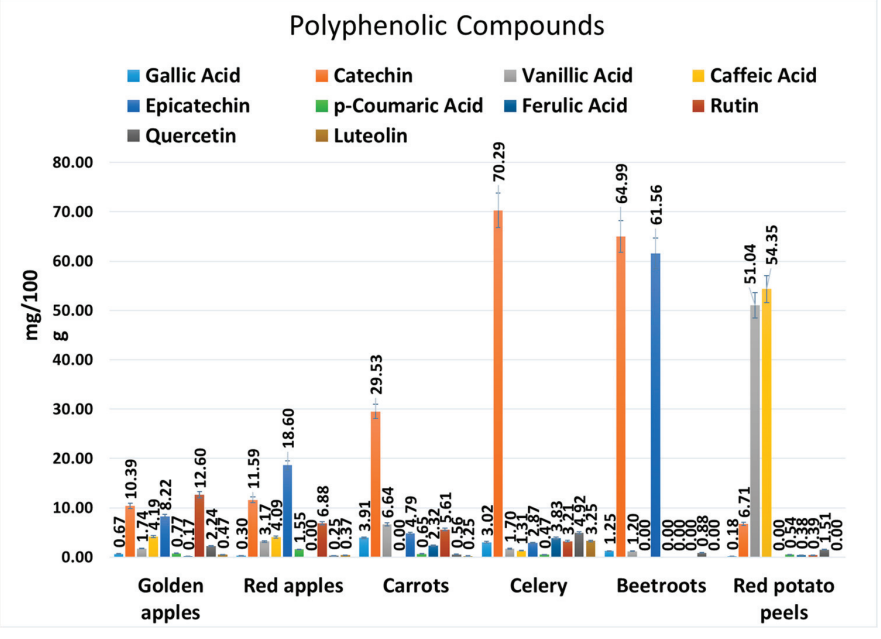

Figure 3. The content of individual polyphenolic compounds (mean ± SD) in fruit and vegetable waste.

3.3. Determination of Total Phenolic Content and Antioxidant Capacity

The Total Phenolic Content (TPC) and antioxidant capacity were determined using the DPPH, ABTS, and FRAC radical scavenging activities of methanol extracts of the fruit and vegetable wastes. The results are shown in Table 3.

Table 3. Total phenolic content and antioxidant capacity of fruit and vegetable wastes.

	Golden Apples	Red Apples	Carrots	Celery	Beetroots	Red Potato Peels
TPC	6.69 ± 0.03	10.64 ± 0.12	4.69 ± 0.06	3.72 ± 0.02	15.51 ± 0.24	7.75 ± 0.14
DPPH	2066.28 ± 10.63	4075.81 ± 8.49	1438.91 ± 8.13	516.60 ± 4.16	6622.28 ± 35.31	2046.02 ± 13.82
ABTS	1941.81 ± 7.32	3852.48 ± 16.59	1838.29 ± 12.52	1443.91 ± 9.90	7334.98 ± 33.22	3669.92 ± 11.30

TPC, total phenolic content, expressed as gallic acid-equivalent (mg GAE/g dry weight). The DPPH and ABTS assays are expressed as μmol Trolox/100 g dry sample. The values are presented as the mean ± standard deviation (SD).

The highest TPC (mg GAE/g dry weight) was found for beetroots, at 15.51, followed by red apples, at 10.64, and red potato peels, at 7.75. The TPC in the celery samples was 3.72 mg GAE/g dry weight.

For the apple samples, the determined values (mg GAE/g dry weight) were 6.69 for golden apples and 10.64 for red apples.

In this study, the results of the DPPH and ABTS methods were expressed using the same unit, i.e., Trolox-equivalent antioxidant capacity (micromolar μmol Trolox/100 g), in order to directly compare the results. The highest antioxidant capacity measured with the ABTS assay was found to be 7334.98 for beetroots, followed by 3852.48 for red apples and 3669.92 for red potato peels. For the golden apple, carrot, and celery samples, the amounts found were lower.

Regarding the DPPH antioxidant capacity (micromolar μmol Trolox/100 g), the obtained values followed the same pattern; the highest value was found for beetroots (6622.28), followed by red apples (4075.81). The golden apple and red potato peel samples showed similar values (2066.28 and 2046.02). The lowest value was obtained for celery (516.60).

3.4. Thermogravimetric Analysis (TGA) and Differential Thermal Analysis (DTG)

A TGA was performed in order to examine the thermal degradation behavior of the lyophilized selected waste of vegetable and fruit samples, providing important information regarding the pyrolysis process of these materials (Figure 4). As the temperature increased, a mass loss was observed together with the removal of volatile substances.

In a thermodynamic system analysis, heat gain by a system is considered positive, while heat loss is considered negative. The decomposition stages of the powdered fruit and vegetable samples were investigated under a nitrogen atmosphere at temperatures of up to 1200 °C (Figure 4).

In the case of all the freeze-dried samples, the DTA curves showed four stages of thermal decomposition with endothermic effects. Table 4 presents the temperatures and mass losses corresponding to each stage of decomposition and the final residues.

The first stage of decomposition was between 30 and 98.99 °C, and was accompanied by a mass loss of 2.72% for golden apples, 3.85% for red apples, 4.32% for carrots, 4.61% for celery, 4.09% for beetroots, and 5.87% for red potato peels. In this stage, moisture evaporation and a slight weight loss took place due to the loss of light volatiles.

The second stage occurred between 100 and 260 °C, with a mass loss of 37.22% for golden apples, 37.43% for red apples, 39.26% for carrots, 24.17% for celery, and 39.23% for beetroots.

In the third stage of decomposition between 260 and 600 °C, the mass loss, which was equal to 27.89% for carrots and 44.00% for celery, was due to the decomposition of hemicelluloses (220–315 °C), cellulose (315–400 °C), and lignin.

The fourth stage of decomposition, which involved a mass loss of 11.97% for red apples and 25.96% for beetroots, was due to lignin, which decomposes slowly. The total mass losses were in the range of 89.28–96.57% under a nitrogen atmosphere.

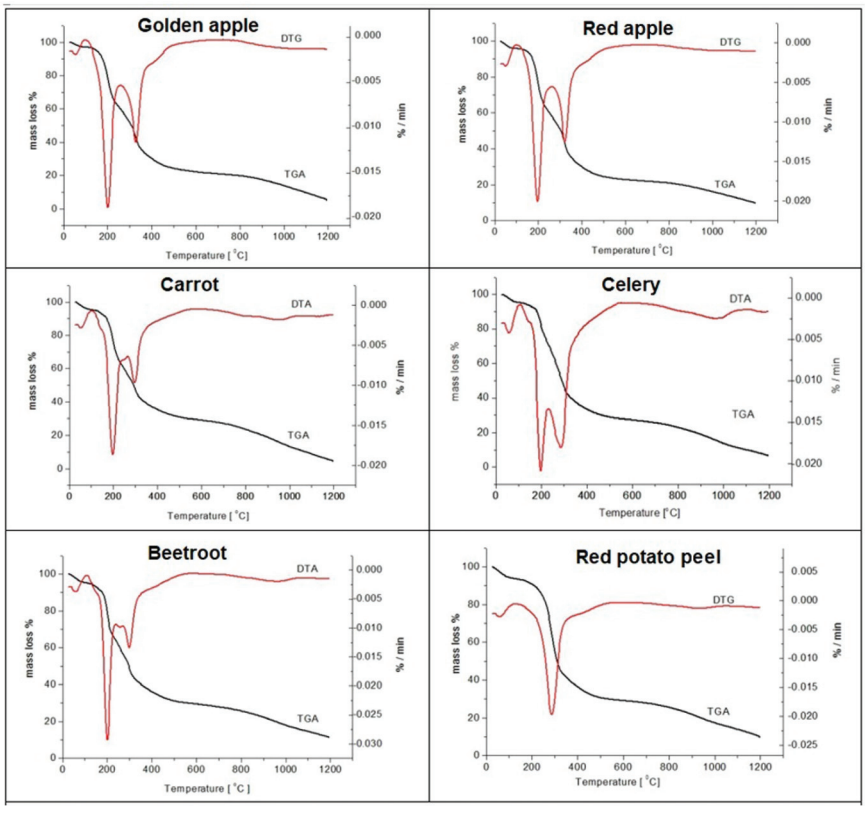

Figure 4. TGA-DTG of the studied fruit and vegetable waste.

Table 4. The stages of thermal decomposition of the studied samples.

Sample	Tmin °C	Ml I %	T1 °C	Ml II %	T2 °C	Ml III %	T3 °C	Ml IV %	Tmax °C	TMl %
Golden apples	53.79	2.77	199.93	37.43	328.00	38.91	1189.97	15.22	1200	94.11
Red apples	49.52	3.85	196.1	37.22	321.93	37.09	1144.79	11.97	1200	89.69
Carrots	52.44	4.32	196.96	39.26	295.89	27.28	960.08	24.27	1200	95.64
Celery	57.55	4.61	197.18	24.17	286.38	44.00	962.75	20.43	1200	96.57
Beetroots	56.15	4.09	201.27	39.23	296.91	27.89	919.10	25.96	1200	92.84
Red potato peels	56.76	5.87	-	-	286.43	64.66	928.41	18.79	1200	89.28

Mass loss (Ml) stages I, II, III, and IV; TMl, total mass loss.

3.5. FTIR-ATR Analysis of Fruit and Vegetable Waste

The FTIR-ATR spectra of the studied golden apple, red apple, carrot, celery, beetroot, and red potato peel samples are shown in Figure 5. Table 5 presents the wavenumbers (cm^{-1}) for each studied sample and the assignment of vibration bands.

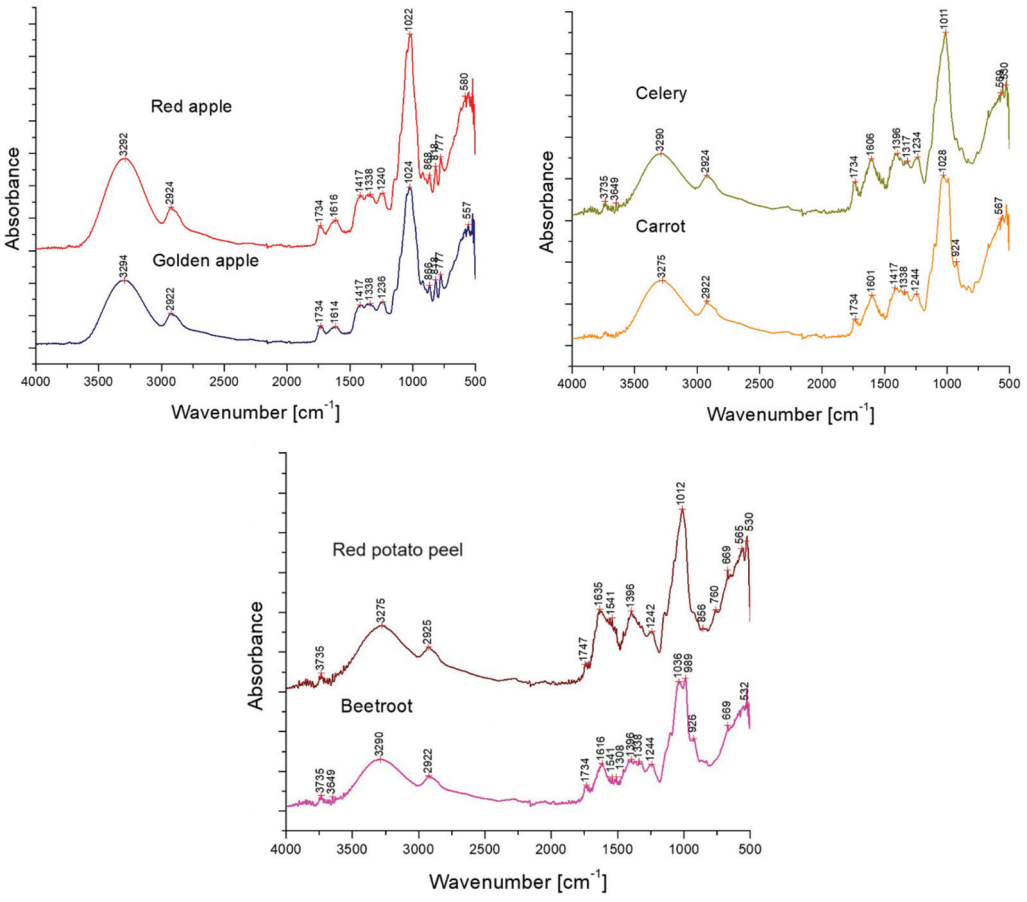

Figure 5. The FTIR-ATR spectra of the studied fruit and vegetable waste.

Table 5. FTIR absorption band assignments.

Golden Apples	Red Apples	Carrots	Celery	Beetroots	Red Potato Peels	Assignment of Vibration Bands	References
Wavenumber [cm^{-1}]							
-	-	3735	3735; 3649	3735	3735; 3649	O-H group stretching from alcohols, which are abundant in polysaccharides.	[41,42]
3294	3292	3275	3290	3290	3275	O-H group stretching and bending from cellulose or pectins.	[41]
2922	2924	2922	2924	2922	2925	CH_3, CH_2, or -CH=CH- aliphatic group asymmetric and symmetric stretching; trans -CH=CH- of beta-carotene and pectins.	[41]
1734	1734	1734	1734	1734	1747	C-O in acetyl group and uranic ester group stretching, or ester groups present in the carboxylic group of ferulic and p-coumaric acids of lignin and/or hemicellulose and pectins.	[41]

Table 5. *Cont.*

Golden Apples	Red Apples	Carrots	Celery	Beetroots	Red Potato Peels	Assignment of Vibration Bands	References
Wavenumber [cm^{-1}]							
1614	1616	1601	1606	1616	1635	C=O esters from free carboxyl groups (acids); C-O stretching of aryl group present in lignin.	[43]
1417	1417	1417	-	1541	1541	C=C vibration of the aromatic ring.	[44]
1338	1338	1338	1317	1396	1396	CH_3 and CH_2 aliphatic groups.	[44]
1236	1240	1244	1234	1244	1242	C-OH, C-O-C, C-C, C-O, and C=O stretching in sugars, alcohols, and ethers.	[45–47]
1024	1022	1028	1011	1036; 989	1012	C-O bond deformation vibrations in secondary alcohols and aliphatic ethers; C-C, C-OH, C-H ring, and side group vibrations; C-O-C stretching of galacturonic acid, starch, cellulose, and phenols.	[44]
866	868	-	-	926	856	C-O out-of-plane band.	[48]
818–777	818–777	-	-	-	-	C-H, C-C, C-OH, COC, CCO, CCH, and N-H bond deformation and stretching vibrations associated with aromatic rings, carbohydrates, and lignin.	[44]
557	580	567	568	568	565	C-O-O and P-O-C group bending in aromatic phosphates.	[48]

3.6. X-Ray Diffraction Analysis

The X-ray diffraction patterns indicated many amorphous compounds and the presence of low organic crystallinity (Figure 6).

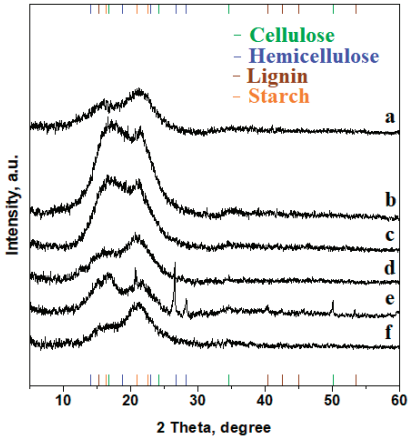

Figure 6. XRD patterns for the wastes of (a) beetroots, (b) red apples, (c) golden apples, (d) carrots, (e) red potato peels, and (f) celery.

The beetroot sample contained starch, hemicellulose, and lignin. In the red apple sample, lignin, cellulose, hemicellulose, and traces of starch were found. The golden apple sample also contained lignin, cellulose, hemicellulose, and traces of starch, and the carrot sample contained starch, cellulose, and lignin. In the red potato peel sample, hemicellulose, starch, cellulose, and lignin were found. The celery sample contained starch, lignin, and cellulose.

3.7. SEM Analysis

SEM is a test procedure that scans a sample with an electron beam to produce a magnified image for an analysis. The image contains microscopic information about the surface or near-surface region of a specimen (Figure 7).

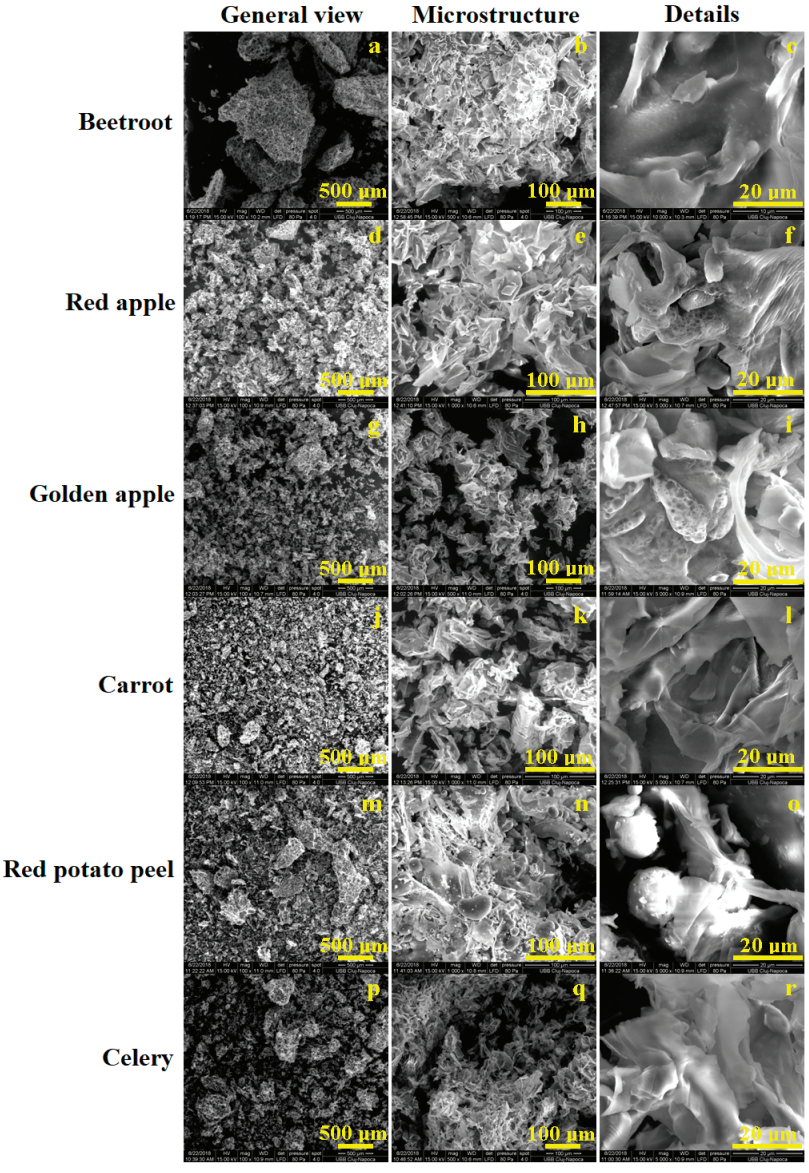

Figure 7. SEM images of the investigated samples. Beetroot sample at different magnifications: (**a**) ×100, (**b**) ×500, and (**c**) ×5000; red apple sample at different magnifications: (**d**) ×100, (**e**) ×1000, and (**f**) ×5000; golden apple sample at different magnifications: (**g**) ×100, (**h**) ×1000, and (**i**) ×5000; carrot sample at different magnifications: (**j**) ×100, (**k**) ×1000, and (**l**) ×5000; red potato peel sample at different magnifications: (**m**) ×100, (**n**) ×1000, and (**o**) ×5000; and celery sample at different magnifications: (**p**) ×100, (**q**) ×500, and (**r**) ×5000.

The beetroot sample presented several conglomerates with irregular shapes of about 700 μm. The microstructural details of the beetroot sample revealed ultrastructural constituents within the flakes.

The apple and carrot powders were very well dispersed, with small particles, while the potato skin and celery samples presented a bimodal aspect, with some clusters of about 350 μm, related mainly to the starch content, surrounded by fine fractions, related to the fiber content. The powders' microstructure revealed a flake-like shape due to the high lignin and cellulose content, giving the powders cohesion and mechanical strength.

The samples from both of the apples revealed a grainy ultrastructural formation (20–40 μm in diameter) of rounded nanoparticles. The details of the carrot sample revealed a lamellar structure, most likely caused by an interlaced texture of lignin and cellulose.

The microstructural detail of the red potato peel sample was heterogeneous due to the presence of starch grains and the lamellar features of hemicellulose interlaced with cellulose and lignin. In the celery sample, there was no evidence of the presence of starch, and well-formed flakes with a fibrous structure were found, induced by lignin and cellulose.

3.8. AFM Analysis

The sample particles were further subjected to water dispersion in order to simulate their initial disaggregation during feeding. The dispersions were deposited on glass slides as thin films, naturally dried, and were further investigated with AFM microscopy (Figure 8). The fine microstructural details were observed for a scanned area of 20 μm × 20 μm and the nanostructural details were observed for an area of 2 μm × 2 μm. The peel flakes seemed to keep their consistency, but their nanostructural features were enhanced, indicating the possibility of their release into a humid environment. The beetroot sample revealed rounded nanoparticles of about 90 nm, with starch disaggregation due to the wet interactions. These starch nanostructural fractions are more effective in the digestive process, facilitating nutrient absorption. The red and golden apple samples featured a complex ultrastructure based on round nanoparticles of about 35–40 nm that might be related to degraded starch and pectin nanoparticles. The carrot flakes presented a very fine ultrastructure containing fine nanoparticles of about 20 nm that might be related to the presence of beta carotene. The red potato peel ultrastructure showed evidence of starch disaggregation into ultrastructural features consisting of submicron clusters of about 150 nm surrounded by nanoparticles in the range of 60–90 nm. The celery ultrastructure was very interesting, revealing a smooth and uniform surface with a lignin and cellulose texture.

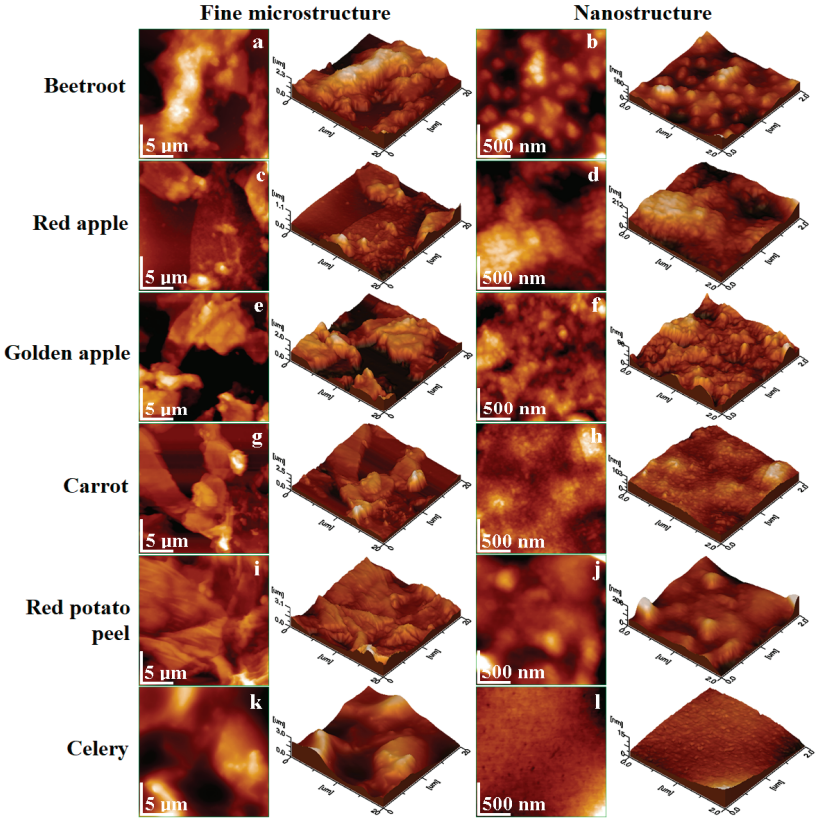

Figure 8. AFM topographic images of fruit and vegetable waste: (**a**) beetroot fine microstructure, (**b**) beetroot nanostructure, (**c**) red apple fine microstructure, (**d**) red apple nanostructure, (**e**) golden apple fine microstructure, (**f**) golden apple nanostructure, (**g**) carrot fine microstructure, (**h**) carrot nanostructure, (**i**) red potato peel fine microstructure, (**j**) red potato peel nanostructure, (**k**) celery fine microstructure, and (**l**) celery nanostructure.

4. Discussion

4.1. Evaluation of Chemical Composition and Fiber Content of Fruit and Vegetable Waste

The DM content is an important parameter affecting the storage stability of the powders.

The lyophilization method was selected for analytical purposes only. Another stage of this study will focus on methods for the preservation and use of fruit and vegetable by-products. Many feedstuffs are used in various forms: fresh, ensiled (fermented) using various techniques, or dried using various techniques. The current study focused on the nutritional potential of fresh samples. Thus, the values of the DM for the studied samples ranged from 11.85 to 20.88% at a temperature of 65 °C. The highest protein concentration was found for beetroots (16.08%) and red potato peels (15.58%), with similar values of protein to those presented by other authors for potato peels, at 13.15% [49].

Moreover, the ash content of the samples indicated the mineral quantity. Therefore, a low ash content indicates a low metal content [50]. Celery presented the highest amount of CA (10.38%), and golden apples presented the lowest value (1.48%). Both NDFs and ADFs included cellulose and lignin present in the plant material. The NDF content varied from 46.29% in red potato peels to 14.19% in carrots. The CC content of the studied waste samples was in agreement with the literature data [49,51]. The CC content of the golden

apple, red apple, and beetroot samples presented the highest values, at 14.49 and 13.09 and 13.07%, respectively. Different apple cultivars can influence the physico-chemical composition of apple residues [52]. Moreover, these studied vegetable and fruit wastes are a valuable source of macro- and micro-elements.

4.2. HPLC Determination of the Carbohydrates, Organic Acids, and Individual Polyphenols of Fruit and Vegetable Waste

The carbohydrate constituents in the studied pomaces are valuable substances due to their positive health effects. The total studied carbohydrates were found in the largest quantities (g/100 g) in the golden apple samples, at 50.38, and the carrot samples, at 46.89. The carbohydrates reported by Luca et al. for carrot pomace represent a rich source of fibers, carbohydrates, and minerals, which suggests its capacity to improve the nutritional value of food products, into which it can be incorporated [53]. The celery waste contained 15.35 g/100 g of the studied carbohydrates, which is within range of literature data on celery waste reporting 5.7–5.9% of the total carbohydrates as sucrose and 33.5–39.3% of the total carbohydrates as mannitol [54].

The studied waste samples are a good source of malic, citric, succinic, and oxalic acids, which behave as antioxidants because they have the ability to chelate metals. The organic acid content in the celery and carrot samples represented the largest amounts, at 59.38 mg/100 g and 43.81 mg/100 g, respectively. In addition, these organic acids enhance appetite; facilitate digestion; and improve potassium, copper, zinc, iron, and calcium absorption [55]. In the fermentation process of rapeseed meal, it was reported that the content of organic acids decreased to varying degrees, while that of succinic acid increased [36].

The content of individual flavonoids and phenolic acids had values between 41.44 mg/100 g in golden apple samples and 129.88 mg/100 g in beetroot samples. The results obtained show that the vegetables waste samples had a larger quantity of flavonoids and phenolic acids than the fruit waste samples.

Javed et al. reported that potato peels are an important source of polyphenols, with their extract comprising protocatechuic acid, caffeic acid, gallic acid, and chlorogenic acid [56]. Some authors reported the content of polyphenols in an aqueous extract of beetroot peels at 70% chlorogenic acid, 21% gallic acid, 6% syringic acid, 2% ferulic acid, and 1% caffeic acid [57].

4.3. Evaluation of Total Polyphenolic Content and Antioxidant Capacity of Fruit and Vegetable Waste

Waste pomace contains substantial concentrations of polyphenols, which are located mainly in the skin. These valuable active compounds have many health benefits for both animals and humans. In piglets, for example, they improve digestive and fermentative processes [58]. The highest TPC (mg GAE/g dry weight) was found for beetroots at 15.51. The TPC in the celery samples of 3.72 was in agreement with the amounts found in the literature of 3.36–3.50 g GAE/kg [59], 3.3 g GAE/kg [60], and 10.8 g GAE/kg in celery roots [61]. For the apple samples, the determined values (mg GAE/g dry weight) of 6.69 for golden apples and 10.64 for red apples were higher than the values obtained for commercial apple varieties from Croatia, at 2.88–5.72 mg GAE/g dry weight [62]. Some authors reported TPC values in red and purple potatoes of between 7.72 and 40.45 mg GAE/g [63], which are similar to the quantity found in our samples of 7.75 mg GAE/g. The TPC in red-skinned potatoes may be correlated with a high amount of anthocyanins, which are the coloring pigments in red potato varieties [22].

The antioxidant capacities obtained using the DPPH and ABTS assays were expressed in μmol Trolox/100 g in order to facilitate a direct comparison of the results. The highest antioxidant capacity measured using the ABTS assay was 7334.98 for beetroots, followed by 3852.48 for red apples and 3669.92 for red potato peels. The scavenging capacity (ABTS assay) of red beetroot varieties was determined by T. Sawicki et al. to be within the range from 37.68 to 49.71 μmol Trolox/g dry matter; the difference in the antioxidant capacity of

red beetroots depends on the type of root [64]. Regarding the DPPH antioxidant capacity (μmol Trolox/100 g), the obtained values followed the same pattern, with the highest value being found for beetroots (6622.28) and the lowest value being obtained for celery (516.60). W.K. Lau et al. found that the antioxidant capacity of carrots could be significantly reduced through drying or treatment methods [65]. In addition, P.D. Drogoudi et al. showed that the highest antioxidant capacity was found in apple peel tissues and was lower in the flesh tissue, and golden apples showed a lower value in comparison with red apples [66]. The literature findings show that apple and carrot pomaces contain important amounts of bioactive substances with many benefits for both animal and human health. Among these are polyphenols and organic acids, which prevent the multiplication of pathogenic bacteria in the intestine and reduce the diarrhea incidence in piglets after weaning [58]. In addition, polyphenols are recognized as anti-inflammatory compounds. Both apples and carrots, and their by-products, demonstrated antioxidative activity [58]. For example, Sehm et al. (2007) reported a beneficial effect on catalase, glutathione peroxidase, and superoxide dismutase antioxidant enzyme activity by including 3.5% apple pomace in the diet of mice, thus increasing their defense against oxidative stress [67]. The same antioxidant effect was demonstrated for β-carotene, one of the most bioactive compounds found in carrots and their by-products.

Therefore, the obtained results for the studied fruit and vegetable wastes demonstrate that they could be used as bioactive foods due to their strong antioxidant activity.

4.4. Evaluation of Fruit and Vegetable Waste Using TGA and DTG

The TGA provided important information regarding the pyrolysis process of these materials. As the temperature increased, a mass loss was observed together with the removal of volatile substances.

The first stage of decomposition was between 30 and 98.99 °C, and was accompanied by a mass loss of 2.72% for golden apples and 3.85% for red apples. The same results were obtained by Guerrero et al. [68]. A.C. Gowman et al. found a 2% mass loss for apple pomaces under the same conditions [69]. In this stage, moisture evaporation and a slight weight loss took place due to the loss of light volatiles. According to the literature, a weight loss that occurs at 200 °C is related to the beginning of lignin and hemicellulose pyrolysis [70,71]. The second stage occurred with a mass loss between 24.17% for celery and 39.26% for carrots. In the case of potato skins, starch decomposition took place with a distinct peak at 286.43 °C and a mass loss of 64.66% [44]. In the third stage of decomposition (260–600 °C), the mass loss was due to the decomposition of hemicelluloses (220–315 °C), cellulose (315–400 °C), and lignin. The fourth stage of decomposition, which involved a mass loss of 11.97% for red apples and 25.96% for beetroots, was due to lignin, which decomposes slowly over the entire temperature range up to 1200 °C [44,72].

The thermal degradation stages of the fruit and vegetable samples were in agreement with their compositions.

4.5. Evaluation of Fruit and Vegetable Waste Using ATR-FTIR

The FTIR analysis provided data on the absorption regions of the characteristic groups, along with a rapid process for interpreting these data [42].

The FTIR-ATR spectra of the samples showed intense bands at 3294–3292 cm^{-1}, corresponding to O-H group stretching and bending from cellulose or pectins, and at 2922–2924 cm^{-1}, corresponding to CH_3, CH_2, or -CH=CH- aliphatic group asymmetric and symmetric stretching, as well as the trans-CH=CH- of beta-carotene and pectins [41,42]. The bands at 1734 cm^{-1} resulted from C-O, the most widespread group in hemicellulose and pectin [41]. The absorption at 1614–1616 cm^{-1} was attributed to C=O groups from esters from the free carboxyl groups (acids) and/or C-O stretching of the aryl groups present in lignin [43]. The band detected at 1635 cm^{-1} was assigned to the C=N vibration group in the potato spectra. A closer examination of the spectra revealed the presence of a band at 1541 cm^{-1}, which was characteristic of the deformation of the C-H bond vibration and C=C

vibration of the aromatic ring [44]. The region of the ATR-FTIR spectra between 800 and 1300 cm^{-1} was considered to be the specific region for carbohydrates, which allowed for the identification of the major characteristic chemical groups for certain polysaccharides [45]. The most intense peak, with a maximum in the area of 1024–1022 cm^{-1}, belonged to the C-O deformation vibration bonds in secondary alcohols and aliphatic ethers; the C-C, C-OH, C-H ring, and side group vibrations of cellulose and hemicellulose; and the C-O-C stretching of galacturonic acid [46,47]. The bands between 1065 and 989 cm^{-1} corresponded to -C-C, -C-OH, and -C-H group vibrations from cellulose and phenols [48].

4.6. Evaluation of Fruit and Vegetable Waste Using X-Ray Diffraction, SEM, and AFM

The observed patterns indicated many amorphous compounds, with the decomposition of cellulose indicating an amorphous carbon structure with randomly oriented aromatic carbon sheets and a low organic crystallinity [73]. Microscopic information about the surface or near-surface region showed that the fruit and vegetable waste contained starch, cellulose, hemicellulose, and lignin [74].

The surface SEM morphology analysis of the fruit and vegetable waste revealed differently shaped particles with an agglomerated cluster-like morphology. The microstructure of the powders revealed a flake-like shape due to the high lignin and cellulose contents, giving them cohesion and mechanical strength. According to Anukriti et al., a morphology and surface analysis of solar-dried vegetables through XRD and SEM showed the effect of solar drying on the nutritional value of green leafy vegetables [74]. The structure of the celery leaves was dense and uniform, without any detectable gel breaks, and the pores were smaller and fewer [75].

The fine microstructural information revealed using AFM is in good agreement with the SEM observations of the microstructure details. The peel flakes seemed to maintain their consistency, but their nanostructural features were enhanced, indicating the possibility of their release into a humid environment. Ultrastructure AFM provides images with a near-atomic resolution for measuring the surface topography of dried fruits and vegetables, revealing their surfaces and textures. These are properties that influence the behavior of these wastes as an animal feed additive. In the literature, AFM analyses have been used to clarify the influences of blanching treatments on carrot texture [76].

5. Conclusions

The abundance of the biologically active substances in vegetable and fruit waste potentially makes these residues valuable products for the livestock industry. The results obtained show that the CP content in the beetroots and red potato peels was between 16.08 and 15.58%. The CA content was higher in the celery samples, at 10.38%, and the NDF and ADF content was 18.85 and 14.96%, respectively. All the samples contained fibers in the form of lignin, cellulose, hemicellulose, starch, and pectin. The highest values of macro- and micro-elements in celery included Zn at 40.250%, Mn at 21.190%, and Cu at 10.480 ppm. In addition, the beetroot samples contained 4.502% K, and the red potato peel samples contained 249.400 ppm, representing the highest Fe content of all the samples.

The total studied carbohydrates were found in the largest quantities (g/100 g) in the golden apple samples at 50.38, followed by the carrot samples at 46.89. The celery samples contained 15.35 g/100 g of the studied carbohydrates. Regarding the organic acid content, the celery and carrot samples had the largest amounts, at 59.38 mg/100 g and 43.81 mg/100 g, respectively. The content of individual flavonoids and phenolic acids was between 41.44 mg/100 g (for the golden apple samples) and 129.88 mg/100 g (for the beetroot samples). The vegetable samples, including from beetroots, red potato peels, and celery, presented a larger quantity of flavonoids and phenolic acids than the fruit samples, which included golden and red apples.

The highest TPC (mg GAE/g dry weight) was found for beetroots at 15.51, followed by red apples at 10.64, and red potato peels at 7.75. The highest antioxidant capacities (micromolar μmol Trolox/100 g), as measured using the ABTS assay, were found in beetroots

at 7334.98; in red apples at 3852.48; and in red potato peels at 3669.92. Regarding the DPPH antioxidant capacity, the obtained values (micromolar μmol Trolox/100 g) were the highest for the beetroot (6622.28) and red apple (4075.81) samples.

TGA-DTG analysis was used to determine the thermal stability and weight loss, which confirmed the composition of the solid samples. FTIR analysis showed the presence of C-H, O-H, C=O, and N-H functional groups in non-starchy carbohydrates, organic acids, and proteins. The SEM and XRD microstructural analyses revealed the particle size and the semicrystalline profile of the samples. The AFM ultrastructure of celery consisted of a smooth and uniform surface with a lignin and cellulose texture.

These results indicate the importance of fruit and vegetable waste as an alternative source of essential nutrients and bioactive compounds for livestock feeds.

Author Contributions: Conceptualization, M.V. and M.F.; methodology, M.F., M.V., I.P. (Ioan Petean), D.M., I.P. (Ioana Perhaiță), D.P. and G.B.; software, M.F., I.Ț. and C.D.; validation, M.F., I.P. (Ioan Petean) and D.P.; formal analysis, I.P. (Ioan Petean), D.M., I.P. (Ioana Perhaiță), D.P. and G.B.; investigation, M.F., M.V., I.P. (Ioan Petean), D.M., I.P. (Ioana Perhaiță), D.P. and G.B.; resources, M.V. and C.D.; data curation, I.Ț., D.M. and C.D.; writing—original draft preparation, M.V. and M.F.; writing—review and editing, M.V. and C.D.; visualization, I.Ț.; supervision, I.Ț. and M.V.; project administration, M.V.; funding acquisition, C.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Romanian Ministry of Research, Innovation and Digitization under grant No. PN2320-0201.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Girotto, F.; Alibardi, L.; Cossu, R. Food Waste Generation and Industrial Uses: A Review. *Waste Manag.* **2015**, *45*, 32–41. [CrossRef] [PubMed]
2. Esparza, I.; Jiménez-Moreno, N.; Bimbela, F.; Ancín-Azpilicueta, C.; Gandía, L.M. Fruit and Vegetable Waste Management: Conventional and Emerging Approaches. *J. Environ. Manag.* **2020**, *265*, 110510. [CrossRef] [PubMed]
3. Lau, K.Q.; Sabran, M.R.; Shafie, S.R. Utilization of Vegetable and Fruit By-Products as Functional Ingredient and Food. *Front. Nutr.* **2021**, *8*, 661693. [CrossRef] [PubMed]
4. Pathania, S.; Kaur, N. Utilization of Fruits and Vegetable By-Products for Isolation of Dietary Fibres and Its Potential Application as Functional Ingredients. *Bioact. Carbohydr. Diet. Fibre* **2022**, *27*, 100295. [CrossRef]
5. Sagar, N.A.; Pareek, S.; Sharma, S.; Yahia, E.M.; Lobo, M.G. Fruit and Vegetable Waste: Bioactive Compounds, Their Extraction, and Possible Utilization. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 512–531. [CrossRef]
6. Shurson, G.C.; Dierenfeld, E.S.; Dou, Z. Rules are meant to be broken—Rethinking the regulations on the use of food waste as animal feed. *Resour. Conserv. Recycl.* **2023**, *199*, 107273–107283. [CrossRef]
7. Ajila, C.M.; Brar, S.K.; Verma, M.; Tyagi, R.D.; Godbout, S.; Valéro, J.R. Bio-Processing of Agro-Byproducts to Animal Feed. *Crit. Rev. Biotechnol.* **2012**, *32*, 382–400. [CrossRef]
8. European Commission. COMMISSION REGULATION (EU) No 68/2013 of 16 January 2013 on the Catalogue of feed materials. *Off. J. Eur. Union* **2013**, *29*, 1–64. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:029:0001:0064:EN:PDF (accessed on 15 July 2024).
9. European Commission. COMMISSION REGULATION (EU) 2017/1017 of 15 June 2017 amending Regulation (EU) No 68/2013 on the Catalogue of feed materials. *Off. J. Eur. Union* **2017**, *159*, 48–119. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R1017 (accessed on 15 July 2024).
10. Georganas, A.; Giamouri, E.; Pappas, A.C.; Papadomichelakis, G.; Galliou, F.; Manios, T.; Tsiplakou, E.; Fegeros, K.; Zervas, G. Bioactive Compounds in Food Waste: A Review on the Transformation of Food Waste to Animal Feed. *Foods* **2020**, *9*, 291. [CrossRef]
11. Lee, K.; Malerba, F. Catch-up Cycles and Changes in Industrial Leadership:Windows of Opportunity and Responses of Firms and Countries in the Evolution of Sectoral Systems. *Res. Policy* **2017**, *46*, 338–351. [CrossRef]
12. Sharma, K.D.; Karki, S.; Thakur, N.S.; Attri, S. Chemical Composition, Functional Properties and Processing of Carrot—A Review. *J. Food Sci. Technol.* **2012**, *49*, 22–32. [CrossRef] [PubMed]
13. Bao, B.; Chang, K.C. Carrot Pulp Chemical Composition, Color, and Water-holding Capacity as Affected by Blanching. *J. Food Sci.* **1994**, *59*, 1159–1161. [CrossRef]

14. Shyamala, B.N.; Jamuna, P. Nutritional Content and Antioxidant Properties of Pulp Waste from *Daucus carota* and *Beta vulgaris*. *Malays. J. Nutr.* **2010**, *16*, 397–408. [PubMed]
15. Ikram, A.; Rasheed, A.; Ahmad Khan, A.; Khan, R.; Ahmad, M.; Bashir, R.; Hassan Mohamed, M. Exploring the Health Benefits and Utility of Carrots and Carrot Pomace: A Systematic Review. *Int. J. Food Prop.* **2024**, *27*, 180–193. [CrossRef]
16. Hashem, N. The Use of Dried Carrot Processing Waste in Broiler Diets. *J. Anim. Poult. Prod.* **2012**, *3*, 423–435. [CrossRef]
17. Vulić, J.J.; Ćebović, T.N.; Čanadanović-Brunet, J.M.; Ćetković, G.S.; Čanadanović, V.M.; Djilas, S.M.; Tumbas Šaponjac, V.T. In Vivo and in Vitro Antioxidant Effects of Beetroot Pomace Extracts. *J. Funct. Foods* **2014**, *6*, 168–175. [CrossRef]
18. Costa, A.P.D.; Hermes, V.S.; Rios, A.O.; Flôres, S.H. Minimally Processed Beetroot Waste as an Alternative Source to Obtain Functional Ingredients. *J. Food Sci. Technol.* **2017**, *54*, 2050–2058. [CrossRef]
19. Singh, A.; Ganesapillai, M.; Gnanasundaram, N. Optimizaton of Extraction of Betalain Pigments from *Beta vulgaris* Peels by Microwave Pretreatment. *IOP Conf. Ser. Mater. Sci. Eng.* **2017**, *263*, 032004. [CrossRef]
20. Jasmin, K.J.; Somanath, B. Carrot Waste and Beetroot Waste Supplemented Diet Promoting Carotenoid Changes in Freshwater Goldfish *C. auratus*. *Int. J. Life Sci. Res.* **2016**, *4*, 105–113.
21. Egüés, I.; Hernandez-Ramos, F.; Rivilla, I.; Labidi, J. Optimization of Ultrasound Assisted Extraction of Bioactive Compounds from Apple Pomace. *Molecules* **2021**, *26*, 3783. [CrossRef] [PubMed]
22. Sepelev, I.; Galoburda, R. Industrial Potato Peel Waste Application in Food Production: A Review. *Res. Rural Dev.* **2015**, *1*, 130–136.
23. Wu, Z.G.; Xu, H.Y.; Ma, Q.; Cao, Y.; Ma, J.N.; Ma, C.M. Isolation, Identification and Quantification of Unsaturated Fatty Acids, Amides, Phenolic Compounds and Glycoalkaloids from Potato Peel. *Food Chem.* **2012**, *135*, 2425–2429. [CrossRef] [PubMed]
24. Ncobela, C.N.; Kanengoni, A.T.; Hlatini, V.A.; Thomas, R.S.; Chimonyo, M. A Review of the Utility of Potato By-Products as a Feed Resource for Smallholder Pig Production. *Anim. Feed Sci. Technol.* **2017**, *227*, 107–117. [CrossRef]
25. Li, M.Y.; Hou, X.L.; Wang, F.; Tan, G.F.; Xu, Z.S.; Xiong, A.S. Advances in the Research of Celery, an Important Apiaceae Vegetable Crop. *Crit. Rev. Biotechnol.* **2018**, *38*, 172–183. [CrossRef]
26. Aşkın Uzel, R. Sustainable Green Technology for Adaptation of Circular Economy to Valorize Agri-Food Waste: Celery Root Peel as a Case Study. *Manag. Environ. Qual. Int. J.* **2023**, *34*, 1018–1034. [CrossRef]
27. Taranu, I.; Marin, D.E.; Manda, G.; Motiu, M.; Neagoe, I.; Tabuc, C.; Stancu, M.; Olteanu, M. Assessment of the potential of a boron–fructose additive in counteracting the toxic effect of Fusarium mycotoxins. *Br. J. Nutr.* **2011**, *106*, 398–407. [CrossRef]
28. *ISO 16472:2006*; Animal Feeding Stuffs—Determination of Amylase-Treated Neutral Detergent Fiber Content (aNDF). ISO: Geneva, Switzerland, 2006. Available online: https://www.iso.org/standard/37898.html (accessed on 15 July 2024).
29. *ISO 13906:2008*; Animal Feeding Stuffs—Determination of Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) CONTENTS. ISO: Geneva, Switzerland, 2008. Available online: https://www.iso.org/standard/43032.html (accessed on 15 July 2024).
30. Anzano, J.M.; Perise, E.; Belarra, M.A.; Castillo, J.R. Determination of Calcium and Copper in Feedstuffs by Atomic Absorption Spectrometry Following a Digestion Procedure with H_2SO_4 + H_2O_2. *Microchem. J.* **1995**, *52*, 268–273. [CrossRef]
31. *ISO 7485:2000*; Animal Feeding Stuffs—Determination of Potassium and Sodium Contents—Methods Using Flame-Emission Spectrometry. ISO: Geneva, Switzerland, 2000. Available online: https://www.iso.org/standard/32070.html (accessed on 15 July 2024).
32. COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009, Laying down the Methods of Sampling and Analysis for the Official Control of Feed. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32009R0152 (accessed on 22 July 2024).
33. Filip, M.; Vlassa, M.; Coman, V.; Halmagyi, A. Simultaneous Determination of Glucose, Fructose, Sucrose and Sorbitol in the Leaf and Fruit Peel of Different Apple Cultivars by the HPLC-RI Optimized Method. *Food Chem.* **2016**, *199*, 653–659. [CrossRef]
34. Filip, M.; Moldovan, M.; Vlassa, M.; Sarosi, C.; Cojocaru, I. HPLC Determination of the Main Organic Acids in Teeth Bleaching Gels Prepared with the Natural Fruit Juices. *Rev. Chim.* **2016**, *67*, 2440–2445.
35. Filip, M.; Silaghi-Dumitrescu, L.; Prodan, D.; Codruţa, S.; Moldovan, M.; Cojocaru, I. Analytical Approaches for Characterization of Teeth Whitening Gels Based on Natural Extracts. *Key Eng. Mater.* **2017**, *752 KEM*, 24–28. [CrossRef]
36. Vlassa, M.; Filip, M.; Ţăranu, I.; Marin, D.; Untea, A.E.; Ropotă, M.; Dragomir, C.; Sărăcilă, M. The Yeast Fermentation Effect on Content of Bioactive, Nutritional and Anti-Nutritional Factors in Rapeseed Meal. *Foods* **2022**, *11*, 2972. [CrossRef] [PubMed]
37. Zielińska, D.; Turemko, M. Electroactive Phenolic Contributors and Antioxidant Capacity of Flesh and Peel of 11 Apple Cultivars Measured by Cyclic Voltammetry and HPLC–DAD–MS/MS. *Antioxidants* **2020**, *9*, 1054. [CrossRef] [PubMed]
38. Duda-Chodak, A.; Tarko, T.; Tuszyński, T. Antioxidant Activity of Apples—An Impact of Maturity Stage and Fruit Part. *Acta Sci. Pol. Technol. Aliment.* **2011**, *10*, 443–454. [PubMed]
39. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
40. Boultif, A.; Louër, D. Powder Pattern Indexing with the Dichotomy Method. *J. Appl. Crystallogr.* **2004**, *37*, 724–731. [CrossRef]
41. Canteri, M.H.G.; Renard, C.M.G.C.; Le Bourvellec, C.; Bureau, S. ATR-FTIR Spectroscopy to Determine Cell Wall Composition: Application on a Large Diversity of Fruits and Vegetables. *Carbohydr. Polym.* **2019**, *212*, 186–196. [CrossRef]
42. Hong, T.; Yin, J.Y.; Nie, S.P.; Xie, M.Y. Applications of Infrared Spectroscopy in Polysaccharide Structural Analysis: Progress, Challenge and Perspective. *Food Chem. X* **2021**, *12*, 100168. [CrossRef]

43. Kamnev, A.A.; Colina, M.; Rodriguez, J.; Ptitchkina, N.M.; Ignatov, V.V. Comparative Spectroscopic Characterization of Different Pectins and Their Sources. *Food Hydrocoll.* **1998**, *12*, 263–271. [CrossRef]
44. Liang, S.; McDonald, A.G. Chemical and Thermal Characterization of Potato Peel Waste and Its Fermentation Residue as Potential Resources for Biofuel and Bioproducts Production. *J. Agric. Food Chem.* **2014**, *62*, 8421–8429. [CrossRef]
45. Muhammad, K.; Nur, N.I.; Gannasin, S.P.; Adzahan, N.M.; Bakar, J. High Methoxyl Pectin from Dragon Fruit (*Hylocereus polyrhizus*) Peel. *Food Hydrocoll.* **2014**, *42*, 289–297. [CrossRef]
46. Zlatanović, S.; Ostojić, S.; Micić, D.; Rankov, S.; Dodevska, M.; Vukosavljević, P.; Gorjanović, S. Thermal Behaviour and Degradation Kinetics of Apple Pomace Flours. *Thermochim. Acta* **2019**, *673*, 17–25. [CrossRef]
47. Fan, M.; Dai, D.; Huang, B. Fourier Transform Infrared Spectroscopy for Natural Fibres. In *Fourier Transform*; Salih, S.M., Ed.; IntechOpen: Rijeka, Croatia, 2012.
48. Šoštarić, T.; Simić, M.; Lopičić, Z.; Zlatanović, S.; Pastor, F.; Antanasković, A.; Gorjanović, S. Food Waste (Beetroot and Apple Pomace) as Sorbent for Lead from Aqueous Solutions—Alternative to Landfill Disposal. *Processes* **2023**, *11*, 1343. [CrossRef]
49. Singh, L.; Kaur, S.; Aggarwal, P.; Kaur, N. Characterisation of Industrial Potato Waste for Suitability in Food Applications. *Int. J. Food Sci. Technol.* **2023**, *58*, 2686–2694. [CrossRef]
50. Boadi, N.O.; Badu, M.; Kortei, N.K.; Saah, S.A.; Annor, B.; Mensah, M.B.; Okyere, H.; Fiebor, A. Nutritional Composition and Antioxidant Properties of Three Varieties of Carrot (*Daucus carota*). *Sci. Afr.* **2021**, *12*, e00801. [CrossRef]
51. Hussain, S.; Jõudu, I.; Bhat, R. Dietary Fiber from Underutilized Plant Resources—A Positive Approach for Valorization of Fruit and Vegetable Wastes. *Sustainability* **2020**, *12*, 5401. [CrossRef]
52. Taranu, I.; Filip, M.; Vlassa, M.C.; Marin, D.; Untea, A.; Oancea, A.; Pertea, A.M. Assessing Comparatively The Bioactive Compounds Composition Of Apple Pomace Obtained From Three Apple Cultivars After Juice Extraction. *Anim. Food Sci. J. Iasi* **2023**, *80*, 29–38.
53. Luca, M.I.; Ungureanu-Iuga, M.; Mironeasa, S. Carrot Pomace Characterization for Application in Cereal-Based Products. *Appl. Sci.* **2022**, *12*, 7989. [CrossRef]
54. Rupérez, P.; Toledano, G. Celery By-Products as a Source of Mannitol. *Eur. Food Res. Technol.* **2003**, *216*, 224–226. [CrossRef]
55. Bouhlali, E.d.T.; Derouich, M.; Meziani, R.; Bourkhis, B.; Filali-Zegzouti, Y.; Alem, C. Nutritional, Mineral and Organic Acid Composition of Syrups Produced from Six Moroccan Date Fruit (*Phoenix dactylifera* L.) Varieties. *J. Food Compos. Anal.* **2020**, *93*, 103591. [CrossRef]
56. Javed, A.; Ahmad, A.; Tahir, A.; Shabbir, U.; Nouman, M.; Hameed, A. Potato Peel Waste—Its Nutraceutical, Industrial and Biotechnological Applacations. *AIMS Agric. Food* **2019**, *4*, 807–823. [CrossRef]
57. Abdo, E.M.; Allam, M.G.; Gomaa, M.A.E.; Shaltout, O.E.; Mansour, H.M.M. Valorization of Whey Proteins and Beetroot Peels to Develop a Functional Beverage High in Proteins and Antioxidants. *Front. Nutr.* **2022**, *9*, 984891. [CrossRef] [PubMed]
58. Pistol, G.C.; Pertea, A.M.; Taranu, I. The Use of Fruit and Vegetable By-Products as Enhancers of Health Status of Piglets after Weaning: The Role of Bioactive Compounds from Apple and Carrot Industrial Wastes. *Vet. Sci.* **2024**, *11*, 15. [CrossRef] [PubMed]
59. Nićetin, M.; Pezo, L.; Pergal, M.; Lončar, B.; Filipović, V.; Knežević, V.; Demir, H.; Filipović, J.; Manojlović, D. Celery Root Phenols Content, Antioxidant Capacities and Their Correlations after Osmotic Dehydration in Molasses. *Foods* **2022**, *11*, 1945. [CrossRef] [PubMed]
60. Priecina, L.; Karklina, D. Natural Antioxidant Changes in Fresh and Dried Spices and Vegetables. *Int. J. Biol. Biomol. Agric. Food Biotechnol. Eng.* **2014**, *8*, 492–496.
61. Golubkina, N.A.; Kharchenko, V.A.; Moldovan, A.I.; Sekara, A.; Tallarita, A.; Caruso, G. Yield, Growth, Quality, Biochemical Characteristics and Elemental Composition of Plant Parts of Celery Leafy, Stalk and Root Types Grown in the Northern Hemisphere. *Plants* **2020**, *9*, 484. [CrossRef]
62. Lončarić, A.; Matanović, K.; Ferrer, P.; Kovač, T.; Šarkanj, B.; Babojelić, M.S.; Lores, M. Peel of Traditional Apple Varieties as a Great Source of Bioactive Compounds: Extraction by Micro-Matrix Solid-Phase Dispersion. *Foods* **2020**, *9*, 80. [CrossRef]
63. Ru, W.; Pang, Y.; Gan, Y.; Liu, Q.; Bao, J. Phenolic Compounds and Antioxidant Activities of Potato Cultivars with White, Yellow, Red and Purple Flesh. *Antioxidants* **2019**, *8*, 419. [CrossRef]
64. Sawicki, T.; Bączek, N.; Wiczkowski, W. Betalain Profile, Content and Antioxidant Capacity of Red Beetroot Dependent on the Genotype and Root Part. *J. Funct. Foods* **2016**, *27*, 249–261. [CrossRef]
65. Lau, W.K.; Van Chuyen, H.; Vuong, Q.V. Physical Properties, Carotenoids and Antioxidant Capacity of Carrot (*Daucus carota* L.) Peel as Influenced by Different Drying Treatments. *Int. J. Food Eng.* **2018**, *14*, 20170042. [CrossRef]
66. Drogoudi, P.D.; Michailidis, Z.; Pantelidis, G. Peel and Flesh Antioxidant Content and Harvest Quality Characteristics of Seven Apple Cultivars. *Sci. Hortic.* **2008**, *115*, 149–153. [CrossRef]
67. Sehm, J.; Lindermayer, H.; Dummer, C.; Treutter, D.; Pfaffl, M.W. The influence of polyphenol rich apple pomace or red-wine pomace diet on the gut morphology in weaning piglets. *J. Anim. Physiol. Anim. Nutr.* **2007**, *91*, 289–296. [CrossRef] [PubMed]
68. Guerrero, M.R.B.; Marques Da Silva Paula, M.; Zaragoza, M.M.; Gutiérrez, J.S.; Velderrain, V.G.; Ortiz, A.L.; Collins-Martínez, V. Thermogravimetric Study on the Pyrolysis Kinetics of Apple Pomace as Waste Biomass. *Int. J. Hydrogen Energy* **2014**, *39*, 16619–16627. [CrossRef]
69. Gowman, A.C.; Picard, M.C.; Rodriguez-Uribe, A.; Misra, M.; Khalil, H.; Thimmanagari, M.; Mohanty, A.K. Physicochemical Analysis of Apple and Grape Pomaces. *BioResources* **2019**, *14*, 3210–3230. [CrossRef]

70. Munir, S.; Daood, S.S.; Nimmo, W.; Cunliffe, A.M.; Gibbs, B.M. Thermal Analysis and Devolatilization Kinetics of Cotton Stalk, Sugar Cane Bagasse and Shea Meal under Nitrogen and Air Atmospheres. *Bioresour. Technol.* **2009**, *100*, 1413–1418. [CrossRef]
71. Elkhalifa, S.; Parthasarathy, P.; Mackey, H.R.; Al-Ansari, T.; Elhassan, O.; Mansour, S.; McKay, G. Biochar Development from Thermal TGA Studies of Individual Food Waste Vegetables and Their Blended Systems. *Biomass Conv. Bioref.* **2022**. [CrossRef]
72. Khosrowshahi, M.S.; Mashhadimoslem, H.; Emrooz, H.B.M.; Ghaemi, A.; Hosseini, M.S. Green Self-Activating Synthesis System for Porous Carbons: Celery Biomass Wastes as a Typical Case for CO_2 Uptake with Kinetic, Equilibrium and Thermodynamic Studies. *Diam. Relat. Mater.* **2022**, *127*, 109204. [CrossRef]
73. Mujtaba, G.; Hayat, R.; Hussain, Q.; Ahmed, M. Physio-chemical Characterization of Biochar, Compost and Co-composted Biochar Derived from Green Waste. *Sustainability* **2021**, *13*, 4628. [CrossRef]
74. Anukriti; Singh, N.; Upadhyay, D. XRD and SEM, ED Analysis of Solar Dried Vegetables. *Asian Food Sci. J.* **2022**, *21*, 25–37. [CrossRef]
75. Yi, S.; Lv, K.; Zhang, S.; Wang, W.; Li, X.; Li, J. Gel Quality and in Vitro Digestion Characteristics of Celery Nemipterus Virgatus Fish Sausages. *IOP Conf. Ser. Earth Environ. Sci.* **2020**, *512*, 012074. [CrossRef]
76. Imaizumi, T.; Szymańska-Chargot, M.; Pieczywek, P.M.; Chylińska, M.; Kozioł, A.; Ganczarenko, D.; Tanaka, F.; Uchino, T.; Zdunek, A. Evaluation of Pectin Nanostructure by Atomic Force Microscopy in Blanched Carrot. *LWT* **2017**, *84*, 658–667. [CrossRef]

agriculture

MDPI

Article

Effect of *Bacillus licheniformis* on Growth, Bone Mineralization, and Intestinal Microbiota in Broilers Fed Cowpea Diets

Mihaela Dumitru [1,*], Nicoleta Aurelia Lefter [1], Georgeta Ciurescu [1] and Reta Draghici [2]

[1] Laboratory of Animal Nutrition and Biotechnology, National Research Development Institute for Biology and Animal Nutrition, 077015 Balotesti, Romania; nicoleta.ciuca@ibna.ro (N.A.L.); ciurescu@ibna.ro (G.C.)
[2] Research-Development Station for Plant Culture on Sands Dăbuleni, 217, Petre Banită Street, 207170 Călărasi, Romania; retadraghici@yahoo.com
* Correspondence: mihaela.dumitru@ibna.ro

Abstract: This study investigates the effects of the *Bacillus licheniformis* (BL) ATCC 21424 strain, as a potential bacterial probiotic in broiler diets based on soybean meal (SBM) or cowpea seeds (CWP), on growth performance (GP), bone mineralization, and intestinal/fecal microbiota status (0 to 42 d age). A 2 × 2 factorial arrangement was employed in a completely randomized design, with four dietary treatments: SBM and CWP diets with or without BL supplementation (1.0×10^{11} CFU spores g^{-1} feed). A total of 480 one-day-old mixed-sex Ross 308 broiler chickens were randomly assigned to the treatments, with 6 pens of 20 chicks each. The results showed that broilers fed with CWP diets showed comparable body weight gain (BWG), feed intake (FI), and feed conversion rate to those fed the SBM diet ($p > 0.05$). The inclusion of BL improved BWG during the grower and finisher periods ($p = 0.01$) and overall study ($p < 0.001$), resulting in a numerical increase in FI ($p = 0.054$). In addition, BL in birds' diets reduced abdominal fat ($p = 0.032$) and influenced cecum weight ($p = 0.040$). Additionally, BL improved tibia iron (Fe) and phosphorus (P) bone mineralization and reduced the calcium–phosphorus (Ca:P) ratio ($p = 0.0001$). Microbial analysis revealed that BL inclusion decreased *Coliforms* counts in the CWP diet ($p = 0.073$), reduced *E. coli* in the ileum ($p \leq 0.05$), and lowered *Clostridium* spp. and *Enterococcus* spp. in the cecum broilers on SBM diets ($p \leq 0.05$). The presence of *Staphylococcus* spp. in broiler feces was also reduced in both SBM and CWP groups ($p < 0.05$). In conclusion, the addition of BL to broiler diets enhanced growth performance and bone mineralization and positively influenced gut and excreta bacterial populations in both SBM and CWP diets.

Keywords: protein sources; *Bacillus* probiotic; performance; poultry

Citation: Dumitru, M.; Lefter, N.A.; Ciurescu, G.; Draghici, R. Effect of *Bacillus licheniformis* on Growth, Bone Mineralization, and Intestinal Microbiota in Broilers Fed Cowpea Diets. *Agriculture* **2024**, *14*, 2013. https://doi.org/10.3390/agriculture14112013

Academic Editor: Secundino López

Received: 16 September 2024
Revised: 31 October 2024
Accepted: 6 November 2024
Published: 8 November 2024

1. Introduction

The escalating cost of feed ingredients, particularly cereal grains, and legume seeds, poses a significant challenge within the animal industry, notably in poultry feed manufacturing. This increase is fueled by their expanding use in human food and biofuel production, resulting in limited availability and higher prices. Consequently, the overall expense of poultry feed continues to rise, exerting pressure on poultry producers and impacting production efficiency [1]. Nutrition plays an important role in determining the profitability of animal production, with protein being a key component affecting the costs of feed mixes for poultry [2]. To tackle the challenge of reducing poultry feed costs [3], improving feed efficiency, maximizing growth performance, and minimizing the use of antibiotic growth promoters, nutritionists have been prompted to explore alternative strategies in poultry feed [2]. Therefore, the selected alternative protein source for broiler diets should contain highly digestible protein to ensure efficient utilization for meat production and minimize nitrogen excretion into the environment [4].

Soybean meal (SBM) serves as the main source of protein in broiler diets [5]. Among potential plant protein sources, cereal grains, and legumes, such as cowpeas, could serve

as excellent alternatives to SBM due to their comparable amino acid (AA) profiles [6,7]. Recent studies have highlighted the promising potential of cowpea (CWP) as an alternative protein source in poultry feed [5,8,9].

Cowpea [*Vigna unguiculata* (L.) Walp.], commonly known as "beans", is cultivated across diverse regions of the tropics and subtropics, including Asia, Oceania, the Middle East, Southern Europe, Africa, the Southern USA, and Central and South America [10]. This low-input, herbaceous annual plant pulse crop can grow either as an erect plant or as a climber. CWP is a versatile crop [11], with both its grains and leaves serving as valuable nutritional resources for humans and animals [12]. Due to its high economic importance, this plant is a vital leguminous cover crop rich in AAs and high-quality protein [13]. It provides essential nutrients, including soluble and insoluble dietary fibers [14], polyunsaturated fatty acids (PUFAs), vitamins (such as A, C, riboflavin, thiamine, niacin, B6, pantothenic acid, and a small amount of folate) [15], minerals (like calcium), carbohydrates, antioxidants, and polyphenolic compounds [4,16]. Besides the presence of beneficial bioactive compounds, cowpea seeds contain anti-nutritional factors like protease inhibitors, phytic acid, lectins, and tannins which can induce some limits for utilization in animal feeding [3,17,18].

According to the literature, diets supplemented with probiotics can enhance production performance, reduce chicken mortality, and decrease environmental pollution [18,19]. As feed additives, probiotics improve the health of the digestive system by regulating the intestinal microbiota structure [20], strengthening immunity to improve disease resistance, ensuring quicker nutrient absorption, and promoting faster growth [21]. These benefits result in improved production performance and positive physiological effects on the host [22,23]. Defined as non-pathogenic living organisms, probiotic bacteria can withstand gastric acid, bile, and digestive enzymes, adhere to the intestinal wall, and combat pathogens, improving by the way, the bioavailability of feed ingredients [4,22]. Different probiotics have been shown to inhibit pathogens effectively based on in vitro and in vivo experiments. Specifically, for broiler chickens, previous studies have demonstrated significant benefits from including species such as *Lactobacillus* (*LAB*), *Bacillus*, *Streptococcus*, *Saccharomyces*, and *Aspergillus* in their diet [19,21,24].

Among probiotic bacteria, *Bacillus* spp. received significant attention due to their thermophilic, spore-forming nature and status as Gram-positive aerobic bacteria with a strong safety profile [25]. Known for their ability to produce various compounds, including digestive enzymes and vitamins, *Bacillus* may improve the weight gain and feed conversion ratios in poultry [26]. Generally considered non-pathogenic to humans and animals, these species have shown promising results when used as supplements in poultry diets. For example, the addition of different species such as *B. subtilis*, B. coagulans, B. amyloliquefaciens, and *B. licheniformis* has been demonstrated to promote growth effectively [21,27].

To the best of our knowledge, there has been limited research on the utilization of *Bacillus licheniformis* ATCC 21424 (BL) in broiler chicks, whose diets are based on CWP and SBM inclusion. Therefore, the objective of this study was to assess the effects of BL inclusion in broiler diets containing various protein sources on GP, bone mineralization, and microbial populations (intestinal and excreta content).

2. Materials and Methods

2.1. Preparation of Probiotic Strain

Bacillus licheniformis ATCC 21424 was purchased from the American Tissue Culture Collection (Manassas, VA, USA). A previous study provided BL product preparation details [28].

2.2. Ethical Statement

The care and use protocol for the birds was approved by the Animal Care and Use Committee at the National Research–Development Institute for Biology and Animal Nutrition (INCDBNA-IBNA) from Balotesti, Romania. The protocol followed the guidelines set

forth by the EU Directive 2010/63/EU and complied with Romanian Animal Protection Law [9].

2.3. Experimental Design and Husbandry Bird

A total of 480 healthy, one-day-old mixed-sex Ross 308 broiler chickens (starting body weight 46.5 ± 0.23 g) were sourced from a local commercial hatchery. Two protein-based diets were tested: one using local CWP as a potential substitute for SBM. These diets were evaluated in the presence (+) or absence (−) of BL, arranged in a 2 × 2 factorial design within a completely randomized framework. Broiler chicks were randomly assigned to four dietary treatments, with six replicates per treatment and twenty chicks per replicate. BL inclusion was 1×10^{11} CFU/g^{-1} feed. The study lasted 42 days, with mash feed and freshwater provided ad libitum. The feeding program consisted of three phases: starter (days 1–10), grower (days 11–24), and finisher (days 25–42). Birds were fed isocaloric and isonitrogenous diets with a similar content of total lysine, total sulfur amino acids (TSAAs; Table 1), calcium (Ca), and available phosphorous (P). The diets were formulated to meet or surpass breeder guidelines specified for Ross 308 (Aviagen Ltd., Midlothian, UK). Diets were produced in mash form and did not contain any growth promoters or antibiotics. Nevertheless, all four experimental diets included narasin as a coccidiostat (Monteban G100, Elanco GmbH, Cuxhaven, Germany) and phytase (Axtra PHY 5000 L, Danisco Animal Nutrition, Marlborough, UK) as exogenous enzymes in their premixes. Pens with dimensions of 1.75 × 1.55 m were used. The temperature was initially set at 34 °C for the first 5 days and gradually decreased following standard management protocols. Thermostatically controlled heaters, fans, and adjustable sidewall inlets were utilized to achieve 22 °C. The lighting system provided 23:1 h light/dark conditions per day from day 1 to day 7, then 20:4 h light/dark conditions until the end of the experimental trial. The relative humidity was consistently maintained at approximately 55–60% throughout the entire duration of the trial. This schedule adhered to European Union legislation (EU Council Directive 2007/43/EC). Upon hatching, immediately, the broiler received vaccinations for Marek's disease, Newcastle disease, and Infectious Bronchitis Disease.

Table 1. Ingredients and chemical compositions of the experimental diets.

	Starter		**Grower**		**Finisher**	
	(0 to 10 d)		**(11 to 24 d)**		**(25 to 42 d)**	
	SBM	**CWP**	**SBM**	**CWP**	**SBM**	**CWPs**
Ingredients (%)						
Corn	55.73	46.12	56.66	47.28	64.26	54.73
Soybean meal (45%)	33.10	27.20	31.56	25.50	25.10	19.10
Corn gluten	4.30	4.30	4.00	4.00	3.50	3.50
Cowpea (24%)	0.00	15.00	0.00	15.00	0.00	15.00
Soybean oil	1.50	2.10	2.90	3.40	2.50	3.10
Monocalcium phosphate	1.67	1.63	1.66	1.65	1.47	1.44
Calcium carbonate	1.71	1.72	1.46	1.46	1.27	1.28
Salt (NaCl)	0.28	0.28	0.28	0.28	0.28	0.28
L-lysine HCl	0.32	0.24	0.17	0.10	0.29	0.22
DL-methionine	0.31	0.33	0.23	0.25	0.26	0.28
Choline chloride (50%)	0.08	0.08	0.08	0.08	0.07	0.07
Vitamin–mineral supplement [1]	1.00	1.00	1.00	1.00	1.00	1.00
Bacillus licheniformis ATCC 21424 [2]	−/+	−/+	−/+	−/+	−/+	−/+
Total ingredients	**100**	**100**	**100**	**100**	**100**	**100**
Calculated chemical composition						
ME (MJ/kg)	12.56	12.57	12.98	12.97	13.20	13.21
Crude protein (%)	23.0	23.0	22.0	22.0	19.50	19.50
Lysine, total (%)	1.40	1.40	1.32	1.24	1.16	1.16

Table 1. *Cont.*

	Starter		Grower		Finisher	
	(0 to 10 d)		(11 to 24 d)		(25 to 42 d)	
	SBM	**CWP**	**SBM**	**CWP**	**SBM**	**CWPs**
Lysine, digestible	1.34	1.34	1.18	1.16	1.05	1.04
Methionine + cysteine, total	1.05	1.05	0.95	0.95	0.91	0.91
Methionine + cysteine, digestible	0.97	0.97	0.87	0.86	0.84	0.83
Calcium (%)	1.00	1.00	0.90	0.90	0.79	0.79
Available phosphorus (%)	0.45	0.45	0.45	0.45	0.40	0.40
Crude fat	4.38	5.10	5.77	6.40	5.57	6.29
Crude fiber	2.85	3.27	2.79	3.20	2.61	3.02
Analyzed chemical composition (%)						
Dry matter	88.97	88.50	88.20	88.73	89.95	89.98
Crude protein	23.09	22.90	22.05	22.10	19.58	19.60
Crude fat	4.40	5.05	5.69	6.36	5.40	6.19
Crude fiber	2.90	3.30	2.82	3.25	2.70	3.10
Calcium	0.98	0.96	0.88	0.89	0.80	0.82
Total phosphorous	0.79	0.75	0.76	0.78	0.73	0.75

Abbreviations: SBM, soybean meal; CWP, cowpea. [1] Supplied per kg diet: 12,000 IU vitamin A, 5000 IU vitamin D3, 75 mg vitamin E, 3 mg vitamin K_3, 3 mg vitamin B_1, 8 mg vitamin B_2, 5 mg vitamin B_6, 0.016 mg vitamin B_{12}, 13 mg pantothenic acid, 55 mg nicotinic acid, 2 mg folic acid, 0.2 mg biotin, 120 mg Mn, 100 mg Zn, 40 mg Fe, 16 mg Cu, 1.25 mg I, 0.3 mg Se, 70 mg Monteban G100, 0.2 g Axtra PHY 5000 L (1000 FTU). [2] − = probiotic not included in the diet; + = probiotic included in the diet.

2.4. Feed Analyses and Amino Acid Contents

Samples of ingredients and feeds were analyzed in duplicate for dry matter (DM), crude protein (CP), ether extract (EE), and ash content, using standard procedures outlined in Commission Regulation (EC) no. 152. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) levels were measured with a Fibertec system (automatic Foss-Tecator system, Höganäs, Sweden). Carbohydrate content was calculated as a nitrogen-free extract. The apparent metabolizable energy (AME) content of the diets was estimated using the energy values of individual ingredients, following the European poultry feedstuff energy tables equation (WPSA, 1989). Amino acids (excluding tryptophan, which was not determined) were analyzed using a high-performance liquid chromatography system (HPLC Thermo Fisher Scientific Inc., San Jose, CA, USA) [27]. Trypsin inhibitor activity (TIA) in CWP was measured and expressed as trypsin-inhibited units (TIUs) [29]. All data and AME values are reported on a DM basis.

2.5. Growth Performance Data

The body weights (BWs) of the chicks and their feed intakes (FIs) were weighed at 1, 10, 24, and 42 days of age in each pen at the same times. BW gain (BWG) and feed conversion ratio (FCR) were calculated during different periods. Mortality was recorded daily, and FI was corrected for mortality as it occurred. FCR was calculated by dividing the FI by the BWG.

2.6. Carcass Traits and Bone Mineralization

At the end of the experimental period, on day 42, the six chickens from each treatment group, with a BW close to the respective group averages, after 12 h of fasting for complete gut evacuation were selected randomly for euthanasia and individual weighing. After removing the head, neck, feet, and viscera, the carcasses, breast, legs, abdominal fat, liver, spleen, pancreas, heart, gizzard, and small intestine were weighed individually and expressed as a percentage of their carcass weight [30]. Also, the length of the small intestine (duodenum, jejunum, ileum, and cecum) was measured and recorded. The relative weight and size of internal organs were quantified as percentages of the hot carcass weight. Until analyzed, the tibia from the left leg was removed, deboned, packed into polyethylene bags,

sealed, and promptly stored in the deep freezer at −20 °C. In brief, the tibias of euthanized broilers were autoclaved to remove tissues and cartilage caps, enabling the determination of relative bone weight and length [9]. After microwave digestion, the fat-free bones were ground, dried, and analyzed using flame atomic absorption spectrometry (FAAS) at the following specific wavelengths: 422.7 nm for Ca, 213.9 nm for zinc (Zn), 372.0 nm for Fe, and 403.1 nm for manganese (Mn). P concentration in the bones was determined via UV-VIS spectrometry, while ash content was measured using a gravimetric method. Mineral content in the samples was expressed in milligrams (mg) or micrograms (μg) per gram of ash.

2.7. Intestinal and Feces Bacterial Counts

The same batch of slaughtered broilers (n = 6 per treatment) underwent microbial analysis. The intestinal content, comprising the ileum (1 cm distal to Meckel's diverticulum to the ileocecal junction) and the cecum, was aseptically collected and stored in sterile plastic bags on ice. In the laboratory, 10-fold serial dilutions of 1 g of sample from both the ileum and cecum were prepared. These dilutions were homogenized with 7.0 mL of Brain Heart Infusion (BHI) broth supplemented with 2.0 mL of glycerol and then immediately frozen at −20 °C for subsequent analysis [31]. Upon thawing, decimal dilutions in Phosphate-Buffered Saline (PBS; Oxoid LTD, Basingstoke, UK) were performed. Subsequently, the samples were evaluated for Lactic Acid Bacteria (LABs), *Escherichia coli* (*E. coli*; biotype β-hemolytic), *Salmonella* spp., *Clostridium* spp., Coliforms, *Bacillus* spp., and *Enterococcus* spp. LABs were cultured on de Man, Rogosa, and Sharpe agar (MRS; Oxoid CM0361) under anaerobic conditions at 37 °C for 48 h (Oxoid jar with Anaerogen 2.5 L). Coliforms were cultured on MacConkey agar (Oxoid CM0007) incubated aerobically at 37 °C for 24 h. *E. coli* was determined by inoculating 0.01 mL from 10^{-1} dilution onto sheep blood agar [trypticase soy agar (TSA) 5% (w/v)] and incubating at 37 °C for 24 h under aerobic conditions [32]. *Clostridium* spp. was cultured on Reinforced Clostridial Agar (Oxoid CM0151) incubated anaerobically at 37 °C for 48 h. *Enterococcus* spp. were enumerated on Slanetz–Bartley agar (Oxoid CM0377) incubated at 37 °C for 48 h in anaerobic conditions [31]. *Bacillus* spp. and *Salmonella* spp. were counted on a nutritive agar medium, respectively, with *Salmonella-Shigella* agar (Oxoid CM0099) incubating aerobically at 37 °C for 24 h. Every sample's procedure was repeated three times. Finally, the microbiota enumerations were reported as a mean of 10 logarithm colony-forming units ($\log_{10}$ CFUs) per gram.

The fecal microbial count was assessed at d 42 (n = 24 samples). Paper drop-sheets were placed in each pen to collect fresh excreta samples using sterile plastic containers. The fecal sample was well homogenized by vortexing for 1 min. with PBS (1:10, w/v), and used for bacterial counting. LABs and *Salmonella* spp. followed the same protocol method from the intestinal bacterial count. *Enterobacteriaceae* were enumerated using Levine medium agar (g/L: pancreatic digest of gelatine 10; lactose 10; potassium phosphate 2; eosin Y 0.4; methylene blue 0.065; bacteriological agar 15; pH 7.1 ± 0.2) and *Staphylococcus* spp. on Baird-Parker Agar supplemented with an egg yolk tellurite emulsion (BPA; Oxoid LTD, Basingstoke, UK); both determinations were incubated at 37 °C for 48 h in aerobic conditions.

2.8. Measurement of Intestinal pH

To determine the pH of intestinal content (ileum and cecum; n = 6 per treatment), approximately 1 g of digesta from each bird was collected and thoroughly homogenized in distilled water (DW, 1:10, w/v). The pH was measured using a portable pH meter (series pH 7.0 + DHS, XS Instruments, Carpi, Italy), with the final pH value representing the average of three readings.

2.9. Statistical Analysis

Statistical analysis was performed using a two-way ANOVA with the GLM procedure of SPSS, version 20.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed as a 2 × 2 factorial design of dietary treatments. The statistical model included the effects of protein sources (Ps), probiotic supplementation (BL), and their interactions. For the analysis of growth performance (BWG, FI, and FCR), replicate pens were used as the experimental unit, while carcass traits, tibia bone characteristics, pH, and bacterial counts from digesta (ileum and cecum) were analyzed based on individual broilers (n = 6 per treatment). Tukey's post hoc test was used for multiple comparisons. The significant differences between treatments were considered statistically significant at $p < 0.05$, and a p-value between 0.05 and 0.10 was classified as a tendency to be influenced by the treatment. The graphics were generated using GraphPad Prism software V. 9.1.2 (Boston, MA, USA).

3. Results

3.1. Feed Analyses

The main compounds of the chemical composition of CWP seeds (cultivar Aura 26) and AA profile are presented in Table 2. The results show that the variety of CWP has substantial crude protein (288 g/kg DM) and AME value (12.8 MJ kg DM), highlighting its potential as a protein-rich and energy-dense feed source. Regarding the mineral content, the CWP seed showed a high concentration of Ca (1.1 g/kg) and P (5.6 g/kg). The composition can vary based on varietal differences, climatic conditions, and agronomic practices. The protein quality or nutrient value of food depends on its AA content and the physiological utilization of specific AAs after digestion, absorption, and metabolism. The CWP source is rich in essential AAs such as arginine, leucine, lysine, isoleucine, and valine, while the sulfur AAs (methionine, cysteine, and threonine) are found in lower amounts. For non-essential AAs, the results showed that glutamic and aspartic acids are predominant, followed by serine and glycine contributing to the overall AA profile, enhancing, by the end, the nutritional value of CWP. The ratio of essential amino acids (EAAs) to the total non-essential amino acids (NEAAs) in CWP was noted as 0.95. This balance in AA composition can enhance other dietary components, making it a suitable candidate for use as a partial protein source in broiler feed.

Table 2. Composition and amino acid content of cowpea seeds (cultivar Aura 26).

Item	**Cowpea**	
Nutrient (g/kg dry matter)		
Dry matter	910	
Crude protein	288	
Ether extract	12.00	
Crude fiber	51.00	
Ash	45.00	
Nitrogen-free extract	514	
Calcium	1.10	
Phosphorous, total	5.60	
AME (MJ/kg) [1]	12.80	
Amino acids (g/kg dry matter)	**Amount (g/kg)**	**Relative to lysine (%)**
Lysine	19.20	100
Methionine + cysteine	6.60	34
Threonine	12.80	66
Arginine	27.20	142
Leucine	22.50	117
Isoleucine	12.90	67
Phenylalanine	15.60	81
Valine	16.30	85

Table 2. *Cont.*

Item	Cowpea	
Amino acids (g/kg dry matter)	**Amount (g/kg)**	**Relative to lysine (%)**
Essential amino acids (EAAs)	**133.10**	**133.10**
Tyrosine	8.70	45
Serine	17.50	91
Glycine	12.60	65
Alanine	8.80	46
Aspartic acid	37.40	195
Glutamic acid	54.70	285
Non-essential amino acids (NEAAs)	**139.70**	**139.70**
Essential/non-essential AA ratio	0.95	0.95

[1] Calculated value; European Table of Energy Values for Poultry Feedstuffs (WPSA, 1989). AME = metabolizable energy.

3.2. Growth Performance Data

Table 3 shows the main effect of protein source (Ps), probiotic inclusion (BL), and their interaction (Ps × BL) on the performance growth of broiler chickens. The results demonstrate that broilers fed diets with CWP exhibited growth performance (BWG, FI, and FCR) like those fed SBM throughout the study period (days 1 to 42; $p > 0.05$). BL inclusion in broiler diets increased BWG in the grower and finisher period ($p = 0.01$), respectively, during the entire study period ($p < 0.001$). Further, probiotic addition tended to increase FCR during the grower period (d 11–24) and led to a slight increase in FI for the overall period (d 0–42), resulting in a tendency to be influenced by the treatment ($p = 0.054^{T}$). Regarding the main factors (Ps × BL) for all GP variables measured, there was no significant interaction. Mortality was low (less than 3%) and unrelated to the treatments. All deaths occurred within the first week of age and were attributed to transportation-related stress.

3.3. Carcass Traits and Bone Mineralization

Table 4 presents the effects of BL probiotic supplementation on carcass characteristics (breast and leg yield and abdominal fat) and organ weights (heart, liver, gizzard, pancreas, small intestine, and cecum), along with the weight and length of the small intestine and cecum in broilers at 42 days of age. For all carcass characteristics measured, no significant interaction between the main factors (Ps × BL) was noticed. Diets for broilers up to 42 days with SBM and CWP significantly affect the abdominal fat ($p = 0.026$). CWP diets involved an increase in small intestine weight (SIW) for the jejunum section ($p = 0.032$) vs. birds fed SBM diets. Further, the Ps effect was observed as well in the small intestine length (SIL), more exactly in the jejunum portion, whose length was increased ($p = 0.084$). The inclusion of BL in birds' diets decreased the abdominal fat ($p = 0.032$), respectively, affecting the cecum weight ($p = 0.040$) compared to the treatments without BL inclusion.

Tibia traits and bone mineralization results in broiler feed diets with different Ps in the presence (+)/absence (−) of BL are presented in Table 5. Ps diets did not involve significant differences in tibia bone development (relative weight and length) as well as ash, Ca, P, and Zn content ($p > 0.05$). Tibia Fe content registered significant differences between birds fed different diets ($p = 0.044$). Supplementation with BL significantly increased P tibia concentration on day 42 (4.88%; $p = 0.027$) compared to bird diets without probiotic inclusion. A similar but non-significant trend in Zn mineral content was noted ($p = 0.098$). A reduction in the Ca:P ratio (3.7%; $p = 0.0001$) in BL treatments was observed. There was no interaction between the main factors (Ps × BL) for any of the tibia parameters measured at d 42, except for P content ($p = 0.588$), Fe content ($p = 0.377$), and the Ca:P ratio ($p = 0.443$).

Table 3. Effects of the diets with different protein sources without and with *Bacillus licheniformis* ATCC 21424 (BL) on performance variables (mean values [1]) of broilers.

Items	Protein Source	Probiotic Inclusion [2]	Starter (d 0–10)			Grower (d 11–24)			Finisher (d 25–42)			Overall (d 0–42)			
			BWG (g)	FI (g)	FCR (g/g)	BWG (g)	FI (g)	FCR (g/g)	BWG (g)	FI (g)	FCR (g/g)	FBW (g)	BWG (g)	FI (g)	FCR (g/g)
1	SBM	No	244	298	1.22	769 ^{b}	1245	1.62	1707 ^{ab}	3180	1.86 ^{a}	2765 ^{ab}	2720 ^{ab}	4725	1.74
2	CWP	No	243	300	1.23	762 ^{b}	1230	1.61	1677 ^{b}	3140	1.87 ^{a}	2727 ^{b}	2682 ^{b}	4670	1.74
3	SBM	Yes	245	300	1.22	806 ^{a}	1280	1.59	1733 ^{a}	3205	1.86 ^{a}	2783 ^{ab}	2738 ^{ab}	4795	1.75
4	CWP	Yes	247	303	1.23	819 ^{a}	1285	1.57	1785 ^{a}	3225	1.82 ^{b}	2851 ^{a}	2806 ^{a}	4823	1.72
SEM [3]			7.60	9.70	0.01	19.13	25.50	0.02	27.54	36.50	0.03	28.77	23.14	65.50	0.03
Main effects [4]															
Protein source (Ps)															
SBM			244	299	1.22	788	1263	1.61	1720	3192	1.86	2774	2729	4759	1.74
CWP			245	302	1.23	790	1258	1.59	1731	3183	1.85	2789	2744	4747	1.73
Probiotic inclusion (BL)															
No			245	299	1.23	766 ^{b}	1238	1.61	1692 ^{b}	3160	1.87 ^{a}	2746 ^{b}	2701 ^{b}	4697	1.74
Yes			246	302	1.23	812 ^{a}	1282	1.58	1759 ^{a}	3215	1.84 ^{b}	2817 ^{a}	2772 ^{a}	4809	1.73
p-value															
Ps effect			0.577	0.317	0.348	0.764	0.543	0.244	0.234	0.454	0.770	0.343	0.313	0.446	0.654
BL effect			0.656	0.098	0.983	0.010	0.345	0.067 ^{T}	0.010	0.115	0.043	0.024	0.001	0.054 ^{T}	0.535
Ps × BL effect			0.787	0.933	0.757	0.356	0.089	0.798	0.553	0.262	0.755	0.042	0.353	0.558	0.627

Abbreviation: SBM, soybean meal; CWP, cowpea; FBW, final body weight. [1] Data are means of 6 replicate pens with 20 birds per pen. [2] *Bacillus licheniformis* ATCC 21424 strain: 1×10^{11} CFU/g^{-1} feed. [3] SEM, standard error of the mean. [4] Data were analyzed as a 2 × 2 factorial arrangement. ^{a,b} Means with different superscripts in a row differ significantly ($p < 0.05$). ^{T}: the tendency to be influenced by treatment.

Table 4. Effects of the diets with different protein sources without and with *Bacillus licheniformis* ATCC 21424 (BL) on the carcass traits (mean values [1]) of broilers (d 42).

Items	Protein Source	Probiotic Inclusion [2]	Carcass [5]	Breast [6]	Legs [6]	Abdominal Fat [6]	Organs (g)				SIW [7] (g)				SIL [7] (cm)		
							Heart [6]	Liver [6]	Gizzard [6]	Pancreas [6]	Duodenum [6]	Jejunum [6]	Ileum [6]	Cecum [6]	Jejunum [7]	Ileum [7]	Cecum [7]
1	SBM	No	72.45	37.96	27.70	1.78	0.60	2.85	1.81	0.22	0.84	2.92	2.35	0.77	4.29	4.25	1.82
2	CWP	No	71.97	37.25	27.40	1.54	0.53	2.87	1.83	0.26	0.96	3.40	2.43	0.85	4.52	4.39	1.70
3	SBM	Yes	72.55	37.34	27.55	1.72	0.54	3.10	1.90	0.24	0.93	2.94	2.50	0.74	4.22	4.37	1.75
4	CWP	Yes	72.34	37.82	27.85	1.43	0.59	2.79	1.82	0.27	0.90	3.33	2.55	0.70	4.45	4.44	1.69
SEM [3]			0.67	0.53	0.44	0.09	0.01	0.26	0.09	0.05	0.09	0.17	0.16	0.11	0.15	0.17	0.13
Main effects [4]																	
Protein source (Ps)																	

Table 4. *Cont.*

Items	Protein Source	Probiotic Inclusion [2]	Carcass [5]	Breast [6]	Legs [6]	Abdominal Fat [6]	Organs (g)				SIW [7] (g)				SIL [7] (cm)		
							Heart [6]	Liver [6]	Gizzard [6]	Pancreas [6]	Duodenum [6]	Jejunum [6]	Ileum [6]	Cecum [6]	Jejunum [7]	Ileum [7]	Cecum [7]
SBM			72.50	37.65	27.62	1.75 [a]	0.57	2.97	1.86	0.23	0.89	2.93 [b]	2.43	0.76	4.26	4.31	1.78
CWP			72.16	37.54	27.63	1.49 [b]	0.56	2.83	1.83	0.26	0.93	3.37 [a]	2.49	0.78	4.48	4.42	1.70
Probiotic (BL)																	
No			72.21	37.60	27.55	1.66 [a]	0.57	2.86	1.82	0.24	0.90	3.16	2.39	0.81 [a]	4.40	4.32	1.76
Yes			72.44	37.58	26.70	1.57 [b]	0.57	2.95	1.86	0.26	0.91	3.14	2.53	0.72 [b]	4.34	4.41	1.72
p-value																	
Ps effect			0.253	0.998	0.622	0.026	0.858	0.104	0.632	0.152	0.537	0.032	0.554	0.237	0.084	0.619	0.103
BL effect			0.552	0.654	0.122	0.032	0.994	0.235	0.643	0.275	0.866	0.423	0.201	0.040	0.365	0.663	0.118
Ps × BL effect			0.789	0.277	0.505	0.655	0.785	0.196	0.522	0.646	0.352	0.244	0.119	0.275	0.224	0.745	0.587

Abbreviation: SBM, soybean meal; CWP, cowpea; SIW, small intestine weight; SIL, small intestine length. [1] Data are means of 6 birds per treatment; [2] *Bacillus licheniformis*, ATCC 21424 strain: 5.0×10^{11} CFU/g^{-1} feed. [3] SEM, standard error of the mean. [4] Data were analyzed as a 2 × 2 factorial arrangement. [5] Represents the weight (g) without head, neck, feet, and viscera carcass as 100 g of live body weight. [6,7] Calculated as weight or length (g or cm) of organs as 100 g of carcass weight. [a,b] Means with different superscripts in a row differ significantly ($p < 0.05$).

Table 5. Effects of the diets with different protein sources with and without *Bacillus licheniformis* ATCC 21424 (BL) on tibia bone development and mineralization (mean values [1]) of broilers (d 42).

Items	Protein Source	Probiotic Inclusion [2]	Tibia, Weight [5]	Tibia, Length [5]	Ash, %	Mineral Contents				
						Ca, mg/g	P, mg/g	Fe, µg/g	Zn, µg/g	Ca:P Ratio
1	SBM	No	0.63	0.46	57.02	319.89	167.41	174.42	346.37	1.91
2	CWP	No	0.64	0.47	58.20	319.36	158.80	195.45	333.88	2.01
3	SBM	Yes	0.65	0.47	57.38	321.54	166.27	170.29	356.48	1.93
4	CWP	Yes	0.68	0.49	57.57	325.33	175.90	204.03	349.33	1.85
SEM [3]			0.06	0.02	0.44	2.79	3.27	27.85	15.57	0.03
Main effects [4]										
Protein source (Ps)										
SBM			0.64	0.47	57.20	320.72	166.84	172.35 [b]	354.43	1.92
CWP			0.66	0.48	57.89	322.34	167.35	199.74 [a]	341.61	1.93
Probiotic (BL)										
No			0.64	0.47	57.61	319.63	163.11 [b]	184.93	340.12	1.96 [a]
Yes			0.67	0.48	57.48	323.44	171.08 [a]	187.16	352.90	1.89 [b]
p-value										
Ps effect			0.407	0.982	0.762	0.423	0.395	0.044	0.111	0.968
BL effect			0.754	0.987	0.643	0.502	0.027	0.332	0.098 [T]	0.001
Ps × BL effect			0.556	0.771	0.405	0.496	0.588	0.377	0.393	0.443

Abbreviation: SBM, soybean meal; CWP, cowpea. [1] Data are means of 6 birds per treatment. [2] *Bacillus licheniformis*, ATCC 21,424 strain: 5.0×10^{11} CFU/g^{-1} feed. [3] SEM, standard error of the mean. [4] Data were analyzed as a 2 × 2 factorial arrangement. [5] Represents the weight (g) without head, neck, feet, and viscera carcass as 100 g of live body weight. [a,b] Means with different superscripts in a row differ significantly ($p < 0.05$). [T]: the tendency to be influenced by treatment.

3.4. Intestinal and Feces Bacterial Counts

The treatment effects of the diets using different protein sources (SBM and CWP) with and without BL (log_{10} CFU/g) on the microbial population of broilers (d 42) are presented in Figure 1.

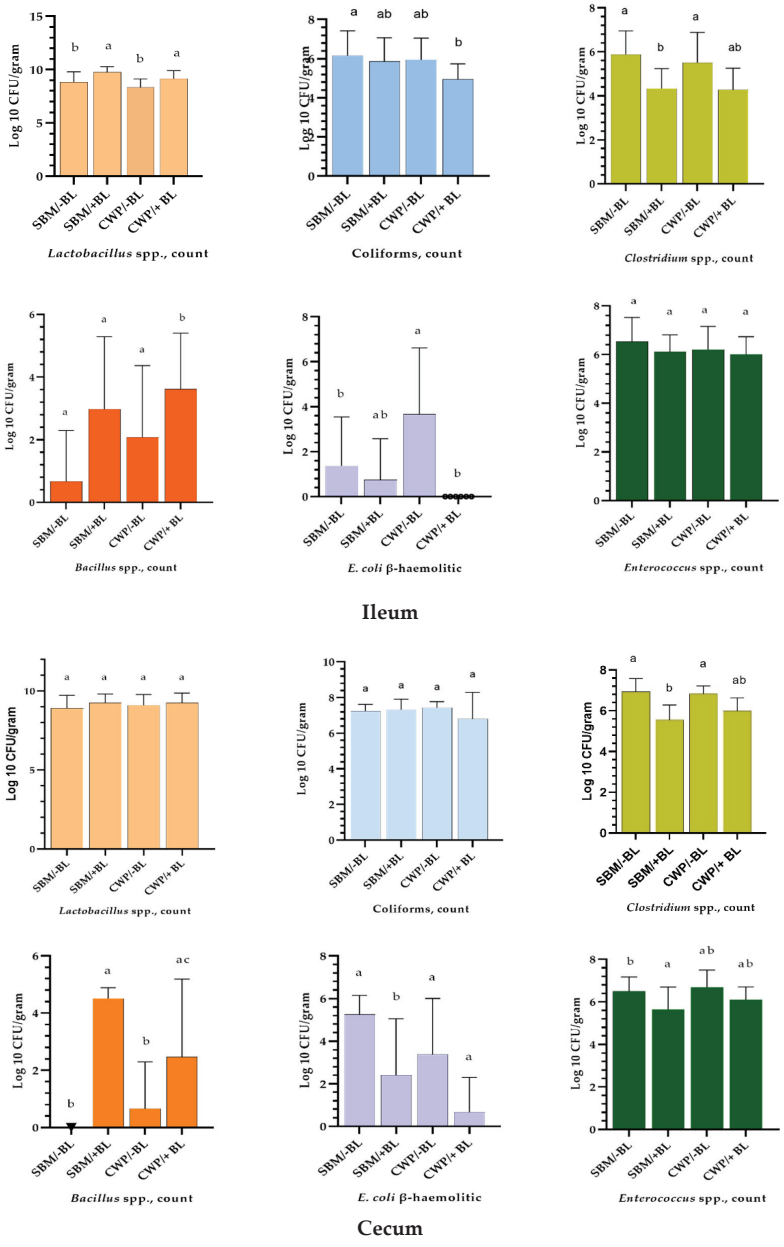

Figure 1. Effects of dietary supplementation with *Bacillus* on the intestinal microbiota count of broiler chickens. Bars represent the mean SD. The use of distinct lowercase letters in the graph corresponds to the results of Tukey's post hoc comparisons.

At d 42, the inclusion of BL in birds' diets showed a significant increase in LABs ($p \leq 0.05$) from the ileum content of broiler chickens. Moreover, the Lactobacilli count from SBM/+BL compared with the SBM/-BL group was significantly influenced by BL supplementation (9.76 vs. 8.82 Log10 CFU/g, $p \leq 0.05$). The BL addition increased CWP vs. CWP/−BL in birds' diets ($p = 0.087$). The presence of BL in CWP broilers' diet demonstrates significantly stronger impacts on microbial populations, exhibiting a significant decrease in Coliforms ($p = 0.073$) vs. the SBM/-BL group. Notably, the *Clostridium* spp. from ileum content decreased by 27.12% in SBM/+BL vs. CWP/−BL ($p = 0.078$), respectively, and by 28.84% in CWP/+BL compared to CWP/−BL ($p = 0.065$). As expected, the probiotic inclusion had a more pronounced effect on *Bacillus* spp. in the SBM/+BL group ($p = 0.063$); also, in the CWP/+BL treatment, an increase of 21.70% vs. the SBM/+BL group was noted. Harmful microbes such as *E. coli* in ileum content were significantly influenced by BL addition ($p < 0.05$). An exhibited decrease was noted in CWP/+BL (CWP/+BL, $p = 0.005$). Further, the presence of BL seems to counteract the potential reduction in *Enterococcus* spp. ($p > 0.05$). However, the interaction between BL and protein diet sources reaches statistical significance ($p \leq 0.05$) and is particularly more evident for LAB, Coliforms, *Bacillus* spp., and *E. coli* counts. This indicates that the combination of BL with SBM and CWP develops a significant impact on microbial populations in the ileum content of broilers.

Regarding the cecum area, the population size of LABs from treatment groups/+BL inclusion involves a slow improvement ($p > 0.05$). Also, the product inclusion led to a decrease in the number of Coliforms ($p > 0.05$), *Clostridium* spp. (SBM/±BL, $p = 0.001$; CWP/−BL vs. SBM/+BL, $p = 0.002$), and *Enterococcus* spp. (SBM/± BL, $p \leq 0.05$). The inclusion of BL in bird diets involved lower microbial counts, especially for *E. coli* (SBM/−BL vs. SBM/+BL, $p = 0.001$). More significant differences were noted for *Bacillus* spp. generated by BL administration (CWP/+BL vs. CWP/−BL, $p = 0.065$; SBM/+BL vs. SBM/−BL, $p = 0.0001$) than those in SBM and CWP groups without product addition ($p > 0.05$).

The results of the feces bacterial population of broilers at 42 d are shown in Figure 2. As can be observed, the LAB and *Enterobacteriaceae* populations in all treatment groups showed similar growth rates ($p > 0.05$). Regarding the *Staphylococcus* spp., the feed supplementation with BL decreased the count number ($p = 0.0001$).

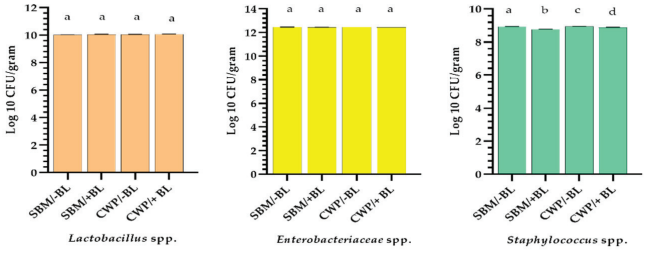

Figure 2. Effects of dietary supplementation with *Bacillus* on the fecal microbiota count of broiler chickens. Bars represent the mean SD. The use of distinct lowercase letters in the graph corresponds to the results of Tukey's post hoc comparisons.

3.5. Measurement of Intestinal pH

The inclusion of BL as a dietary probiotic resulted in higher pH values in the ileum content of broilers compared to SBM/-BL ($p > 0.05$) (Figure 3). Conversely, the pH values of CWP/−BL were significantly lower ($p < 0.05$) vs. CWP/+BL. Moreover, the pH differed between SBM groups in the cecum broiler segment ($p < 0.05$); the pH data from CWP/+BL involved a decrease vs. CWP/−BL ($p > 0.05$).

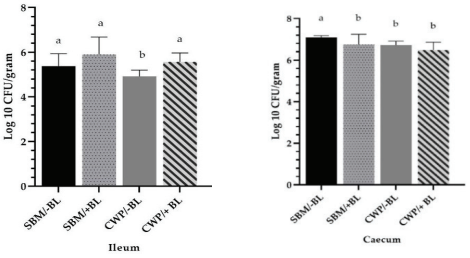

Figure 3. Effects of dietary supplementation with *Bacillus* on the intestinal pH of broiler chickens. Bars represent the mean SD. The use of distinct lowercase letters in the graph corresponds to the results of Tukey's post hoc comparisons.

4. Discussion

In recent decades, probiotics have been widely studied and incorporated into chicken feed as natural additives to support poultry health [33]. These probiotics enhance the viability of beneficial bacteria, promoting a balanced intestinal microbiota that is crucial for digestion and nutrient absorption [6,9,18,21]. Additionally, protein source supplementation has emerged as another important factor in improving poultry growth and feed efficiency [27,30]. The combined use of probiotics and protein sources may offer synergistic effects, promoting both the balance of intestinal microbiota and efficient nutrient utilization, thereby improving overall poultry health and productivity [31].

Bacillus licheniformis ATCC 21424, the strain used in this study, was selected as a potential candidate based on its properties and capabilities and was prepared to act as probiotic bacteria in broilers' diets [32]. The present study was focused on evaluating the efficacy of BL in promoting a balance of microbiota and intestinal pH values, supporting, by the end, as a natural alternative, the growth and health of broilers. By exploring these aspects, the aim was to provide valuable insights into using BL as a sustainable and effective probiotic solution for enhancing poultry health and productivity.

Cowpeas, like other legumes, are recognized for their relatively high protein content, which enhances the nutritional profile of starchy tuber-based diets. The crude protein (CP) level of 28.8% and fiber content of 5.1% found in the recent study align well with values reported in other studies in the literature, which generally range from 20% to 30% for protein content [8,34–36]. Instead, the high protein content of CWP makes them an excellent substrate for producing protein hydrolysates providing peptides and free AAs, which are essential nutrients for the growth and metabolic activity of *Bacillus* probiotics. These nutrients help the probiotics to thrive in the gut, enhancing their ability to maintain a balanced microbial population [14]. Moreover, the hydrolysates, by offering a readily available source of nitrogen and energy, promote the proliferation of *Bacillus* species, which in turn enhances digestion and nutrient absorption through enzyme production [37]. This interaction directly supports the previously mentioned benefits of spore-based probiotics, such as improved feed conversion efficiency, better growth rates, and overall enhanced health of poultry. The synergy between CWP protein hydrolysates and *Bacillus* probiotics thus contributes to maintaining gut health and boosting immune function, ultimately leading to higher productivity and better-quality meat in broiler chickens. Moreover, environmental factors, CWP variety, and growing conditions influence the chemical composition of hydrolysates, adding another dimension to their effectiveness in poultry diets [38]. In addition to their direct benefits, CWP seeds improve the overall nutritional quality of diets when combined with cereals, creating complementary protein mixtures [8]. Furthermore, as indicated in Table 2, the apparent metabolizable energy (AME) values of CWP seeds observed in the recent study are comparable to those reported in earlier literature [35]. This similarity in AME values underscores the consistency of CWP seeds as a reliable energy source in animal feed, supporting their continued use in diverse dietary formulations.

The AA composition of CWP further highlights its value as a dietary ingredient in poultry nutrition. In addition to high levels of arginine, leucine, and lysine, which are crucial for growth, immune function, and overall health, cowpea seeds also contain significant amounts of other essential amino acids (EAAs) such as valine, isoleucine, and threonine [39]. Analyzing the AA concentrations revealed that essential amino acids (EAAs) comprised 133.10% of the total AAs in CWP, while non-essential amino acids (NEAAs) accounted for 139.70%, yielding an EAA-to-NEAA ratio of 0.95. Our ratio suggests a good balance, making them a versatile protein source that can be successfully incorporated into poultry diets. Consistent with the typical characteristics of legumes, CWP was found to have high levels of arginine, leucine, and lysine (27.20%, 22.50%, and 19.20% of DM), which are essential for promoting growth, supporting immune function, and maintaining overall health in poultry [40]. However, to achieve optimal nutrition, it may be necessary to supplement these seeds with other protein sources to balance AAs such as methionine, which is relatively lower in cowpea seeds, enhancing overall dietary quality.

Previous studies have indicated that supplementing with probiotics can enhance growth performance and improve feed utilization in chickens [9,41–43]. Known for their stability and resistance to harsh environmental conditions [44], these probiotics have been shown to significantly improve the feed conversion efficiency, growth rates, bird health [8,9,41], and better-quality meat of broiler chickens [35,45].

Spore-based *Bacillus* probiotics, such as *B. licheniformis*, have been used successfully in poultry feed due to their high environmental tolerance, easy storage, and ability to enhance growth performance and optimize the microbial profile within the gastrointestinal tract (GIT) of broilers [46]. This bacterium is particularly effective due to its capacity to produce a variety of beneficial compounds [47], including enzymes, antimicrobials, and other bioactive substances that promote gut health, improving digestion and nutrient absorption [48]. Moreover, these bacteria can form endospores (spores) involving a highly resilient structure, allowing them to survive the gastric barrier and enzymatic challenges of the broiler's GIT [49]. In the present study, we observed that the BW of broilers fed with BL was improved during the overall period. Our data showed consistency with ref. [9], who found that the inclusion of *Bacillus* spp. promotes an increase in broiler performances. The absence of notable differences in BW, FI, and FCR, as presented in Table 3, aligns with findings from comparable studies investigating the effects of different types of CWP used as alternative protein sources or probiotics on broiler growth [8,41]. Instead, ref. [8] affirmed that different levels of CWP (0–20%) did not affect broiler performance, indicating that CWP can be used as a protein source and can successfully replace the SBM. Moreover, Embaye et al. [8] found that including different levels of CWP in broiler diets, up to 20%, did not negatively affect their performance. Also, probiotic addition in broiler diets containing CWP increased BW and FCR compared to the birds without probiotic inclusion. Moreover, other studies have demonstrated the efficiency of dietary *B. licheniformis* or *Bacillus subtilis* supplementation, showing that it can improve broiler growth parameters [21,50]. One hypothesis suggests that enhanced growth performance could be linked to beneficial metabolites produced by *B. licheniformis*, including its ability to produce a broad range of extracellular enzymes that enhance digestive processes. Additionally, our results showed that improvements in broilers' BW over the entire period were significantly greater in the BL supplement groups than those without probiotic inclusion. This suggests that CWP inclusion of up to 15% can be effectively used as a partial substitute for SBM in poultry diets without compromising growth, feed efficiency, or overall broiler health [9]. However, if a higher replacement level is desired, the feed formulation may need to be adjusted with AA supplementation, anti-nutritional factor reduction, and energy-balancing strategies to avoid any potential negative effects on poultry performance.

Carcass characteristics provide important indices in broiler production [51], especially when following the meat performance of broilers [41]. In our report, BL significantly decreased the abdominal fat of broiler chickens, a result that is in line with other studies [9,51,52]. Moreover, abdominal fat is an important index used to measure lipid

deposition in broilers [53]. The addition of BL to broiler diets did not significantly alter the breast and leg yield compared to diets without probiotics. According to our data, Sarangi et al. [54] reported that the percentage of carcass yield in broiler chickens did not significantly increase with probiotic inclusion compared to the control group. Various other studies showed that *Bacillus* spp. or other probiotic products involve beneficial effects on different organ weights [21,43,55]. Our results showed that BL inclusion was more effective on birds' cecum weight.

Introducing *Bacillus* species into broiler diets can positively influence bone mineralization, which is essential for maintaining skeletal health and preventing leg disorders [56]. *Bacillus* species produce enzymes like phytases [57] that enhance the bioavailability of minerals such as Ca and P, which are vital for the growth and development of broilers, particularly in bone formation and mineralization. The enhanced bioavailability and distribution of these minerals, facilitated by the enzymatic activity of *Bacillus* bacteria, contribute to stronger and healthier bone structures. This aspect is very important for bone tibial growth, a key indicator of skeletal health in broilers [58]. In the current research, the inclusion of BL improved several bone quality traits such as increased Fe, P, and Zn content, and a reduction in the Ca:P ratio. Further, the Ca:P ratio plays a crucial role in broiler health, working synergically to create strong bone development, skeletal integrity, and overall metabolic function. If the Ca is in excess, P absorption can be impaired, which can lead to chondroplasia and/or osteoporosis in broiler chickens, as shown by Liu et al. 2023 [59]. Conversely, if the Ca:P ratio is deficient (excess P), Ca may not be adequately available for bone mineralization, causing conditions like rickets, where bones become soft and brittle, due to the disorders in the body's mineral metabolism [59]. These improvements could result from the probiotic's ability to secrete exogenous enzymes. Consistent with our findings, several studies have reported that dietary probiotic supplements containing *Bacillus* spp. benefit tibial bone growth [9,56,60]. The inclusion of *Bacillus* in animal diets promotes the secretion of exogenous enzymes, like phytases and proteases, that increase mineral availability. These enzymes are important in breaking down phytic acid and anti-nutritional factors commonly present in plant-based feeds [61]. When *Bacillus* species produce phytases, they break down phytic acid, freeing up Ca and P so they can be absorbed more effectively in the gut. Once absorbed, Ca and P are essential for bone mineralization and strength, particularly in the tibia, a key bone for structural integrity in broilers. Consistently, the addition of BL leads to better bone density, stronger tibial structures, and overall bone development in broilers.

Establishing microbial equilibrium within the GIT is crucial for maintaining optimal digestive function and effectively controlling potentially pathogenic microorganisms in the intestine of the host [62]. Each section of the GIT provides unique niches, with distinct environmental conditions like pH, oxygen availability, and nutrient composition, which shape the microbiota composition. For example, the inclusion of spore-forming probiotics (like *Bacillus* species) in poultry diets can significantly impact bacteria communities, potentially enhancing health, growth performance, and feed efficiency [63]. In the present study, it was found that 1×10^{11} CFUs of BL-ATCC 21424/g feed for 42 days provided beneficial effects on the concentration of LABs in the ileum content of both corn wheat protein (CWP) and soybean meal (SBM) groups. Several studies [33,41,44] have demonstrated that the inclusion of probiotics based on *Bacillus* spp. can significantly increase the proliferation of lactobacilli in the intestinal content of poultry. This positive effect is attributed to the ability of *Bacillus*-based probiotics and *Bacillus* species-fermented products to produce catalase enzyme, which helps create a more favorable environment for lactobacilli growth (10^6 to 10^8 CFU/g of intestinal content). This level of lactobacilli increase promotes better intestinal health by enhancing pathogen inhibition and nutrient absorption, leading to stronger immune responses and overall improved performance in broilers [64,65]. Additionally, the presence of lactobacilli supports balanced gut microbiota by outcompeting harmful bacteria, helping to maintain intestinal stability and prevent infections [66]. The diet supplemented with BL exerted a decrease in harmful bacteria. So, a balanced intestinal microbiota is es-

sential for maintaining the structural integrity of the intestinal lining. *Coliforms*, *Clostridium* spp., *E. coli* β-hemolytic, and *Enterococcus* spp. developed a lower growth, especially in the groups with BL diet inclusions. Our study's findings align closely with recent research that demonstrates the positive impact of probiotic supplementation on the gut microbiota of broilers [62,67]. Probiotic inclusion, particularly involving spore-forming bacteria, has been shown to significantly increase the population of viable LABs while concurrently reducing the number of pathogenic microorganisms in the animal GIT [67].

The cecum is an essential intestinal organ in broilers, hosting the most abundant and concentrated intestinal microbiota, which plays critical roles in various physiological and metabolic functions [68]. It is a key site for biological fermentation processes, including the production of short-chain fatty acids (SCFAs). Additionally, the gut microbiota in the cecum can ferment feed through diverse pathways, leading to the production of a range of metabolites [21]. In our study, the abundance of *Clostridium* spp. and *Enterococcus* spp. in the cecum area was significantly decreased by BL addition in the SBM/+BL treatment group. This is in accordance with previous studies that reported similar outcomes in broiler diets [44,69,70]. It has been demonstrated that disruptions to the normal intestinal microbiota can be restored with probiotic administration, which also helps animals resist infections by pathogenic bacteria [71]. Furthermore, previous studies found that *Bacillus licheniformis* can inhibit the proliferation of harmful bacteria by secreting some antimicrobial peptides or inhibiting biofilm formation [72,73]. The findings of the current research on intestinal microbiota populations showed that the dynamics of microorganisms are affected by the addition of BL as a probiotic in broiler diets, which significantly reduces the growth of pathogens. Once they reach the intestine, these spores germinate and colonize the gut effectively, establishing a beneficial microbial community that supports digestive health and inhibits the growth of pathogenic bacteria. This colonization not only enhances nutrient absorption and growth performance but also contributes to the overall health and well-being of the broilers, leading to improved production outcomes in poultry farming.

Dietary treatments did not involve a notable impact on the count of Lactobacilli and *Enterobacteriaceae* in broiler feces. Similarly, other probiotics did not have significant modulation on LAB count in broiler chickens [74]. Instead, the inclusion of BL in broiler diets involved a more pronounced effect on the size of *Staphylococcus* spp. in SBM and CWP groups at 42 d. These findings align with studies in the literature indicating that including *Bacillus* probiotics in broiler diets can reduce the number of *E. coli*, *Salmonella*, *Clostridium*, and *Campylobacter* counts, ultimately inhibiting their growth, followed by reducing the incidence of infections [75–78]. Also, all samples were found to be negative for *Salmonella* spp.

The pH of the poultry GIT is a dynamic characteristic influenced by multiple factors, such as feed composition, endogenous secretions (e.g., gastric acid, bile salts, pancreatic enzymes), and the gut microbiome [79]. The pH levels vary significantly across different sections of the GIT, ranging from 2.6 in the gizzard to 6.3 in the large intestine [80]. These variations are not only a normal physiological feature but also play a crucial role in the health and functionality of the gut, as many pathogens are highly sensitive to low pH levels. This sensitivity directly impacts their survival and proliferation, emphasizing the importance of maintaining appropriate pH conditions within the GIT. Our findings align with these observations and highlight the role of *Bacillus*-based probiotics (BL) in modulating pH levels in different parts of the poultry GIT. Specifically, the results indicate that the addition of *Bacillus*-based probiotics to diets containing SBM and CWP increased the pH in the ileum by 10% to 13% compared to groups without supplementation. This increase in ileum pH could be attributed to the metabolic activities of *Bacillus* spp. which may alter the fermentation profile and microbial composition within this segment, leading to a shift in pH. Conversely, a different effect was observed in the cecum, where the same *Bacillus*-based probiotic treatments resulted in a gradual pH reduction of 3% to 5%. This pH decrease can be explained by the production of lactic acid and other organic acids by *Bacillus* species during fermentation. The production of these acids lowers the pH of the intestinal

environment, creating conditions that inhibit the growth of pathogenic bacteria and favor the proliferation of beneficial microbiota, such as Lactobacilli [81]. This shift in microbial populations can improve gut integrity, immune function, and nutrient utilization over time. In terms of long-term effects, maintaining a stable and favorable gut environment through pH modulation has been linked to improved FCR, reduced disease incidence, and enhanced overall productivity in broilers. By lowering pathogenic load and improving nutrient absorption, these pH changes contribute to better growth rates, healthier flocks, and higher-quality meat production. Thus, while pH changes alone may seem minor, they are integral to sustaining long-term gut health and productivity in broilers. The ability of probiotics to manage pH levels and reduce the population of harmful pathogens is a critical aspect of maintaining a balanced gut microbiome. By enhancing the gut's natural acidity, probiotics create an environment less favorable for pathogenic bacteria such as *E. coli* and more conducive to beneficial bacteria like LABs [82]. Although *Bacillus licheniformis*, a commonly used probiotic, is not a native component of the chicken's intestinal flora and does not colonize the GIT for extended periods, it quickly consumes oxygen and reduces pH levels. The findings of our study suggest that managing the pH levels within the poultry GIT through targeted dietary strategies, including the use of probiotics, is essential for promoting gut health and reducing the risk of infections. However, further research is needed to fully understand the mechanisms by which probiotics influence pH in various sections of the GIT and to determine the optimal types and dosages of probiotics for specific dietary formulations. Additionally, studies should explore the long-term effects of such dietary interventions on poultry health and productivity. In conclusion, our study supports the use of *Bacillus*-based probiotics as an effective strategy for modulating gut pH and promoting a healthier gut environment in poultry. By creating conditions that inhibit pathogens and support beneficial microbiota, these probiotics can play a significant role in improving overall gut health and potentially enhancing poultry performance.

5. Conclusions

Cowpea seeds (*V. unguiculata* [L.] Walp, cv. Aura) can effectively serve as an alternative protein source to replace SBM in broiler chick diets, with inclusion levels of up to 15% showing no negative effects on bird growth. Cowpea is an important source of nutrients (AAs such as lysine and tryptophan) and bioactive compounds, such as fiber. The addition of *B. licheniformis* ATCC 21424 (10 Log10 CFUs) improved GP, which was associated with a slight increase in FI and a reduction in abdominal fat. The probiotic affected bone mineralization by increasing tibia Fe and P content. Additionally, the inclusion of BL positively influenced the GIT microbiota by promoting the growth of beneficial bacteria (*Lactobacillus* and *Bacillus* spp.), while suppressing potential pathogens (*E. coli* and Coliforms). Considering these findings, supplementation with *BL* demonstrates promising potential as a probiotic in poultry feed by enhancing growth performance, supporting bone mineralization, and positively impacting the microbiota profile.

Author Contributions: Conceptualization, M.D. and G.C.; methodology, M.D. and G.C.; software, M.D., N.A.L. and G.C.; validation, M.D. and G.C.; formal analysis, M.D., N.A.L. and G.C.; investigation, M.D., G.C. and R.D.; resources, M.D., R.D. and G.C.; data curation M.D.; writing—original draft preparation, M.D.; writing—review and editing, M.D.; project administration, M.D. and G.C.; funding acquisition, M.D. and G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Romanian Ministry of Agriculture and Rural Development (Project ADER 8.1.7) and the Ministry of Research, Innovation, and Digitalization (Project PN23-20.04.01).

Institutional Review Board Statement: This study was conducted according to the experimental protocol (no. 5183/08.05.2018) approved by the Ethics Commission of the National Research–Development Institute for Animal Biology and Nutrition and complied with European Directive (2010/63/EU) and Law 43/04.2014 on the protection of animals used for scientific purposes.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Muscat, A.; De Olde, E.M.; de Boer, I.J.; Ripoll-Bosch, R. The battle for biomass: A systematic review of food-feed-fuel competition. *Glob. Food Secur.* **2019**, *25*, 100330. [CrossRef]
2. Zhai, W.; Peebles, E.D.; Zumwalt, C.D.; Mejia, L.; Corzo, A. Effects of dietary amino acid density regimens on growth performance and meat yield of Cobb × Cobb 700 broilers. *J. Appl. Poult. Res.* **2013**, *22*, 447–460. [CrossRef]
3. Maidala, A.; Dass, Y.H. Utilization of cowpea seeds (*Vigna Unguiculata* (L) Walp) by Broiler chickens: An overview. *Res. J. Food Sci. Qual. Control* **2017**, *3*, 50–56.
4. Bumhira, E.; Madzimure, J. Effect of incorporating Cowpea (*Vigna unguiculata*) Meal into broiler diets on environmental pollution by nitrogen from poultry excreta: A Review. *J. Agric. Chem. Environ.* **2023**, *12*, 84–92. [CrossRef]
5. Ciurescu, G.; Idriceanu, L.; Gheorghe, A.; Ropotă, M.; Drăghici, R. Meat quality in broiler chickens fed on cowpea (*Vigna unguiculata* [L.] Walp) seeds. *Sci. Rep.* **2022**, *12*, 9685. [CrossRef] [PubMed]
6. Kana, J.R.; Teguia, A.; Fomekong, A. Effect of substituting soybean meal with cowpea (*Vigna unguiculata* WAL) supplemented with natural plant charcoals in broiler diet on growth performances and carcass characteristics. *Iran. J. Appl. Anim. Sci.* **2012**, *2*, 377–381.
7. Abdelgani, A.A.; Abdelatti, K.A.; Elamin, K.M.; Dafalla, K.Y.; Malik, H.; Dousa, B.M. Effect of dietary cowpea (*Vigna unguiculata*) seeds on the performance of broiler chicks. *Wayamba J. Anim. Sci.* **2013**, *1*, 678–684.
8. Embaye, T.N.; Ameha, N.; Yusuf, Y. Effect of cowpea (*Vigna unguiculata*) grain on growth performance of Cobb 500 broiler chickens. *Int. J. Livest. Prod.* **2018**, *9*, 326–333. [CrossRef]
9. Ciurescu, G.; Dumitru, M.; Gheorghe, A.; Untea, A.E.; Drăghici, R. Effect of *Bacillus subtilis* on growth performance, bone mineralization, and bacterial population of broilers fed with different protein sources. *Poult. Sci.* **2020**, *99*, 5960–5971. [CrossRef]
10. Soul, W. Effects of *Vigna unguiculata* and *Lablab purpureus* on Methanogenesis, Haematological Parameters and the Quality of Meat from Xhosa Lop Ear Goats. Ph.D. Thesis, University of Fort Hare, Alice, South Africa, 17 April 2024.
11. Pottorff, M.; Ehlers, J.D.; Fatokun, C.; Roberts, P.A.; Close, T.J. Leaf morphology in Cowpea [*Vigna unguiculata* (L.) Walp]: QTL analysis, physical mapping and identifying a candidate gene using synteny with model legume species. *BMC Genom.* **2012**, *13*, 234. [CrossRef]
12. Gutema, T.; Tolesa, G.N. Effects of traditional processing techniques on nutritional quality and sensory acceptability of value-added products made from cowpea (*Vigna unguiculata* L. walp.) produced in Ethiopia. *J. Food Nutr. Res.* **2024**, *8*, 32–43. [CrossRef]
13. Akuru, E.A.; Ani, A.O.; Orji, J.A.; Oyeagu, C.E.; Osita, C.O.; Idamokoro, M.E.; Ogwuegbu, M.C.; Lewu, F.B. Nutrient digestibility, growth, carcass, and bio-marker traits of weaner rabbits fed diets containing graded levels of cowpea (*Vigna unguiculata*) hull meal. *J. Appl. Anim. Res.* **2021**, *49*, 39–45. [CrossRef]
14. Tolba, S.A.; Amer, S.A.; Gouda, A.; Osman, A.; Sherief, W.R.; Ahmed, A.I.; El-Rahmanf, G.I.A.; Abdel-Warith, A.-W.A.; Younis, E.M.; Davies, S.J.; et al. Potential use of cowpea protein hydrolysate as a dietary supplement in broiler chickens: Effects on growth, intestinal morphology, muscle lipid profile, and immune status. *Ital. J. Anim. Sci.* **2023**, *22*, 1204–1218. [CrossRef]
15. Asare, A.T.; Agbemafle, R.; Adukpo, G.E.; Diabor, E.; Adamtey, K.A. Assessment of functional properties and nutritional composition of some cowpea (*Vigna unguiculata* L.) genotypes in Ghana. *J. Appl. Behav. Sci.* **2013**, *8*, 465–469.
16. Baptista, A.; Pinho, O.; Pinto, E.; Casal, S.; Mota, C.; Ferreira, I.M. Characterization of protein and fat composition of seeds from common beans (*Phaseolus vulgaris* L.), cowpea (*Vigna unguiculata* L. Walp) and bambara groundnuts (*Vigna subterranea* L. Verdc) from Mozambique. *J. Food Meas. Charact.* **2017**, *11*, 442–450. [CrossRef]
17. Duranti, M. Grain legume proteins and nutraceutical properties. *Fitoterapia* **2006**, *77*, 67–82. [CrossRef]
18. Dumitru, M.; Hăbeanu, M.; Sorescu, I.; Tabuc, C. Effects of *Bacillus* spp. as a supplemental probiotic in diets for weaned piglets. *S. Afr. J. Anim. Sci.* **2021**, *51*, 578–586. [CrossRef]
19. Naghibi, F.; Aliakbarpour, H.R.; Rezaeipour, V. Effects of different sources of probiotics on performance, carcass, intestinal morphology and bone characteristics in broiler chickens. *Iran. J. Appl. Anim. Sci.* **2023**, *13*, 535–543.
20. Aziz Mousavi, S.M.A.; Mahmoodzadeh Hosseini, H.; Mirhosseini, S. A review of dietary probiotics in poultry. *J. Appl. Biotechnol. Rep.* **2018**, *5*, 48–54. [CrossRef]
21. Xu, Y.; Yu, Y.; Shen, Y.; Li, Q.; Lan, J.; Wu, Y.; Zhang, R.; Cao, G.; Yang, C. Effects of *Bacillus subtilis* and *Bacillus licheniformis* on growth performance, immunity, short chain fatty acid production, antioxidant capacity, and cecal microflora in broilers. *Poult. Sci.* **2021**, *100*, 101358. [CrossRef]
22. Alagawany, M.; Abd El-Hack, M.E.; Farag, M.R.; Sachan, S.; Karthik, K.; Dhama, K. The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. *Environ. Sci. Pollut. Res.* **2018**, *25*, 10611–10618. [CrossRef] [PubMed]
23. Santacroce, L.; Charitos, I.A.; Bottalico, L. A successful history: Probiotics and their potential as antimicrobials. *Expert Rev. Anti-Infect. Ther.* **2019**, *17*, 635–645. [CrossRef] [PubMed]

24. Czech, A.; Merska-Kazanowska, M.; Całyniuk, Z. Redox status, biochemical parameters and mineral elements content in blood of turkey hens fed a diet supplemented with *Yarrowia lipolytica* yeast and two *Bacillus* species. *Animals* **2020**, *10*, 459. [CrossRef] [PubMed]
25. Payne, J.; Bellmer, D.; Jadeja, R.; Muriana, P. The Potential of *Bacillus* species as probiotics in the food industry: A Review. *Foods* **2024**, *13*, 2444. [CrossRef]
26. Hollensteiner, J.; Wemheuer, F.; Harting, R.; Kolarzyk, A.M.; Diaz Valerio, S.M.; Poehlein, A.; Brzuszkiewicz, E.B.; Nesemann, K.; Braus-Stromeyer, S.A.; Braus, G.H.; et al. *Bacillus thuringiensis* and *Bacillus weihenstephanensis* inhibit the growth of phytopathogenic *Verticillium* species. *Front. Microbiol.* **2017**, *7*, 2171. [CrossRef]
27. Ciurescu, G.; Toncea, I.; Ropota, M.; Habeanu, M. Seeds composition and their nutrients quality of some pea (*Pisum sativum* L.) and lentil (*Lens culinaris* medik.) cultivars. *Rom. Agric. Res.* **2018**, *35*, 101–108. [CrossRef]
28. Dumitru, M.; Sorescu, I.; Habeanu, M.; Tabuc, C.; Jurcoane, Ș. Preliminary characterization in vitro of *Bacillus licheniformis* strain for used as a dietary probiotic. *Sci. Bull. Ser. F Biotechnol.* **2019**, *23*, 164–172.
29. Valdebouze, P.; Bergeron, E.; Gaborit, T.; Delort-Laval, J. Content and distribution of trypsin inhibitors and haemaglutinins in some legume seeds. *Can. J. Plant Sci.* **1980**, *60*, 695–701. [CrossRef]
30. Elleithy, E.M.; Bawish, B.M.; Kamel, S.; Ismael, E.; Bashir, D.W.; Hamza, D.; Fahmy, K.N.E.D. Influence of dietary *Bacillus coagulans* and/or *Bacillus licheniformis*-based probiotics on performance, gut health, gene expression, and litter quality of broiler chickens. *Trop. Anim. Health Prod.* **2023**, *55*, 38. [CrossRef]
31. Ren, H.; Vahjen, W.; Dadi, T.; Saliu, E.M.; Boroojeni, F.G.; Zentek, J. Synergistic effects of probiotics and phytobiotics on the intestinal microbiota in young broiler chicken. *Microorganisms* **2019**, *7*, 684. [CrossRef]
32. Dumitru, M.; Sorescu, I.; Hăbeanu, M.; Tabuc, C.; Idriceanu, L.; Jurcoane, S. Preliminary characterisation of *Bacillus subtilis* strain use as a dietary probiotic bio-additive in weaning piglet. *Food Feed. Res.* **2018**, *45*, 203–211. [CrossRef]
33. Al-Khalaifa, H.; Al-Nasser, A.; Al-Surayee, T.; Al-Kandari, S.; Al-Enzi, N.; Al-Sharrah, T.; Ragheb, G.; Al-Qalaf, S.; Mohammed, A. Effect of dietary probiotics and prebiotics on the performance of broiler chickens. *Poult. Sci.* **2019**, *98*, 4465–4479. [CrossRef] [PubMed]
34. Abdel-Shafi, S.; Al-Mohammadi, A.R.; Osman, A.; Enan, G.; Abdel-Hameid, S.; Sitohy, M. Characterization and antibacterial activity of 7S and 11S globulins isolated from cowpea seed protein. *Molecules* **2019**, *24*, 1082. [CrossRef] [PubMed]
35. Ciurescu, G.; Vasilachi, A.; Ropotă, M. Effect of dietary cowpea (*Vigna unguiculata* [L] walp) and chickpea (*Cicer arietinum* L.) seeds on growth performance, blood parameters and breast meat fatty acids in broiler chickens. *Ital. J. Anim. Sci.* **2022**, *21*, 97–105. [CrossRef]
36. Manole, M.; Ciurescu, G.; Dumitru, M. The use of cowpeas ([L] Walp) in poultry diets: A review. *Arch. Zootech.* **2024**, *27*, 23–47. [CrossRef]
37. Gómez, A.; Gay, C.; Tironi, V.; Avanza, M.V. Structural and antioxidant properties of cowpea protein hydrolysates. *Food Biosci.* **2021**, *41*, 101074. [CrossRef]
38. Mfeka, N.; Mulidzi, R.A.; Lewu, F.B. Growth and yield parameters of three cowpea (*Vigna unguiculata* L. Walp) lines as affected by planting date and zinc application rate. *S. Afr. J. Sci.* **2019**, *115*, 1–9. [CrossRef]
39. Affrifah, N.S.; Phillips, R.D.; Saalia, F.K. Cowpeas: Nutritional profile, processing methods and products—A review. *Legum. Sci.* **2022**, *4*, e131. [CrossRef]
40. Abebe, B.K.; Alemayehu, M.T. A review of the nutritional use of cowpea (*Vigna unguiculata* L. Walp) for human and animal diets. *J. Agric. Food Res.* **2022**, *10*, 100383. [CrossRef]
41. Lefter, N.A.; Gheorghe, A.; Habeanu, M.; Ciurescu, G.; Dumitru, M.; Untea, A.E.; Vlaicu, P.A. Assessing the effects of microencapsulated *Lactobacillus salivarius* and cowpea seed supplementation on broiler chicken growth and health status. *Front. Vet. Sci.* **2023**, *10*, 1279819. [CrossRef]
42. Ahmat, M.; Cheng, J.; Abbas, Z.; Cheng, Q.; Fan, Z.; Ahmad, B.; Zhang, R. Effects of *Bacillus amyloliquefaciens* LFB112 on growth performance, carcass traits, immune, and serum biochemical response in broiler chickens. *Antibiotics* **2021**, *10*, 1427. [CrossRef] [PubMed]
43. Abed, A.H.; Radwan, I.A.; Orabi, A.; Abdelaziz, K. The combined effects of probiotic CLOSTAT and Aviboost supplement on growth performance, intestinal morphology, and immune response of broiler chickens. *Ger. J. Vet. Res.* **2023**, *3*, 7–18. [CrossRef]
44. Dumitru, M.; Vodnar, D.C.; Elemer, S.; Ciurescu, G.; Habeanu, M.; Sorescu, I.; Georgescu, S.E.; Dudu, A. Evaluation of non-encapsulated and microencapsulated lactic acid bacteria. *Appl. Sci.* **2021**, *11*, 9867. [CrossRef]
45. Neveling, D.P.; Dicks, L.M.T. Probiotics: An antibiotic replacement strategy for healthy broilers and productive rearing. *Probiotics Antimicrob. Proteins* **2021**, *13*, 1–11. [CrossRef] [PubMed]
46. Bromfield, J.I.; Niknafs, S.; Chen, X.; von Hellens, J.; Horyanto, D.; Sun, B.; Yu, L.; Tran, V.H.; Navarro, M.; Roura, E. The evaluation of next-generation probiotics on broiler growth performance, gut morphology, gut microbiome, nutrient digestibility, in addition to enzyme production of *Bacillus* spp. in vitro. *Anim. Nutr.* **2024**, *18*, 133–144. [CrossRef]
47. Rambu, D.; Dumitru, M.; Ciurescu, G.; Vamanu, E. Solid-state fermentation using *Bacillus licheniformis*-driven changes in composition, viability and in vitro protein digestibility of oilseed cakes. *Agriculture* **2024**, *14*, 639. [CrossRef]
48. Saggese, A.; Baccigalupi, L.; Ricca, E. Spore formers as beneficial microbes for humans and animals. *Appl. Microbiol.* **2021**, *1*, 498–509. [CrossRef]

49. Fakhry, S.; Sorrentini, I.; Ricca, E.; De Felice, M.; Baccigalupi, L. Characterization of spore forming Bacilli isolated from the human gastrointestinal tract. *J. Appl. Microbiol.* **2008**, *105*, 2178–2186. [CrossRef]
50. Sokale, A.O.; Menconi, A.; Mathis, G.F.; Lumpkins, B.; Sims, M.D.; Whelan, R.A.; Doranalli, K. Effect of *Bacillus subtilis* DSM 32315 on the intestinal structural integrity and growth performance of broiler chickens under necrotic enteritis challenge. *Poult. Sci.* **2019**, *98*, 5392–5400. [CrossRef]
51. Tang, X.; Liu, X.; Liu, H. Effects of dietary probiotic (*Bacillus subtilis*) supplementation on carcass traits, meat quality, amino acid, and fatty acid profile of broiler chickens. *Front. Vet. Sci.* **2021**, *8*, 767802. [CrossRef]
52. Khajeh Bami, M.; Afsharmanesh, M.; Ebrahimnejad, H. Effect of dietary *Bacillus coagulans* and different forms of zinc on performance, intestinal microbiota, carcass and meat quality of broiler chickens. *Probiotics Antimicrob. Proteins* **2020**, *12*, 461–472. [CrossRef] [PubMed]
53. Wan, X.; Yang, Z.; Ji, H.; Li, N.; Yang, Z.; Xu, L.; Yang, H.; Wang, Z. Effects of lycopene on abdominal fat deposition, serum lipids levels and hepatic lipid metabolism-related enzymes in broiler chickens. *Anim. Biosci.* **2021**, *34*, 385–392. [CrossRef] [PubMed]
54. Sarangi, N.R.; Babu, L.K.; Kumar, A.; Pradhan, C.R.; Pati, P.K.; Mishra, J.P. Effect of dietary supplementation of prebiotic, probiotic, and synbiotic on growth performance and carcass characteristics of broiler chickens. *Vet. World* **2016**, *9*, 313–319. [CrossRef] [PubMed]
55. Park, J.H.; Kim, I.H. Supplemental effect of probiotic *Bacillus subtilis* B2A on productivity, organ weight, intestinal *Salmonella* microflora, and breast meat quality of growing broiler chicks. *Poult. Sci.* **2014**, *93*, 2054–2059. [CrossRef]
56. Mohammed, A.A.; Zaki, R.S.; Negm, E.A.; Mahmoud, M.A.; Cheng, H.W. Effects of dietary supplementation of a probiotic (*Bacillus subtilis*) on bone mass and meat quality of broiler chickens. *Poult. Sci.* **2021**, *100*, 100906. [CrossRef]
57. Latorre, J.D.; Hernandez-Velasco, X.; Wolfenden, R.E.; Vicente, J.L.; Wolfenden, A.D.; Menconi, A.; Bielke, L.R.; Hargis, B.M.; Tellez, G. Evaluation and selection of *Bacillus* species based on enzyme production, antimicrobial activity, and biofilm synthesis as direct-fed microbial candidates for poultry. *Front. Vet. Sci.* **2016**, *3*, 1–9. [CrossRef]
58. Moyo, S.; Jaja, I.F.; Mopipi, K.; Masika, P.; Muchenje, V. Effect of graded levels of Imbrasia belina meal on blood lipid profile, bone morphometric and mineral content of broiler chickens. *Anim. Feed. Sci. Technol.* **2021**, *271*, 114736. [CrossRef]
59. Liu, K.L.; He, Y.F.; Xu, B.W.; Lin, L.X.; Chen, P.; Iqbal, M.K.; Mehmood, K.; Huang, S.C. Leg disorders in broiler chickens: A review of current knowledge. *Anim. Biotechnol.* **2023**, *34*, 5124–5138. [CrossRef]
60. Rizzoli, R.; Biver, E. Are probiotics the new calcium and vitamin D for bone health? *Curr. Osteoporos. Rep.* **2020**, *18*, 273–284. [CrossRef]
61. Rizwanuddin, S.; Kumar, V.; Naik, B.; Singh, P.; Mishra, S.; Rustagi, S.; Kumar, V. Microbial phytase: Their sources, production, and role in the enhancement of nutritional aspects of food and feed additives. *J. Agric. Res.* **2023**, *12*, 100559. [CrossRef]
62. Song, J.; Xiao, K.; Ke, Y.L.; Jiao, L.F.; Hu, C.H.; Diao, Q.Y.; Zou, X.T. Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poult. Sci.* **2014**, *93*, 581–588. [CrossRef] [PubMed]
63. Yadav, S.; Jha, R. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 2. [CrossRef] [PubMed]
64. Bonos, E.; Giannenas, I.; Sidiropoulou, E.; Stylianaki, I.; Tzora, A.; Skoufos, I.; Barbe, F.; Demey, V.; Christaki, E. Effect of *Bacillus pumilus* supplementation on performance, intestinal morphology, gut microflora and meat quality of broilers fed different energy concentrations. *Anim. Feed Sci. Technol.* **2021**, *274*, 114859. [CrossRef]
65. Chen, J.Y.; Yu, Y.H. *Bacillus subtilis*-fermented products ameliorate the growth performance and alter cecal microbiota community in broilers under lipopolysaccharide challenge. *Poult. Sci.* **2021**, *100*, 875–886. [CrossRef]
66. Ma, T.; Shen, X.; Shi, X.; Sakandar, H.A.; Quan, K.; Li, Y.; Jin, H.; Kwok, L.Y.; Zhang, H.; Sun, Z. Targeting gut microbiota and metabolism as the major probiotic mechanism-An evidence-based review. *Trends Food Sci. Technol.* **2023**, *138*, 178–198. [CrossRef]
67. Fang, S.; Fan, X.; Xu, S.; Gao, S.; Wang, T.; Chen, Z.; Li, D. Effects of dietary supplementation of postbiotic derived from *Bacillus subtilis* ACCC 11025 on growth performance, meat yield, meat quality, excreta bacteria, and excreta ammonia emission of broiler chicks. *Poult. Sci.* **2024**, *103*, 103444. [CrossRef]
68. Kamada, N.; Kim, Y.G.; Sham, H.P.; Vallance, B.A.; Puente, J.L.; Martens, E.C.; Núñez, G. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. *Science* **2012**, *336*, 1325–1329. [CrossRef]
69. Mountzouris, K.C.; Tsirtsikos, P.; Kalamara, E.; Nitsch, S.; Schatzmayr, G.; Fegeros, K. Evaluation of the efficacy of a probiotic containing *Lactobacillus, Bifidobacterium, Enterococcus,* and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult. Sci.* **2007**, *86*, 309–317. [CrossRef]
70. Park, J.H.; Yun, H.M.; Kim, I.H. The effect of dietary *Bacillus subtilis* supplementation on the growth performance, blood profile, nutrient retention, and caecal microflora in broiler chickens. *J. Appl. Anim. Res.* **2018**, *46*, 868–872. [CrossRef]
71. Martin, F.P.J.; Wang, Y.; Sprenger, N.; Yap, I.K.S.; Lundstedt, T.; Lek, P.; Rezzi, S.; Ramadan, Z.; Nicholson, J.K. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Mol. Syst. Biol.* **2008**, *4*, 157. [CrossRef]
72. Lin, Y.; Xu, S.; Zeng, D.; Ni, X.; Zhou, M.; Zeng, Y.; Li, G. Disruption in the cecal microbiota of chickens challenged with *Clostridium perfringens* and other factors was alleviated by *Bacillus licheniformis* supplementation. *PLoS ONE* **2017**, *12*, 182426. [CrossRef] [PubMed]

73. Arif, M.; Akteruzzaman, M.; Islam, S.S.; Das, B.C.; Siddique, M.P.; Kabir, S.L. Dietary supplementation of *Bacillus*-based probiotics on the growth performance, gut morphology, intestinal microbiota and immune response in low biosecurity broiler chickens. *Vet. Anim. Sci.* **2021**, *14*, 100216. [CrossRef] [PubMed]
74. Hahn-Didde, D.; Purdum, S.E. Prebiotics and probiotics used alone or in combination and effects on pullet growth and intestinal microbiology. *J. Appl. Poult. Res.* **2016**, *25*, 1–11. [CrossRef]
75. Zhang, L.; Cao, G.T.; Zeng, X.F.; Zhou, L.; Ferket, P.R.; Xiao, Y.P.; Chen, A.G.; Yang, C.M. Effects of *Clostridium butyricum* on growth performance, immune function, and cecal microflora in broiler chickens challenged with *Escherichia coli* K88. *Poult. Sci.* **2014**, *93*, 46–53. [CrossRef]
76. Ding, J.; Dai, R.; Yang, L.; He, C.; Xu, K.; Liu, S.; Zhao, W.; Xiao, L.; Luo, L.; Zhang, Y.; et al. Inheritance and Establishment of gut microbiota in chickens. *Front. Microbiol.* **2017**, *8*, 1967. [CrossRef]
77. Castañeda, C.D.; Gamble, J.N.; Wamsley, K.G.S.; McDaniel, C.D.; Kiess, A.S. In ovo administration of *Bacillus subtilis* serotypes effect hatchability, 21-day performance, and intestinal microflora. *Poult. Sci.* **2021**, *100*, 101125. [CrossRef]
78. Luise, D.; Bosi, P.; Raff, L.; Amatucci, L.; Virdis, S.; Trevisi, P. *Bacillus* spp. probiotic strains as a potential tool for limiting the use of antibiotics, and improving the growth and health of pigs and chickens. *Front. Microbiol.* **2022**, *13*, 801827. [CrossRef]
79. Reis, M.P.; Fassani, E.J.; Júnior, A.G.; Rodrigues, P.B.; Bertechini, A.G.; Barrett, N.; Persia, M.E.; Schmidt, C.J. Effect of *Bacillus subtilis* (DSM 17299) on performance, digestibility, intestine morphology, and pH in broiler chickens. *J. Appl. Poult. Res.* **2017**, *26*, 573–583. [CrossRef]
80. Svihus, B. Function of the digestive system. *J. Appl. Poult. Res.* **2014**, *23*, 306–314. [CrossRef]
81. Wang, J.; Yao, L.; Su, J.; Fan, R.; Zheng, J.; Han, Y. Effects of *Lactobacillus plantarum* and its fermentation products on growth performance, immune function, intestinal pH, and cecal microorganisms of Lingnan yellow chicken. *Poult. Sci.* **2023**, *102*, 102610. [CrossRef]
82. Wu, B.Q.; Zhang, T.; Guo, L.Q.; Lin, J.F. Effects of *Bacillus subtilis* KD1 on broiler intestinal flora. *Poult. Sci.* **2011**, *90*, 2493–2499. [CrossRef]

agriculture

MDPI

Article

Supplemental Xylooligosaccharide Attenuates Growth Retardation and Intestinal Damage in Broiler Chickens Challenged by Avian Pathogenic *Escherichia coli*

Lulu Ren †, Qingyun Cao †, Hui Ye, Zemin Dong, Changming Zhang, Dingyuan Feng, Jianjun Zuo * and Weiwei Wang *

Guangdong Provincial Key Laboratory of Animal Nutrition Control, College of Animal Science, South China Agricultural University, No. 483 of Wushan Road, Guangzhou 510642, China; deermany@163.com (L.R.)

* Correspondence: zuoj@scau.edu.cn (J.Z.); wangweiwei@scau.edu.cn (W.W.); Tel.: +86-135-7046-4890 (J.Z.); +86-180-4652-4795 (W.W.)

† These authors contributed equally to this work.

Abstract: This study was conducted to investigate the protective effects of xylooligosaccharide (XOS) on the growth performance and intestinal health of broilers challenged by avian pathogenic *Escherichia coli* (APEC). A total of 144 newly hatched male Lingnan yellow-feathered broilers were randomly divided into three groups (six replicates/group): a control (CON) group, an APEC group and an XOS group (APEC-challenged broilers supplemented with 1600 mg/kg XOS). Birds in the APEC and XOS groups were orally challenged with APEC from 7 to 12 d of age. Growth performance and intestinal health-related parameters were determined on d 13 and 17. The reductions ($p < 0.05$) in final body weight, average daily gain and elevation ($p < 0.05$) in intestinal APEC colonization in challenged broilers were counteracted by the XOS addition, which also alleviated the APEC-induced reductions ($p < 0.05$) in jejunal goblet cell count and density in broilers on d 17. Supplementing with XOS increased ($p < 0.05$) jejunal villus height and crypt depth, coupled with *occludin* and *zonula occluden-1* expression, on d 17, and diminished the change ($p < 0.05$) in the jejunal inflammatory cytokine expression profile in a time-dependent manner. Moreover, cecal counts of total bacteria and Lactobacillus in challenged broilers were augmented ($p < 0.05$) by the XOS addition, which also mitigated APEC-induced reductions ($p < 0.05$) in cecal acetate, butyrate and valerate concentrations in broilers on d 13 or 17. Supplementing with XOS blocked the increases ($p < 0.05$) in the expression of cecal *E. coli* virulence genes *relA* and *ompR* on d 13 along with the expression of *fimH* and *csgA* on d 17. XOS alleviated APEC-induced growth retardation and intestinal disruption in broilers partially by restraining the intestinal colonization of APEC. Furthermore, the improvements in cecal microbiota and fermentation pattern, along with attenuation of cecal *E. coli* virulence resulting from XOS supplementation, could also support the maintenance of intestinal health in APEC-challenged broilers.

Keywords: avian pathogenic *Escherichia coli*; broiler; growth performance; intestinal health; xylooligosaccharide

Citation: Ren, L.; Cao, Q.; Ye, H.; Dong, Z.; Zhang, C.; Feng, D.; Zuo, J.; Wang, W. Supplemental Xylooligosaccharide Attenuates Growth Retardation and Intestinal Damage in Broiler Chickens Challenged by Avian Pathogenic *Escherichia coli*. *Agriculture* **2024**, *14*, 1684. https://doi.org/10.3390/agriculture14101684

Academic Editors: Tatiana Dumitra Panaite and Mihaela Hâbeanu

Received: 23 August 2024
Revised: 22 September 2024
Accepted: 24 September 2024
Published: 26 September 2024

1. Introduction

As one of the most prevalent pathogenic bacteria in poultry, avian pathogenic *Escherichia coli* (APEC), such as serogroups O1, O2 and O78, account for a range of diseases. Both broilers and laying hens are susceptible to APEC, with economic losses from APEC-contaminated carcasses reaching USD 40 million in the U.S. APEC serves as either a primary pathogen or secondary pathogen to viral infections, immunosuppressive disease, or environment stress, leading to colisepticemia, hemorrhagic septicemia and enteritis. Although antibiotics and vaccines are employed to combat APEC infections, the emergence of resistance and the variety of serotypes limit their effectiveness [1]. Although APEC belongs to

the extra-intestinal pathogens, it mainly inhabits and develops in the gut [1]. Avian isolates clustered with human extra-intestinal pathogenic *Escherichia coli* (ExPEC) at the genomic level, indicating a horizontal exchange of mobile genetic elements between APEC and ExPEC [2]. This interaction contributes to their survival and virulence. Therefore, APEC is characterized as a potential zoonotic pathogen that poses a threat to public health [3]. Moreover, APEC still has intestinal pathogenicity with the potential to trigger numerous intra-intestinal disorders in chickens [1]. Indeed, oral administration of APEC has been verified to cause intestinal damage, leading to retardation of growth in broilers [4]. During the past few decades, antibiotics have been widely used in feeds to prevent or control bacteria-related disorders in animals. However, the efficacy of antibiotic treatment for APEC is diminished by the horizontal spread of genetic elements, such as islands of resistance and virulence genes in APEC and commensal *E. coli* [5]. Moreover, the in-feed antibiotic prohibition results in a demand for exploring natural additives to mitigate APEC damage. There is an interest in characterizing prebiotics as potential alternatives to alleviate the detrimental effects of the APEC challenge in poultry [6].

Xylooligosaccharide (XOS) represents an important type of prebiotic that can pass through the proximal intestine and selectively stimulate the proliferation of certain beneficial bacteria as well as facilitate the production of short-chain fatty acids (SCFAs) in the hindgut of animals [7]. As a key nutritional and energy component for enterocytes, SCFAs sustain cell renewal and repair to enhance the anti-inflammation response and barrier function of intestinal epithelia [8]. In addition, SCFAs inhibit the virulence genes and colonization ability of ExPEC, making them versatile and effective antimicrobial agents produced during microbial degradation of oligosaccharides [9]. Additionally, XOS is hypothesized to exert a similar effect to other oligosaccharides in inhibiting the adhesion of specific pathogenic bacteria to host intestinal epithelia, due to its potential ability to bind to bacterial surfaces, probably by acting as decoy receptors for bacterial adhesins [10]. Other ways that XOS suppresses pathogens, such as interference with gene expression associated with bacterial virulence (e.g., adhesion), are less investigated but might be equally impactful [11]. The aforementioned actions of XOS are speculated to optimize the gut microbiome and diminish bacterial pathogenicity, which may subsequently enhance the production performance and intestinal health of chickens. Indeed, it has been reported that an XOS addition improved growth performance and intestinal health as well as modulated the immune responses of broilers that were free of challenges [12]. Nevertheless, it is unknown whether dietary XOS could protect broilers against an APEC challenge. Therefore, this study aimed to investigate the effect of XOS on intestinal integrity, cecal microbial composition and SCFA production, with the goal of decreasing APEC colonization and the expression of virulence, thus protecting yellow-feathered broiler chicks from APEC. The findings of this research may offer insights into the application of XOS in broiler farming and present an environmentally friendly and effective approach to address microbial infections.

2. Materials and Methods

2.1. Animals and Experimental Design

The experimental animal protocols of this study were approved by the Animal Care and Use Committee of the South China Agricultural University (Protocol Number: 2023F240). In this study, we selected yellow-feathered broilers, which are medium-growing breeds favored in China [13]. A total of 144 1 d old male Lingnan yellow-feathered broiler chicks were purchased from Xinwang Poultry Co., Ltd. (Guangzhou, China), vaccinated with live chicken Marek's disease vaccine and did not receive any other vaccinations or probiotics during the trial period. Chicks (paired as AF × DB, with A and F representing the heavy-duty sire lines of the Lingnan yellow-feathered chicken, while D and B denote the high-yielding female lines of the same breed) were randomly allocated into 3 groups: a control (CON) group (birds received a basal diet without challenge), an APEC group (birds received a basal diet with APEC challenge) and an XOS group (APEC-challenged birds supplemented with 1600 mg/kg XOS). Each group involved 6 replicates with 8 birds per

replicate. The initial body weight was similar across replicates. The corncob-derived XOS (95% purity) was obtained from Shandong Longlive Bio-Technology Co. Ltd. (Dezhou, China). The percentage of each component was as follows: xylose 2.2%, xylobiose 40%, xylotriose 33%, xylotetraose 12%, xylotentaose 5%, xylhexaose and xylheptaose 5.5%, glucose and arabinose 2%. The additive dosage of XOS in the broiler diet was selected based on our preliminary experiment, and another study used 2000 mg/mg of XOS additions to mitigate pathogenic bacterial infections [5,14]. Feed ingredients were crushed and mixed with the corresponding weight of XOS to make a ground diet for broiler rearing.

The nutritional composition and nutrient levels of the basal diet based on the Chinese Feeding Standard of Yellow-feathered Chickens (NY/T 3645-2020) are presented in Table 1 [15]. Broilers were housed in a windowed room measuring 40 square meters, and the humidity was kept at approximately 65%. Eighteen wire cages were used in this study, and each cage contained eight chickens. The dimensions of the cages were 70 cm in length, 32 cm in width and 43 cm in depth. The room temperature was kept at 34 °C during the first three days using heaters and then gradually reduced to 26 °C on d 17. Birds were exposed to continuous white lighting and had free access to the diets and fresh water.

Table 1. Composition and nutrient levels of basal diet (air-dry basis).

Ingredients	Contents (%)
Corn	60.88
Soybean meal	35.21
Limestone	1.44
Dicalcium phosphate	1.56
Salt	0.34
Choline chloride (50%)	0.35
DL-Methionine (98%)	0.12
Premix [(1)]	0.10
Total	100.00
Nutrient levels [(2)]	
Metabolizable energy (Mcal/kg)	2.86
Crude protein (%)	21.06
Calcium (%)	1.00
Available phosphorus (%)	0.40
Digestible lysine (%)	1.05
Digestible methionine (%)	0.42
Digestible methionine + cysteine (%)	0.71

[(1)] Supplied per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 600 IU; tocopherol, 45 IU; menadione, 2.5 mg; thiamin, 2.2 mg; riboflavin, 8 mg; niacin, 40 mg; pantothenic acid, 10 mg; pyridoxine, 4 mg; biotin, 0.4 mg; folic acid, 1.0 mg; cobalamin, 0.013 mg; Fe, 80 mg; Cu, 8.0 mg; Zn, 60 mg; Mn, 110 mg; Se, 0.3 mg; I, 1.1 mg. [(2)] Values represent calculated levels of nutrients.

2.2. Construction of APEC O78 Recombinant Strain with Antibiotic Resistance

In order to detect the colonization in the intestine by resistance plate screening, we constructed APEC O78 with a spectinomycin-resistant biofilm self-inducible promoter [16]. The pMB1-spect-PthrC3_8-eGFP plasmid (Addgene#107411) that exerts no impacts on the growth, virulence and physiological activities of *E. coli* [16] was extracted from DH5α strain in agar stab (Addgene Plasmid Repository) by using the SanPrep Column Plasmid Mini-preps Kit (Sangon Biotech. Co., Ltd., Shanghai, China). The concentration of extracted plasmid was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purity and integrity of the plasmid were verified by measuring the ratio of absorbance at 260 nm to 280 nm (A260/A280 greater than 1.8) and using agarose gel electrophoresis, respectively. The recovery and purification of plasmid were performed using DNA gel recovery kits (Tsingke Biotech. Co., Ltd., Beijing, China).

The APEC O78 strain (CVCC1570), provided by China Veterinary Culture Collection Center (Beijing, China), was plated in LB agar. Single colonies of APEC O78 were picked and cultured in LB medium (37 °C, 180 rpm) until the OD_{600} value reached 0.3~0.5, followed

by centrifugation for 8 min (4 °C, 3000 rpm). After discarding the supernatant, the resulting precipitate was washed twice with calcium chloride solution and then resuspended with sterile water containing 15% glycerol. The resultant competent cells of APEC O78 (100 μL) were electro-transformed with pMB1-spect-PthrC3_8-eGFP plasmid (5 μL, guaranteed plasmid mass greater than 1 μg) through a micropulser (Bio-Rad, Hercules, CA, USA) with voltage of 1.8 kv, capacitance of 25 μF and resistance of 200 Ω. The successfully recombinant bacteria were screened by plating on spectinomycin (50 μg/mL, solarbio Tech. Co., Ltd., Beijing, China)-resistant LB agar.

2.3. Oral Administration of Recombinant APEC O78

The above recombinant APEC O78 strain was inoculated in LB broth and cultured at 37 °C overnight. To enumerate bacteria, the inoculum was diluted and plated on LB broth agar at 37 °C for 24 h. From 7 to 12 d of age, each bird in the APEC and XOS groups was orally gavaged with 2 mL of recombinant APEC culture (total 4.0×10^9 CFU), while CON birds were orally gavaged with the same amount of LB broth. The APEC gavage dose was determined by a combination of a previous trial and studies to construct APEC infections [17]. No food or water were administered for 6 h before and 3 h after the APEC challenge in all groups.

2.4. Sample Collection

At 1st and 5th d post challenge (namely 13 and 17 d of age), birds were randomly selected from each replicate (one bird/replicate) and then slaughtered for separating the intestinal tract. Sterile scissors and forceps were used to cut 1 cm of duodenum, jejunum and ileum at their midpoints for determination of intestinal colonization of APEC O78. Further, the samples near the midpoints of the jejunum and ileum were collected and separated into two sections, one of which was fixed in 4% paraformaldehyde solution, while the other one was snap-frozen by liquid nitrogen and kept at −80 °C. Meanwhile, cecal content was harvested from each bird.

2.5. Measurement of Growth Performance

Each replicate was kept in a cage, and they were weighed collectively. The overall weight of each treatment group was then divided by the number of chicks in that group to calculate the average weight. Body weight and feed consumption of broilers were recorded for each replicate at 13 and 17 d of age for calculation of the final body weight (FBW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) during 1–13 d and 1–17 d of age. The collected data on FBW, FCR, mortality rates and rearing duration were utilized to compute the European Production Index (EPI) using the following formula: (FBW × liveability × 100)/(FCR × rearing time).

2.6. Determination of Intestinal Colonization of APEC O78

The experimental method was derived from a previous study [18]. The samples from duodenal, jejunal and ileal tissues were dissected longitudinally and rinsed in sterile phosphate buffer solution (PBS) containing 0.25 mg/mL of spectinomycin (in order to kill the irrelevant bacteria). These tissues were then cryohomogenized by a rapid Sample Grinder (JXFSTPRP-24, Jingxin Industrial Development Co., Ltd., Shanghai, China), and the resulting homogenate was 10-fold gradient-diluted with sterile PBS containing 0.05 mg/mL of spectinomycin, followed by spreading on a MacConkey agar plate containing 0.05 mg/mL of spectinomycin at 37 °C overnight. We recorded the number of colonies grown and multiplied this by the number of dilutions, after which this was processed as $\log_{10}$ to analyze the differences between the groups.

2.7. Histomorphological Examination of Intestinal Tissues

Jejunal samples fixed in formaldehyde solution were subjected to paraffin-embedding procedures. The 4 μm cross-sections of samples were separately stained with hematoxylin–

eosin and periodic acid–Schiff (PAS) stain. For each section, the intact and representative villus–crypt units in each section (at least 8) were selected for analyzing intestinal morphology under microscopic vision fields with Image J software (1.54d). Villus height (VH) was defined as the length from the tip of the villus to the villus–crypt junction, while crypt depth (CD) was defined as the depth of emboli between adjacent villi, and the ratio of VH to CD (VCR) was then calculated. Further, goblet cells were detected in PAS-stained sections, and the count and density of goblet cells were expressed as the total number of goblet cells per villus and per 100 μm of villus, respectively.

2.8. Determination of the Relative mRNA Expression of Intestinal Genes

Total RNA was isolated from jejunal tissue using the FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazyme Biotech., Nanjing, China) according to the manufacturer's protocols. The verification of RNA concentration and quality, reverse transcription as well as quantitative reverse transcription PCR (RT-qPCR) were implemented according to the methods described elsewhere [18]. Primer sequences for the reference gene, glyceraldehyde-phosphate dehydrogenase (*GAPDH*), and the target genes, including interleukin (*IL*)-*1β*, *IL-6*, *IL-8*, *IL-10*, tumor necrosis factor α (*TNF-α*), *claudin-1*, *occludin* and zonula occludens-1 (*ZO-1*) are listed in Table 2. The relative mRNA expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method, $\Delta Ct = Ct_{Target} - Ct_{GAPDH}$, $\Delta\Delta Ct = \Delta Ct_{treat} - \Delta Ct_{CON}$.

Table 2. Primer sequences for relative quantitative PCR.

Species	Genes [(1)]	Primer Sequences (5′-3′)	Product Sizes (bp)
Chicken	*GAPDH*	F: GTGAAGGTCGGAGTGAACGGATTT R: CCCATTTGATGTTGGCGGGAT	187
	IL-1β	F: TGCCTGCAGAAGAAGCCTCG R: GACGGGCTCAAAAACCTCCT	204
	IL-6	F: GCTGCAGTCACAGAACGAGT R: GGACAGGTTTCTGACCAGAGG	167
	IL-8	F: TGAGAAGCAACAACAACAGCA R: CAGCACAGGAATGAGGCATA	129
	IL-10	F: TCAATCCAGGGACGATGAACT R: TCTGTGTAGAAGCGCAGCAT	114
	TNF-α	F: GCATCGCCGTCTCCTACCA R: CCTGCCCAGATTCAGCAAAGT	204
	Claudin-1	F: GTGCAGAAGATGCGGATGG R: TTGGTGTTGGGTAAGATGTTGTTT	253
	Occludin	F: ATCAACAAAGGCAACTCT R: GCAGCAGCCATGTACTCT	157
	ZO-1	F: GAGTTTGATAGTGGCGTT R: GTGGGAGGATGCTGTTGT	298
Escherichia coli	*GAPDH*	F: TCGCATTGTTTTCCGTGCTG R: TCAGCGTCTAACAGGTCGTT	75
	fimH	F: GATGTTTCTGCTCGTGATG R: TACCGCCGAAGTCCCT	261
	csgA	F: ACTGGCCTCATATCAACGGC R: CGTAAAGTAGCATTCGCCGC	98
	hycA	F: CGGCATGATTGATGGCAAGG R: GGCGGTGTATAAGCTGTCGT	100
	luxS	F: TTGGTACGCCAGATGAGCAG R: GCCACACTGGTAGACGTTCA	113
	tolA	F: ATGGTTGATTCAGGTGCGGT R: CTTGCGCTGCTCATCAGAAC	87
	relA	F: GTTCGCCGGATGTTATTGGC R: CCGGCGCATCTTTTACTTCG	100
	ompR	F: GCGTCGCTAATGCAGAACAG R: ATGATCGGCATCGGATTGCT	142

[(1)] The primer sequences were obtained from the genes of the corresponding species. *Gallus gallus*: *GAPDH*, reduced glyceraldehyde-phosphate dehydrogenase; *IL*, interleukin; *TNF*, tumor necrosis factor; *ZO-1*, zonula occludens-1. *Escherichia coli: fimH*, fimbrillin H; *csgA*, curli subunit gene A; *hycA*, formate hydrogenlyase regulator HycA gene; *luxS*, S-ribosylhomocysteine lyase; *tolA*, Tol/Pal system protein TolA gene; *relA*, (p)ppGpp synthetase gene; *ompR*, outer membrane protein R.

2.9. Quantitative Profiling of Cecal Bacterial Counts

Bacterial populations were measured by the absolute RT-qPCR method described previously [19]. Briefly, total genomic DNA was extracted from cecal chyme using the TIANamp Stool DNA kit (TIANGEN Biotech. Co., Ltd., Beijing, China). The concentration and quality of DNA were validated according to our previous study [20]. The extracted DNA was used as a template for PCR amplification using microbe-specific primers (Table 3), and the PCR products were then validated by agarose gel electrophoresis, followed by recovery and purification using DNA gel recovery kits (Tsingke Biotech. Co., Ltd., Beijing, China). The resulting DNA standards for target bacteria with their serial 10-fold dilutions served as the templates for qPCR with the corresponding primers. To construct standard curves for target bacteria, the lg (copy numbers) and their respective Ct values of each concentration of DNA standards were denoted as the X-and Y-axes, respectively. The copy numbers of DNA standards were calculated under the following formula: DNA (copy numbers) = $[(C \times 6.0233 \times 10^{23}\ (\text{copies/mol})]/(S \times 660 \times 10^{6})$, in which C represents the concentration of DNA standards (μg/μL) and S represents the number of product bases corresponding to microbe-specific primers. Finally, individual sample DNA served as the template to obtain the Ct value by quantitative PCR, and the copy number of each bacterium was then calculated according to the standard curve obtained above.

Table 3. Primer sequences for absolute quantitative PCR.

Bacteria	Primer Sequences (5′-3′)	Product Sizes (bp)
Total bacteria	F: GCAGGCCTAACACATGCAAGTC R: TGCTGCCTCCCGTAGGAGT	315
Lactobacillus	F: GAGGCAGCAGTAGGGAATCTTC R: GGCCAGTTACTACCTCTATCCTTCTTC	126
Bifidobacterium	F: TACACCACCACCCGAAGAA R: GGAGTGCTCCTGCAGATTGT	123
Escherichia coli	F: CATGCCGCGTGTATGAAGAA R: CGGGTAACGTCAATGAGCAAA	96
Avain pathogenic E. coli O78	F: CGATGTTGAGCGCAAGGTTG R: TAGGTATTCCTGTTGCGGAG	323
Salmonella	F: AGGCCTTCGGGTTGTAAAGT R: GTTAGCCGGTGCTTCTTCTG	97

2.10. Analysis of SCFA Profile

The concentrations of SCFAs in cecal chyme were determined by the internal standard method of gas chromatography with 2-ethylbutyric acid (2-EB) used as the internal standard. Briefly, cecal chyme was dissolved in 2.5 times (*v*/w) the volume of ultrapure water by vortex shaking for 5 min and then centrifuged (10,000 rpm, 4°C) for 10 min. The supernatant was collected and mixed with 0.2 times the volume of 25% (*v*/*v*) metaphosphate containing 2 g/L 2-EB, followed by incubation in ice water for 30 min and centrifugation (10,000 rpm, 4 °C) for 10 min. The resulting supernatant was then poured into the chromatographic injection bottle of an Agilent 6890 N gas chromatograph (Agilent, Santa Clara, CA, USA) for SCFA analysis following the method of a previous report [21].

2.11. Determination of the Relative mRNA Expression of Cecal E. coli Virulence Genes

Bacterial RNA was isolated from cecal chyme, using the PowerFecal Pro kit (QIAGEN, Hilden, Germany) under the manufacturer's instructions [22]. The confirmation of RNA concentration and quality, reverse transcription as well as RT-qPCR were implemented as described previously [19]. Primer sequences of the reference gene GAPDH and virulence genes (type 1 fimbriae D-mannose specific adhesin (*fimH*), curlin major subunit (*csgA*), formate hydrogenlyase regulatory protein (*hycA*), S-ribosylhomocysteine lyase (*luxS*), Tol/Pal system protein (*tolA*), (p)ppGpp synthetase (*relA*) and DNA-binding transcriptional dual regulator (*ompR*)) of *E. coli* are presented in Table 2. The relative mRNA expression of target

genes was calculated using the $2^{-\Delta\Delta Ct}$ method, $\Delta Ct = Ct_{Target} - Ct_{GAPDH}$, $\Delta\Delta Ct = \Delta Ct_{treat} - \Delta Ct_{CON}$.

2.12. Statistical Analysis

Data are presented as means with their pooled standard error of the mean. All data were analyzed by one-way ANOVA in the general linear model procedure of SPSS 22.0. Differences among groups were detected by Duncan's multiple comparisons. Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance.

3. Results

3.1. Effect of XOS on Growth Performance of Broilers Challenged by APEC

As shown in Table 4, broilers in the APEC group showed reduced ($p < 0.05$) FBW and ADG during both 1–13 d and 1–17 d of age, along with an increased ($p < 0.05$) FCR during 1–13 d of age when compared with those in the CON group. However, supplemental XOS restored the above parameters in challenged broilers to the same ($p > 0.05$) levels as those in the CON group. An XOS addition also elevated ($p < 0.05$) the ADFI in challenged broilers during 1–17 d of age as compared with that in the APEC group.

Table 4. Effect of xylooligosaccharide on growth performance of broilers challenged by avian pathogenic *Escherichia coli* [(1)].

Items	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
Days 1–13					
FBW, g	149 [a]	133 [b]	160 [a]	6.77	<0.001
ADG, g	9.01 [a]	7.59 [b]	9.98 [a]	0.547	<0.001
ADFI, g	14.8 [b]	14.3 [b]	15.4 [a]	0.514	0.037
FCR	1.62 [b]	1.86 [a]	1.68 [b]	0.087	0.004
EPI	705.92 [a]	547.81 [b]	734.20 [a]	62.88	0.020
Days 1–17					
FBW, g	220 [a]	191 [b]	237 [a]	11.6	<0.001
ADG, g	11.23 [a]	9.40 [b]	11.99 [a]	0.624	<0.001
ADFI, g	21.7 [a,b]	19.7 [b]	22.8 [a]	1.17	0.005
FCR	1.90	2.09	1.97	0.143	0.158
EPI	680.21 [a,b]	536.55 [b]	709.83 [a]	89.83	0.012

SEM, pooled standard error of the mean; FBW, final body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio; EPI, European Production Index. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

3.2. Effect of XOS on Intestinal Colonization of APEC in Broilers Challenged by APEC

There was a higher ($p < 0.05$) number of duodenal, jejunal and ileal APEC in broilers on both d 13 and 17 in the APEC group versus the CON group (Table 5). However, the number of duodenal, jejunal and ileal APEC on d 13 and d 17 in the XOS group was reduced to a level comparable to ($p > 0.05$) those in the CON group. Based on the above results (jejunal APEC number was reduced more obviously than duodenal APEC number due to XOS addition), we selected jejunal samples for further analysis.

Table 5. Effect of xylooligosaccharide on intestinal colonization of *Escherichia coli* (O78) in broilers challenged by avian pathogenic *Escherichia coli* on d 13 and 17 [(1)].

Items (CFU)	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
		Day 13			
Duodenum	3.94 [b]	4.28 [a]	3.81 [a,b]	0.124	0.047
Jejunum	3.25 [b]	4.57 [a]	3.85 [a,b]	0.216	0.048
Ileum	4.03 [b]	5.14 [a]	3.87 [b]	0.153	0.016
		Day 17			
Duodenum	3.59 [b]	4.45 [a]	3.91 [a,b]	0.096	0.042
Jejunum	4.23 [b]	4.54 [a]	4.08 [b]	0.115	0.039
Ileum	4.44 [b]	5.23 [a]	3.79 [b]	0.085	0.024

SEM, pooled standard error of the mean. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

3.3. Effects of XOS on Jejunal Histomorphological Measurements of Broilers Challenged by APEC

As shown in Table 6, compared with the CON group, the APEC group had a decrease ($p < 0.05$) in CD coupled with a trend toward a decrease ($p < 0.10$) in VH on d 13. However, VH on d 13 tended to be higher ($p < 0.10$), while VH and CD on d 17 were higher ($p < 0.05$) in the XOS group than those in the APEC group. Furthermore, compared to the APEC group, the CD on d 13 in the XOS group was increased to a level similar to that in the CON group ($p > 0.05$). Goblet cell density on d 13 in the XOS group was higher ($p < 0.05$) than that in either the CON group or the APEC group. Both goblet cell count and density on d 17 were lower ($p < 0.05$) in the APEC group versus either the CON group or the XOS group.

Table 6. Effect of xylooligosaccharide on histological measurements from jejunal tissues of broilers challenged by avian pathogenic *Escherichia coli* [(1)].

Items	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
		Day 13			
Villus height (μm)	575	475	542	60.1	0.088
Crypt depth (μm)	77.2 [a]	60.8 [b]	71.4 [a,b]	9.75	0.043
Villus height-to-crypt depth ratio	7.51	7.74	7.77	1.30	0.963
Goblet cell count [(4)]	72.3	66.5	86.6	20.4	0.313
Goblet cell density [(5)]	8.19 [b]	7.73 [b]	11.22 [a]	1.68	0.013
		Day 17			
Villus height (μm)	648 [a,b]	536 [b]	815 [a]	92.9	0.006
Crypt depth (μm)	82.2 [a,b]	79.6 [b]	104.7 [a]	13.98	0.035
Villus height-to-crypt depth ratio	7.88	6.74	7.83	0.957	0.100
Goblet cell count	88.3 [a]	52.0 [b]	80.8 [a]	9.03	<0.001
Goblet cell density	9.28 [a]	4.75 [b]	8.47 [a]	0.887	<0.001

SEM, pooled standard error of the mean. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance. [(4)] Goblet cell count was calculated as the total number of goblet cells per villus. [(5)] Goblet cell density was calculated as the number of goblet cells per 100 μm of villus. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

3.4. Effect of XOS on the Relative Expression of Jejunal Genes of Broilers Challenged by APEC

As shown in Table 7, the mRNA expression profile of jejunal tight junction (TJ) proteins was similar ($p > 0.05$) between the CON group and the APEC group on both d 13 and 17.

However, the XOS group showed increases ($p < 0.05$) in *occludin* mRNA expression on d 13 as well as the mRNA expression of *occludin* and *ZO-1* on d 17 in comparison with the APEC group.

Table 7. Effect of xylooligosaccharide on the relative mRNA expression of jejunal tight junction proteins in broilers challenged by avian pathogenic *Escherichia coli* on d 13 and 17 [(1)].

Items	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
Day 13					
ZO-1	1.00 [b]	1.05 [ab]	1.32 [a]	0.105	0.036
Occludin	1.07 [a,b]	0.87 [b]	1.45 [a]	0.135	0.041
Claudin-1	1.12	1.35	1.90	0.327	0.279
Day 17					
ZO-1	1.00 [b]	1.12 [b]	1.79 [a]	0.253	0.048
Occludin	1.03 [b]	1.01 [b]	1.66 [a]	0.178	0.027
Claudin-1	1.00 [b]	1.17 [a,b]	2.57 [a]	0.464	0.019

SEM, pooled standard error of the mean; *ZO-1*, zonula occludens-1. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

Compared with the CON group, the APEC group had a lower ($p < 0.05$) expression of *TNF-α* and *IL-8* on d 13 coupled with a higher ($p < 0.05$) expression of *IL-8* on d 17 (Table 8). The XOS group had a higher ($p < 0.05$) expression of *TNF-α* on d 13 with a lower ($p < 0.05$) expression of *IL-1β* on d 17 when compared with the APEC group.

Table 8. Effect of xylooligosaccharide on the relative mRNA expression of jejunal inflammatory cytokines in broilers challenged by avian pathogenic *Escherichia coli* on d 13 and 17 [(1)].

Items	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
Day 13					
IL-6	1.09	1.71	1.81	0.233	0.098
IL-8	1.10 [a]	0.23 [b]	0.28 [b]	0.064	<0.001
IL-10	1.09 [b]	1.32 [a,b]	2.26 [a]	0.108	0.041
IL-1*β*	1.23	1.20	0.64	0.253	0.302
TNF-α	1.02 [a]	0.51 [b]	1.15 [a]	0.079	0.001
Day 17					
IL-6	1.05 [b]	1.91 [a,b]	2.36 [a]	0.409	0.047
IL-8	1.02 [b]	1.92 [a]	2.79 [a]	0.404	0.048
IL-10	0.94 [b]	1.81 [a,b]	2.45 [a]	0.438	0.044
IL-β	0.94 [a,b]	1.42 [a]	0.80 [b]	0.178	0.024
TNF-α	1.00	0.97	0.67	0.137	0.570

SEM, pooled standard error of the mean; *IL*, interleukin; *TNF*, tumor necrosis factor. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

3.5. Effect of XOS on Cecal Bacterial Count of Broilers Challenged by APEC

As presented in Table 9, the APEC group had a lower ($p < 0.05$) count of *Lactobacillus* with a higher ($p < 0.05$) count of *E. coli* O78 in the cecum on d 13 than those in the CON group. Comparatively, the counts of total bacteria and *Lactobacillus* in the cecum on both d 13 and 17 were higher ($p < 0.05$) in the XOS group versus the APEC group. No differences ($p > 0.05$) were observed in the counts of cecal *Bifidobacteria*, *E. coli* and *Salmonella* among groups on either d 13 or 17.

Table 9. Effect of xylooligosaccharide on cecal bacterial counts of broilers challenged by avian pathogenic *Escherichia coli* [(1)].

Items	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
Day 13					
Total bacteria (lg copies/g)	9.95 [a,b]	9.58 [b]	10.19 [a]	0.327	0.041
Lactobacillus (lg copies/g)	7.83 [a]	7.47 [b]	8.27 [a]	0.277	0.003
Bifidobacteria (lg copies/g)	6.87	6.72	7.15	0.486	0.359
E. coli (lg copies/g)	7.77	7.47	7.69	0.124	0.621
E. coli O78 (lg copies/g)	3.44 [b]	4.29 [a]	4.20 [a]	0.453	0.016
Salmonella (lg copies/g)	4.14	4.25	4.17	0.170	0.636
Day 17					
Total bacteria (lg copies/g)	9.71 [a,b]	9.69 [b]	10.11 [a]	0.221	0.035
Lactobacillus (lg copies/g)	8.00 [a,b]	7.49 [b]	8.19 [a]	0.325	0.036
Bifidobacteria (lg copies/g)	6.77	6.58	6.85	0.323	0.420
E. coli (lg copies/g)	7.80	7.74	7.47	0.186	0.552
E. coli O78 (lg copies/g)	3.35	3.46	3.19	0.211	0.212
Salmonella (lg copies/g)	3.87	3.90	3.86	0.180	0.963

SEM, pooled standard error of the mean. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

3.6. Effect of XOS on Cecal SCFA Profile of Broilers Challenged by APEC

Compared with the CON group, the APEC group presented reductions ($p < 0.05$) in cecal acetate and isobutyrate concentrations on d 13, along with the concentrations of cecal acetate, butyrate and valerate on d 17 (Table 10). The propionate concentration on d 13 and isovalerate concentration on d 17 showed a tendency toward reduction ($p < 0.10$) in the APEC group compared to the CON group. The concentration of acetate on both d 13 and 17 in the XOS group was higher ($p < 0.05$) than that in the APEC group but did not differ ($p > 0.05$) from the CON group. Furthermore, the concentrations of butyrate and valerate on d 17 in the XOS group were not different ($p > 0.05$) from both the CON group and the APEC group.

Table 10. Effects of xylooligosaccharide on cecal short-chain fatty acid profile of broilers challenged by avian pathogenic *Escherichia coli* [(1)].

Items (mmol/L)	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
Day 13					
Acetate	24.8 [a]	18.5 [b]	30.5 [a]	3.07	0.001
Propionate	2.26	1.36	1.43	0.589	0.077
Butyrate	4.66	5.33	7.27	2.201	0.200
Isobutyrate	0.26 [a]	0.16 [b]	0.18 [b]	0.039	0.014
Valerate	0.29	0.17	0.28	0.085	0.135
Isovalerate	0.21	0.21	0.20	0.085	0.981
Day 17					
Acetate	22.8 [a]	12.8 [b]	24.1 [a]	5.10	0.008
Propionate	1.53	1.05	1.49	0.685	0.553
Butyrate	7.94 [a]	5.13 [b]	6.13 [a,b]	1.241	0.027
Isobutyrate	0.21	0.10	0.15	0.112	0.411
Valerate	0.38 [a]	0.20 [b]	0.27 [a,b]	0.062	0.004
Isovalerate	0.30	0.14	0.18	0.091	0.055

SEM, pooled standard error of the mean. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Significance was defined as $p < 0.05$, and $0.05 < p < 0.10$ was considered as a tendency toward significance. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

3.7. Effect of XOS on the Relative Expression of Cecal E. coli Virulence Genes of Broilers Challenged by APEC

As displayed in Table 11, the cecal *E. coli* of broilers in the APEC group showed increases ($p < 0.05$) in *relA* and *ompR* expression on d 13 together with *fimH* and *csgA* expression on d 17 compared with those in the CON group. Supplementing XOS to challenged broilers restored the above parameters to the same levels ($p > 0.05$) as those in the CON group. Furthermore, the XOS group had a lower ($p < 0.05$) expression of *fimH* and *hycA* along with a tendency toward lower ($p < 0.10$) expression of *luxS* of cecal *E. coli* on d 13 when compared with the APEC group.

Table 11. Effect of xylooligosaccharide on the relative mRNA expression of virulence genes of cecal *E. coli* in broilers challenged by avian pathogenic *Escherichia coli* on d 13 and 17 [(1)].

Items	CON [(2)]	APEC	XOS	SEM	*p*-Value [(3)]
Day 13					
fimH	0.93 [b]	1.89 [a]	0.60 [b]	0.241	0.045
csgA	1.29	2.17	0.52	0.475	0.134
hycA	0.97 [a,b]	1.68 [a]	0.36 [b]	0.192	0.024
luxS	0.84	2.21	1.03	0.238	0.056
tolA	1.04	1.34	1.49	0.175	0.237
relA	0.88 [b]	3.17 [a]	0.84 [b]	0.521	0.006
ompR	1.16 [b]	2.84 [a]	0.95 [b]	0.412	0.024
Day 17					
fimH	0.90 [b]	2.75 [a]	0.38 [b]	0.337	0.003
csgA	0.87 [b]	2.00 [a]	0.53 [b]	0.236	0.012
hycA	0.84	1.20	0.65	0.287	0.329
luxS	0.87	1.35	0.51	0.178	0.155
tolA	0.85	0.76	0.39	0.191	0.269
relA	1.01	1.73	0.67	0.367	0.153
ompR	1.00	0.97	0.67	0.137	0.141

SEM, pooled standard error of the mean; *fimH*, fimbrillin H; *csgA*, curli subunit gene A; *hycA*, formate hydrogenlyase regulator HycA gene; *luxS*, S-ribosylhomocysteine lyase; *tolA*, Tol/Pal system protein TolA gene; *relA*, (p)ppGpp synthetase gene; *ompR*, outer membrane protein R. [(1)] Values are the mean of six replicates per treatment. [(2)] CON = control (broilers were free of challenge); APEC = broilers were challenged by avian pathogenic *Escherichia coli* from 7 to 12 d of age; XOS = APEC-challenged broilers supplemented with 1600 mg/kg xylooligosaccharide. [(3)] Statistical significance was determined at $p < 0.05$, and trends (tendencies toward significant effects) were measured at $0.05 < p < 0.10$. [a,b] Values within a row with different superscript letters differ significantly ($p < 0.05$).

4. Discussion

Consistent with a previous report [4] that revealed the detrimental effects of the APEC challenge on the growth performance of broilers, the present study showed that an APEC challenge caused reduced FBW and ADG during both 1–13 and 1–17 d of age concurrent with increased FCR during 1–13 d of age without alteration of ADFI in broilers. These results suggest that the poor growth of broilers induced by APEC was likely due to the detected intestinal disruption instead of a reduction in appetite. It has been reported that XOS supplementation could improve the growth performance of broilers [12]. However, contrasting results were also described elsewhere [23,24]. The discrepancies might originate from the differences in the amount of XOS added and the health status of the chickens. In this study, the addition of XOS to diets reversed the decline in growth performance induced by an APEC challenge, which could be partly attributed to the observed effect of XOS in mitigating the APEC-induced intestinal disruption of broilers.

Intestinal colonization of APEC is essential for colonization, invasion and damage to intestinal and extra-intestinal tissues of chickens [25]. Our study revealed that oral administration of APEC O78 increased its colonization of intestinal tissues in broilers, while an XOS addition reduced its colonization in the intestine on d 13 and d 17. Those findings indicated the use of XOS against intestinal colonization of APEC in broilers. Similarly, other researchers reported that feeding XOS reduced intestinal colonization of *Salmonella*

in mice by enriching *Bifidobacterium* in the intestine [7]. Alternatively, XOS might repress adhesion-related gene expression in bacteria. This hypothesis is somewhat supported by the current findings on cecal *E. coli* virulence genes' expression as well as by some previous studies [11], thus supporting that there is reduced intestinal colonization of APEC in broilers that were fed with XOS.

Intestinal histological morphology serves as an indicator of intestinal health. The elongation of intestinal villi increases their surface area, which enhances absorption, strengthens the barrier function and improves the growth performance of animals [4,26]. Impaired intestinal morphology, along with a slow turnover of enterocytes, occurred in broilers during APEC invasion [27]. Likewise, we found that the APEC challenge tended to decrease VH and reduced the CD of jejunum in broilers on d 13, implying compromises of development and turnover of intestinal villi by the APEC challenge. Increasing evidence has demonstrated the benefits of XOS in the intestinal morphological structure of broilers [23,24]. In the present study, the XOS addition tended to increase jejunal VH on d 13 as well as elevating both the VH and CD of the jejunum in APEC-challenged broilers on d 17. These results indicated that the addition of XOS could protect intestinal epithelia, maintain the development of the crypt–villus and repair intestinal villi [26].

Goblet cells are capable of secreting a variety of functional proteins (e.g., mucin-2) that help maintain the intestinal barrier and prevent colonization by pathogenic bacteria [28]. Similar to the previous report, this study observed reductions in jejunal goblet cell count and the density of broilers on d 17 due to an APEC challenge [29]. On the other hand, adding XOS to challenged broilers enhanced the density of jejunal goblet cells on day 13 and offset the decreases in both count and density on day 17, indicating that dietary XOS could potentially shield the intestinal mucosal barrier against an APEC challenge by promoting the growth of goblet cells. This is consistent with the elevation of *Lactobacillus* and SCFAs in the cecum, which is thought to promote *mucin-2* expression [30]. Analogous results were reported in previous studies regarding chickens under unchallenged conditions [23]. Because prebiotics can improve the intestinal histomorphology of animals in a microbiota-associated manner, we hypothesized that the reduced jejunal colonization of APEC due to the XOS addition could partially explain the observed enhancements in intestinal morphology, goblet cell count and density in broilers fed with XOS [24].

Intraepithelial TJ is composed of various proteins, including transmembrane proteins (such as claudin-1 and occludin) and linker proteins (such as ZO-1), which are implicated in maintaining intestinal integrity against paracellular penetration of pathogen-related factors from the intestinal lumen. Thus, they serve as a crucial defense line against enteric infections [31]. In line with a previous study [27], this study revealed minimal changes in the expression of jejunal TJ proteins in broilers following an APEC challenge. Supplementing XOS to challenged broilers improved their intestinal integrity, as evidenced by an increased expression of *occludin* in the jejunum on both d 13 and 17, along with increased expression of *ZO-1* on d 17. Similar results were reported in previous studies, where the addition of XOS fortified intestinal integrity by increasing the expression of specific TJ proteins in chickens [23].

The disruptions in the intestinal structure of chickens due to an APEC challenge are established to be linked with intestinal inflammation [22]. On the one hand, inflammatory cytokine-mediated inflammation benefits the recruitment of phagocytes to clear pathogens, especially at the early stage of infection [32]. On the other hand, sustained inflammation contributes to bacteria-related intestinal injury [33]. There can be varying responses of intestinal inflammatory cytokine expression profiles in broilers to bacterial challenges that likely depend on the time-points post-challenge and the intricate immune feedback of the host [19]. Indeed, we found that the APEC challenge had a complex effect on the expression of jejunal inflammatory cytokines, as evidenced by a time-dependent change in their expression profile. It was possible that the downregulation of *TNF-α*, a multifunctional cytokine that enhances the host immune response against pathogens [34], in challenged broilers at an early stage of APEC infection (d 13) was unfavorable for eliminating the

invading bacteria in the intestine. In contrast, the upregulation of *IL-6* and *IL-8* at a later stage of APEC infection (d 17) was assumed to contribute to the detected damage of the intestinal histomorphology in broilers [22]. It has been shown that feeding XOS attenuated intestinal inflammation in piglets by decreasing the expression of several inflammatory cytokines, such as *TNF-α* and *IL-6* [35]. We observed that supplementing XOS to challenged broilers reversed the reduction in jejunal *TNF-α* expression on d 13 and lowered jejunal *IL-1β* expression on d 17, suggesting a potential of XOS addition in prompting elimination of intestinal pathogens (e.g., APEC) and alleviating intestinal inflammation in broilers at early and later stages of APEC infection, respectively.

The gut microbiota is involved in maintaining intestinal homeostasis and regulating pathological processes in broilers [19]. It has been indicated that APEC disrupt gut microbial composition in broilers, primarily characterized by an increased count of *E. coli* and a reduced count of *Lactobacillus* [25]. In this study, the APEC challenge elevated their abundance and reduced the *Lactobacillus* count in the cecum of broilers on d 13, demonstrating a negative shift in the cecal microbiota of broilers at an early stage of APEC infection. XOS stimulated beneficial bacteria such as *Lactobacillus* in the chicken gut [36]. We found that adding XOS to challenged broilers enhanced the numbers of cecal *Lactobacillus* as well as total bacteria on days 13 and 17. These results validated that an XOS addition optimized cecal microbial composition in broilers partially through the enrichment of *Lactobacillus* [23]. However, a contrasting finding was reported of no changes in cecal bacterial counts of broilers fed with XOS [37]. This inconsistency might be related to the variations in the quantity and duration of the XOS addition. Remarkably, it seemed that the observed increase in *Lactobacillus* upon the XOS addition was not sufficient to fully account for the simultaneous increase in total bacteria, suggesting the potential enrichment of others in broiler cecum [38].

SCFAs are the crucial metabolites during microbial fermentation of carbohydrates in the hindgut and mediate cross-talk between the host and gut microbes; furthermore, they maintain the health and growth of animals, primarily as a key energy component for enterocytes, thus sustaining intestinal renewal [8]. This study revealed reductions or decreasing trends of SCFAs in broilers' responses to APEC. These findings suggested a disturbance in the cecal fermentation pattern in challenged broilers, which was plausibly related to the observed alteration of gut microbiota. Previously, feeding XOS increased cecal concentrations of certain SCFAs in chickens [24,38], although these results showed some variations. In this study, the XOS addition alleviated the APEC-induced reduction in the cecal acetate concentration in broilers on both d 13 and 17, concurrent with reductions in cecal butyrate and valerate concentrations in broilers on d 17, which were presumably associated with the stimulation of SCFA-producing bacteria [38]. These results supported the idea that dietary XOS protects the cecal fermentation pattern from the APEC challenge, in consideration of the abilities of SCFAs to regulate intestinal recovery and immunity, thus defending against bacterial invasion [8].

The cecum of chickens is considered a reservoir for *E. coli* virulence factors, whose expression is pivotal for their pathogenicity [1,22]. The upregulation of virulence genes has been indicated to aggravate intestinal disorders in broilers [1]. In this way, the *fimH* and *csgA* genes are, respectively, responsible for encoding an important subunit of fimbriae type I and curli, prompting adhesion, motility and biofilm formation, thus being prominent for establishing *E. coli* infection in chickens [1]. The *hycA* gene encodes a regulatory protein related to antibiotic resistance in *E. coli* [39]. The *luxS* gene encodes an enzyme impelling the synthesis of autoinducer-2, which can reinforce *E. coli* pathogenicity [1]. The *tolA* gene encodes a cytoplasmic membrane protein required for maintaining the outer membrane integrity of bacteria and, consequently, benefits the motility and adherence of *E. coli* [40]. The *relA* gene encodes a ribosome-associated enzyme that mediates the environmental sustainability of *E. coli*, and enhances its adhesion and survival [41]. The *ompR* acts as a response regulator in bacterial two-component systems, which can facilitate the growth and virulence of *E. coli* [1].

Despite a minor difference in amount, the expression profile of virulence genes in the cecal digesta differed across groups. We recorded an upregulation of *fimH*, *hycA*, *relA* and *ompR* on d 13 along with *fimH* and *csgA* on d 17 following the APEC challenge. These results evidenced a fortification of virulence genes within the cecum in APEC-challenged broilers, which coincided with the findings of Afridi [42]. Previous studies have observed the repressed expression of adhesin-related genes in *Listeria* [11] caused by XOS treatment in in vitro models. Herein, APEC-induced upregulations of cecal *E. coli* virulence factors *relA* and *ompR* expression on d 13, together with *fimH* and *csgA* expression on d 17 in broilers, were reversed by an XOS addition. These effects might be related to elevated concentrations of cecal SCFAs, which could inhibit the expression of *E. coli* O157 virulence factors [9]. The above findings uncovered an influential role of XOS in limiting *E. coli* virulence, which could diminish *E. coli* pathogenicity and thereby protect intestinal health in broilers challenged by APEC.

5. Conclusions

In this experiment with short duration, supplemental XOS attenuated growth retardation and intestinal disruption in APEC-challenged broilers at least partially by inhibiting APEC colonization of the intestine. Moreover, supplemental XOS mitigated APEC-induced perturbations of cecal microbiota and the fermentation product profile along with the increase in *E. coli* virulence, which might also contribute to protecting intestinal health in broilers challenged by APEC. The findings can expand our fundamental knowledge regarding the mechanisms of XOS in protecting the intestinal health of animals. Future experiments with long duration deserve to be conducted to confirm the above benefits of an XOS addition.

Author Contributions: Data curation, L.R.; investigation, L.R.; methodology, L.R., Q.C., H.Y., Z.D. and C.Z.; formal analysis, Q.C., H.Y., Z.D. and C.Z.; conceptualization, L.R., Q.C., H.Y., Z.D., C.Z., D.F., J.Z. and W.W.; resources, D.F., J.Z. and W.W.; supervision, Q.C., D.F., J.Z. and W.W.; visualization, L.R.; writing—original draft, L.R. and Q.C.; writing—review and editing, J.Z. and W.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the National Natural Science Foundation of China (No. 32102584), the Natural Science Foundation of Guangdong Province (No. 2023A1515011112) and the Rural Science and Technology Correspondent Project of Guangzhou City (2024E04J0277).

Institutional Review Board Statement: The experimental animal protocols of this study were approved by the Animal Care and Use Committee of the South China Agricultural University (Protocol Number: 2023F240).

Data Availability Statement: The data are available upon request; Lulu Ren and Weiwei Wang are responsible for data-keeping.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Kathayat, D.; Lokesh, D.; Ranjit, S.; Rajashekara, G. Avian Pathogenic *Escherichia coli* (APEC): An Overview of Virulence and Pathogenesis Factors, Zoonotic Potential, and Control Strategies. *Pathogens* **2021**, *10*, 467. [CrossRef] [PubMed]
2. Shames, S.R.; Auweter, S.D.; Finlay, B.B. Co-evolution and exploitation of host cell signaling pathways by bacterial pathogens. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 380–389. [CrossRef]
3. Logue, C.M.; Wannemuehler, Y.; Nicholson, B.A.; Doetkott, C.; Barbieri, N.L.; Nolan, L.K. Comparative Analysis of Phylogenetic Assignment of Human and Avian ExPEC and Fecal Commensal *Escherichia coli* Using the (Previous and Revised) Clermont Phylogenetic Typing Methods and its Impact on Avian Pathogenic *Escherichia coli* (APEC) Classification. *Front. Microbiol.* **2017**, *8*, 283. [CrossRef] [PubMed]
4. Wang, W.; Li, Z.; Han, Q.; Guo, Y.; Zhang, B.; D'inca, R. Dietary Live Yeast and Mannan-Oligosaccharide Supplementation Attenuate Intestinal Inflammation and Barrier Dysfunction Induced by *Escherichia coli* in Broilers. *Br. J. Nutr.* **2016**, *116*, 1878–1888. [CrossRef]
5. Messerer, M.; Fischer, W.; Schubert, S. Investigation of Horizontal Gene Transfer of Pathogenicity Islands in *Escherichia coli* Using Next-Generation Sequencing. *PLoS ONE* **2017**, *12*, e0179880. [CrossRef] [PubMed]

6. Hashem, M.A.; Hassan, A.E.A.; Abou-Elnaga, H.M.M.; Abdo, W.; Dahran, N.; Alghamdi, A.H.; Elmahallawy, E.K. Modulatory Effect of Dietary Probiotic and Prebiotic Supplementation on Growth, Immuno-Biochemical Alterations, DNA Damage, and Pathological Changes in *E. coli*-Infected Broiler Chicks. *Front. Vet. Sci.* **2022**, *9*, 964738. [CrossRef]
7. Pang, J.; Wang, S.; Wang, Z.; Wu, Y.; Zhang, X.; Pi, Y.; Han, D.; Zhang, S.; Wang, J. Xylo-Oligosaccharide Alleviates Salmonella Induced Inflammation by Stimulating Bifidobacterium Animalis and Inhibiting Salmonella Colonization. *FASEB J.* **2021**, *35*, e21977. [CrossRef]
8. Martin-Gallausiaux, C.; Marinelli, L.; Blottière, H.M.; Larraufie, P.; Lapaque, N. SCFA: Mechanisms and Functional Importance in the Gut. *Proc. Nutr. Soc.* **2021**, *80*, 37–49. [CrossRef]
9. Zhang, S.; Dogan, B.; Guo, C.; Herlekar, D.; Stewart, K.; Scherl, E.J.; Simpson, K.W. Short Chain Fatty Acids Modulate the Growth and Virulence of Pathosymbiont *Escherichia coli* and Host Response. *Antibiotics* **2020**, *9*, 462. [CrossRef]
10. Asadpoor, M.; Peeters, C.; Henricks, P.A.J.; Varasteh, S.; Pieters, R.J.; Folkerts, G.; Braber, S. Anti-Pathogenic Functions of Non-Digestible Oligosaccharides in vitro. *Nutrients* **2020**, *12*, 1789. [CrossRef]
11. Ebersbach, T.; Andersen, J.B.; Bergström, A.; Hutkins, R.W.; Licht, T.R. Xylo-Oligosaccharides Inhibit Pathogen Adhesion to Enterocytes in Vitro. *Res. Microbiol.* **2012**, *163*, 22–27. [CrossRef] [PubMed]
12. Li, S.Z.; Liu, G.H.; Xu, Y.; Liu, J.; Chen, Z.M.; Zheng, A.J.; Cai, H.Y.; Chang, W.H. Comparison of the Effects of Applying Xylooligosaccharides Alone or in Combination with Calcium Acetate in Broiler Chickens. *Anim. Feed Sci. Technol.* **2022**, *290*, 115360. [CrossRef]
13. Wang, H.; Zhang, X.; Wang, G.; Jia, K.; Xu, X.; Zhou, G. Bacterial Community and Spoilage Profiles Shift in Response to Packaging in Yellow-Feather broiler, a Highly Popular Meat in Asia. *Front. Microbiol.* **2017**, *8*, 2588. [CrossRef]
14. Pourabedin, M.; Chen, Q.; Yang, M.; Zhao, X. Mannan-and Xylooligosaccharides Modulate Caecal Microbiota and Expression of Inflammatory-Related Cytokines and Reduce Caecal *Salmonella Enteritidis* Colonization in Young Chickens. *FEMS Microbiol. Ecol.* **2017**, *93*, fiw226. [CrossRef] [PubMed]
15. *NY/T 3645-2020*; Nutrient Requirements of Yellow Chickens. National Animal Husbandry Standardization Technical Committee: Beijing, China, 2020.
16. Anilionyte, O.; Liang, H.; Ma, X.; Yang, L.; Zhou, K. Short, Auto-Inducible Promoters for Well-Controlled Protein Expression in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 7007–7015. [CrossRef] [PubMed]
17. LA Ragione, R.M.; Cooley, W.A.; Woodward, M.J. The Role of Fimbriae and Flagella in the Adherence of Avian Strains of Escherichia coli O78:K80 to Tissue Culture Cells and Tracheal and Gut Explants. *J. Med. Microbiol.* **2000**, *49*, 327–338. [CrossRef]
18. Herigstad, B.; Hamilton, M.; Heersink, J. How to Optimize the Drop Plate Method for Enumerating Bacteria. *J. Microbiol. Methods.* **2001**, *44*, 121–129. [CrossRef]
19. Harshitha, R.; Arunraj, D.R. Real-time quantitative PCR: A Tool for Absolute and Relative Quantification. *Biochem. Mol. Biol. Educ.* **2021**, *49*, 800–812. [CrossRef]
20. Wang, W.; Ou, J.; Ye, H.; Cao, Q.; Zhang, C.; Dong, Z.; Feng, D.; Zuo, J. Supplemental N-acyl Homoserine Lactonase Alleviates Intestinal Disruption and Improves Gut Microbiota in Broilers Challenged by Salmonella Typhimurium. *J. Anim. Sci. Biotechnol.* **2023**, *14*, 7. [CrossRef]
21. Cruwys, J.A.; Dinsdale, R.M.; Hawkes, F.R.; Hawkes, D.L. Development of a Static Headspace Gas Chromatographic Procedure for the Routine Analysis of Volatile Fatty Acids in Wastewaters. *J. Chromatogr. A* **2002**, *945*, 195–209. [CrossRef]
22. Ibrahim, D.; Eldemery, F.; Metwally, A.S.; Abd-Allah, E.M.; Mohamed, D.T.; Ismail, T.A.; Hamed, T.A.; Al Sadik, G.M.; Neamat-Allah, A.N.F.; Abd El-Hamid, M.I. Dietary Eugenol Nanoemulsion Potentiated Performance of Broiler Chickens: Orchestration of Digestive Enzymes, Intestinal Barrier Functions and Cytokines Related Gene Expression with a Consequence of Attenuating the Severity of *E. coli* O78 Infection. *Front. Vet. Sci.* **2022**, *9*, 847580. [CrossRef] [PubMed]
23. Wang, Q.; Wang, X.F.; Xing, T.; Li, J.L.; Zhu, X.D.; Zhang, L.; Gao, F. The Combined Impact of Xylo-Oligosaccharides and Gamma-Irradiated Astragalus Polysaccharides on Growth Performance and Intestinal Mucosal Barrier Function of Broilers. *Poult. Sci.* **2021**, *100*, 100909. [CrossRef]
24. Deng, F.; Tang, S.; Zhao, H.; Zhong, R.; Liu, L.; Meng, Q.; Zhang, H.; Chen, L. Combined Effects of Sodium Butyrate and Xylo-Oligosaccharide on Growth Performance, Anti-Inflammatory and Antioxidant Capacity, Intestinal Morphology and Microbiota of Broilers at Early Stage. *Poult. Sci.* **2023**, *102*, 102585. [CrossRef] [PubMed]
25. Papouskova, A.; RychlikI, I.; Harustiakova, D.; Cizek, A. Research Note: A Mixture of *Bacteroides* spp. and Other Probiotic Intestinal Anaerobes Reduces Colonization by Pathogenic *E. coli* Strain O78:H4-ST117 in Newly Hatched Chickens. *Poult. Sci.* **2023**, *102*, 102529. [CrossRef]
26. Noah, T.K.; Donahue, B.; Shroyer, N.F. Intestinal Development and Differentiation. *Exp. Cell Res.* **2011**, *317*, 2702–2710. [CrossRef]
27. Huang, L.; Luo, L.; Zhang, Y.; Wang, Z.; Xia, Z. Effects of the Dietary Probiotic, *Enterococcus Faecium* ncimb11181, on the Intestinal Barrier and System Immune Status in *Escherichia coli* O78-Challenged Broiler Chickens. *Probiotics Antimicrob. Proteins* **2019**, *11*, 946–956. [CrossRef] [PubMed]
28. Gustafsson, J.K.; Johansson, M.E.V. The Role of Goblet Cells and Mucus in Intestinal Homeostasis. *Nat. Rev. Gastroenterol. Hepatol.* **2022**, *19*, 785–803. [CrossRef]
29. Manafi, M.; Khalaji, S.; Hedayati, M.; Pirany, N. Efficacy of *Bacillus subtilis* and Bacitracin Methylene Disalicylate on Growth Performance, Digestibility, Blood Metabolites, Immunity, and Intestinal Microbiota after Intramuscular Inoculation with *Escherichia coli* in Broilers. *Poult. Sci.* **2017**, *96*, 1174–1183. [CrossRef]

30. Burger-van Paassen, N.; Vincent, A.; Puiman, P.J.; van der Sluis, M.; Bouma, J.; Boehm, G.; van Goudoever, J.B.; van Seuningen, I.; Renes, I.B. The Regulation of Intestinal Mucin MUC2 Expression by Short-Chain Fatty Acids: Implications for Epithelial Protection. *Biochem. J.* **2009**, *420*, 211–219. [CrossRef]
31. Buckley, A.; Turner, J.R. Cell Biology of Tight Junction Barrier Regulation and Mucosal Disease. *Cold Spring Harb. Perspect. Biol.* **2018**, *10*, a029314. [CrossRef]
32. Duess, J.W.; Sampah, M.E.; Lopez, C.M.; Tsuboi, K.; Scheese, D.J.; Sodhi, C.P.; Hackam, D.J. Necrotizing Enterocolitis, Gut Microbes, and Sepsis. *Gut Microbes* **2023**, *15*, 2221470. [CrossRef] [PubMed]
33. Tan, J.; Applegate, T.J.; Liu, S.; Guo, Y.; Eicher, S.D. Supplemental Dietary L-arginine Attenuates Intestinal Mucosal Disruption During a Coccidial Vaccine Challenge in Broiler Chickens. *Br. J. Nutr.* **2014**, *112*, 1098–1099. [CrossRef]
34. Zhang, H.L.; Zhang, Y.J. A Systemic Assessment of the Association between Tumor Necrosis Factor Alpha 308 G/A Polymorphism and Risk of Cervical Cancer. *Tumour Biol.* **2013**, *34*, 1659–1665. [CrossRef]
35. Wang, X.; Xiao, K.; Yu, C.; Wang, L.; Liang, T.; Zhu, H.; Xu, X.; Liu, Y. Xylooligosaccharide Attenuates Lipopolysaccharide-Induced Intestinal Injury in Piglets via Suppressing Inflammation and Modulating Cecal Microbial Communities. *Anim. Nutr.* **2021**, *7*, 609–620. [CrossRef]
36. Pourabedin, M.; Guan, L.; Zhao, X. Xylo-Oligosaccharides and Virginiamycin Differentially Modulate Gut Microbial Composition in Chickens. *Microbiome* **2015**, *3*, 15. [CrossRef] [PubMed]
37. Suo, H.; Lu, L.; Xu, G.; Xiao, L.; Chen, X.; Xia, R.; Zhang, L.; Luo, X. Effectiveness of dietary xylo-oligosaccharides for broilers fed a conventional corn-soybean meal diet. *J. Integr. Agric.* **2015**, *14*, 2050–2057. [CrossRef]
38. De Maesschalck, C.; Eeckhaut, V.; Maertens, L.; De Lange, L.; Marchal, L.; Nezer, C.; De Baere, S.; Croubels, S.; Daube, G.; Dewulf, J.; et al. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. *Appl. Environ. Microbiol.* **2015**, *81*, 5880–5888. [CrossRef]
39. Aunins, T.R.; Erickson, K.E.; Chatterjee, A. Transcriptome-Based Design of Antisense Inhibitors Potentiates Carbapenem Efficacy in CRE *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 30699–30709. [CrossRef] [PubMed]
40. Johnson, C.L.; Ridley, H.; Pengelly, R.J.; Salleh, M.Z.; Lakey, J.H. The Unstructured Domain of Colicin N Kills *Escherichia coli*. *Mol. Microbiol.* **2013**, *89*, 84–95. [CrossRef] [PubMed]
41. Kaspy, I.; Glaser, G. *Escherichia coli* relA Regulation via its C-Terminal Domain. *Front. Microbiol.* **2020**, *11*, 572419. [CrossRef]
42. Afridi, O.K.; Ali, J.; Chang, J.H. Next-Generation Sequencing Based Gut Resistome Profiling of Broiler Chickens Infected with Multidrug-Resistant *Escherichia coli*. *Animals* **2020**, *10*, 2350. [CrossRef] [PubMed]

agriculture

MDPI

Article

White Grape Pomace Effect on Laying Hens' Productivity, Egg Quality Traits, and Antioxidant Capacity Under Normal, Heat, and Cold Thermal Conditions

Gabriela Maria Cornescu [1], Tatiana Dumitra Panaite [1,*], Ana Elena Cișmileanu [1], Mihaela Sărăcilă [2], Arabela Elena Untea [2] and Iulia Varzaru [2]

[1] Nutrition Physiology Department, National Research and Development Institute for Biology and Animal Nutrition, 077015 Balotesti, IF, Romania; gabriela_cornescu@yahoo.com (G.M.C.); ana_cismileanu@yahoo.com (A.E.C.)

[2] Food and Feed Quality Department, National Research and Development Institute for Biology and Animal Nutrition, 077015 Balotesti, IF, Romania; mihaela.saracila@ibna.ro (M.S.); arabela.untea@ibna.ro (A.E.U.); iulia.varzaru@ibna.ro (I.V.)

* Correspondence: tatiana.panaite@ibna.ro; Tel.: +40-21-351-2082

Abstract: This study investigated the effect of white grape pomace (WGP) via a 6% level dietary supplementation on laying hens exposed to varying thermal stress conditions. The experiment was designed as a 2 × 3 factorial study, incorporating two dietary treatments (C and E) and three different thermal conditions: normal (NT: 22 °C), high stress (HST: 35 °C), and low stress (LST: 10 °C). Feed and water were provided ad libitum throughout the experiment. Results showed that the inclusion of 6% WGP in laying hens' diet did not demonstrate beneficial effects under HST conditions, but under LST conditions, the WGP showed higher final body weight (1849.38 g) compared to both groups from the HST conditions (C 1599.40 g and WGP 1592.59 g), and the AEW (average egg weight) was highly significantly higher (p = 0.0001) compared to the C or NT groups (both groups, 2nd week), and the HST conditions (both groups, 2nd, 4th, and 6th weeks). HDEP (hen-day egg production) registered highly significant values (p = 0.0001) for the WGP group under HST conditions compared to HST conditions (both groups, 4th and 6th weeks). The whole egg weight was highly significant (p = 0.0001) for the WGP group under LTS conditions compared to the C group and to HST conditions (both groups, 2nd week and 6th week). The yolk weight parameter registered highly significant (p = 0.0001) values for the WGP group under LTS conditions compared to the C group and HST conditions (both groups, 2nd week; C group, 4th week; both groups 6th week) and the NT conditions (C group, 2nd week). Our study indicates that dietary supplementation with 6% white grape pomace (WGP) has potential benefits in LST conditions but limited efficacy under HST conditions. Further research is needed to explore the mechanisms and optimal inclusion levels of WGP in diets for laying hens exposed to different temperatures, especially in HST conditions.

Keywords: antioxidant capacity; eggs; grape pomace; heat stress; low stress

Citation: Cornescu, G.M.; Panaite, T.D.; Cișmileanu, A.E.; Sărăcilă, M.; Untea, A.E.; Varzaru, I. White Grape Pomace Effect on Laying Hens' Productivity, Egg Quality Traits, and Antioxidant Capacity Under Normal, Heat, and Cold Thermal Conditions. *Agriculture* **2024**, *14*, 2209. https://doi.org/10.3390/agriculture14122209

Academic Editor: Lin Zhang

Received: 11 October 2024
Revised: 24 November 2024
Accepted: 29 November 2024
Published: 3 December 2024

1. Introduction

The worldwide, continuously increasing temperatures and changing climate patterns have raised significant concerns regarding the welfare and productivity of laying hens, particularly during peak summer months when high thermal stress can severely impact their health and egg production, threatening the sustainability of poultry production systems [1].

Heat stress in laying hens leads to reduced feed intake, lower egg production, poor egg quality, compromised immune function, increased oxidative stress, and higher mortality rates [2,3].

Negative effects were also observed in laying hens exposed to low temperatures.

Cold stress in poultry increases dietary energy demand, feed intake, and feed conversion rate [3]. Kim et al. [4] reported changes in antioxidant status and lipid peroxidation under low thermal stress without stress-related responses. Other studies observed elevated antioxidant vitamins, reactive oxygen species, malondialdehyde, and corticosterone, alongside decreased insulin levels in laying hens' serum [5–7].

According to OECD/FAO [8] global food consumption is expected to rise by 1.3% annually, necessitating adaptive strategies and investments to ensure a resilient, sustainable food system while confronting climate change and population growth challenges.

Using agricultural byproducts in animal feed reduces land and water competition, saves resources, and supports a sustainable food system [9].

In the context of a circular economy, grape pomace serves as a sustainable antioxidant resource for farm animal feed, effectively mitigating heat or cold stress when combined with natural dietary additives and advancements in housing and climate control [10].

Grape pomace, a low-cost and available Romanian byproduct of the winemaking process, is a rich source of bioactive compounds (polyphenols, flavonoids, and dietary fibers). These bioactive molecules are known for their antioxidant, anti-inflammatory, and antimicrobial properties, which can offer various health benefits when included in farm animal feed formulations, also positively influencing the egg/meat quality [11].

The polyphenolic compounds present in grape pomace were highlighted for their ability to enhance the antioxidant status of poultry, with tested beneficial effects on gut microbial activity modulation histomorphology and functionality of the gut [12]. Herranz et al. [13] achieved enriched yolks with gallic acid and albumen Haugh units and yolk color score enhancement; although, they registered a reduction in shell thickness when including 50 g/kg of grape pomace in laying hens' diet.

Incorporating waste materials into poultry diets innovatively enhances animal health and productivity while supporting the circular bioeconomy by transforming agricultural byproducts into valuable feed, reducing waste, and promoting sustainable agriculture [14,15].

Furthermore, higher antioxidant enzyme activity and lower lipid peroxidation lead to overall improvements in the health, performance, and egg quality of the hens, particularly during storage, as evidenced by a study in which grape pomace flour was included in the feed of 74-week-old laying hens under heat stress [16].

We hypothesized that incorporating white grape pomace with recognized antioxidant potential into the diets of laying hens will mitigate the effects of different thermal stresses (high and low temperatures), improving productive performance, some quality traits, and the antioxidant capacity of eggs, efficiently enhancing the utilization of Romanian low-cost local natural available resources and reducing the need for synthetic additives.

2. Materials and Methods

2.1. Ethical Statement

The experiment was conducted according to the protocol (No. 601/05.02.2024) approved by the Commission of the National Research Development Institute of Animal Biology and Nutrition (IBNA-Balotesti, Romania) following the Romanian legislation (Law 206/2004, Ordinance 28/31.08.2011, Law 43/11.04.2014, Directive 2010/63/EU) for feeding, handling, and slaughtering procedures.

2.2. White Grape Pomace Purchasing and Their Proximal Chemical Analyses

The white grape pomace used in this study was sourced from a local vineyard farmer in Dâmbovița County, Romania. Traditionally, the waste generated after the grape harvest is considered to have no economic or productive value, becoming a significant disposal challenge for many Romanian farmers. Prior to incorporating the white grape pomace (WGP) into the laying hens' diet, the provided amount was dried in an oven (ECO CELL Blueline Comfort, Nuremberg, Germany) at a constant temperature of 65 °C for 48 h. Once dried, the WGP was weighed to assess its initial moisture content and then ground into powder using a laboratory hammer mill (Pulverisette 11, Fritsch, Industriestr 8, 55743 Idar-

Oberstaein, Germany) equipped with a 1 mm screen. To establish the proximate analyses, samples of white grape pomace (500 g) were analyzed for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF), ash, minerals, including copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), and antioxidant activity, including polyphenols and antioxidant capacity using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity. The entire quantity of WGP was hammer milled and included in the laying hens' diet. The proximate composition, mineral content, and antioxidant activity of white grape pomace are presented in Table 1.

Table 1. The proximate composition, mineral and vitamin content, and antioxidant capacity of white grape pomace.

Parameters	White Grape Pomace
Proximate composition	
Dry matter (DM), %	92.17
Crude protein (CP), %	7.27
Ether extract (EE), %	5.21
Crude fiber (CF), %	14.44
Ash, %	3.33
Mineral content	
Cooper (Cu), ppm	9.27
Iron (Fe), ppm	147.02
Manganese (Mn), ppm	14.57
Zinc (Zn), ppm	10.11
Vitamin E content	
Alpha-tocopherol (mg/kg)	68.93
Delta tocopherol (mg/kg)	6.46
Gamma tocopherol (mg/kg)	19.61
Vitamin E total (mg/kg)	95
Antioxidant activity	
Total phenolic contents (mg GAE/100 g)	1254.78
Total flavonoid contents (mg catechin/100 g)	883.94
Antioxidant activity (DPPH, μmol TE/100 g)	11,020.40
Antioxidant activity (ABTS, μmol TE/100 g)	7923.94

Note: GAE (gallic acid equivalents), TE (Trolox equivalent), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).

The high DM content of the WGP sample suggests that white grape pomace has relatively low moisture, which is beneficial for storage and stability. The high CF content can be attributed to the presence of skins, seeds, stems, and other components. Previous studies have reported total dietary fiber levels for red grape pomace ranging from 51% to 56%, while for white grape pomace, values were found to range between 17% and 28% [17].

2.3. Birds Performances, Housing, and Experimental Diets

The 6-week experiment involved 240 Lohmann Brown laying hens, aged 58 weeks, housed in three experimental rooms. Each room was assigned a Control (C) and an Experimental (E) group, resulting in a total of 6 Control and 6 Experimental groups. This 2×3 factorial design combined two dietary treatments (C and E) with three thermal conditions: normal, high stress, and low stress, ensuring that both diets were tested under each temperature condition, under permanent microclimate conditions controlled by a Big Dutchmann computer, with temperature and humidity parameters recorded

twice a day at 8:00 a.m. and 3:00 p.m. The registered daily average temperature and humidity were 22.44 °C ± 1.85 and 45% ± 1.02 for the normal temperature, NT, condition; 35 °C ± 1.50 and 63.88% ± 6.02 for the high-thermal-stress temperature, HST, condition, and 10 °C ± 1.50 and 40.00% ± 2.00 for the low-thermal-stress temperature, LST, condition.

Each thermal temperature experimental hall housed 80 birds, 40 birds/group, with 10 cages per group with 4 birds per cage (dimensions: front 610 mm; back: 745 mm; height: front 560 mm/back 450 mm; between levels: 688 mm; inclinations: 8°/14%). The lighting regime across all halls was maintained at 16 h of light per 24 h period. This setup was designed to analyze the effects of normal, high, and low thermal stress on the birds' overall productive performance and internal and external egg quality. Each cage was considered an experimental unit, and performance parameters were evaluated per pen. Feed and water were offered ad libitum during the entire experimental period. In the experimental room under high temperatures, the water was changed when it overheated, at 8 a.m. and again at 3 p.m. Each of the three groups from all experimental halls had a similar basal diet formulations shown in Table 2. Compared to the C diet, the E group included 6% white grape pomace. The diet structure was formulated using a specifically designed compound feed formulation software (Brill® Formulation, AGRIFOOD, Spain), in agreement with the feeding requirements of laying hens [18]. The diets were isoenergetic and isonitrogenous containing 18% CP and 2850.00 kcal/kg metabolizable energy (ME) per kg diet (Table 1). Average body weight (BW, g/hen) was measured at the beginning and the end of the experimental period. Daily production parameters were monitored and calculated: average daily feed intake (ADFI; g/hen/day); feed conversion rate (FCR; kg feed/kg egg); hen-day egg production (HDEP; %); average egg weight (AEW; g); and viability (%). No medical treatment was necessary or requested during the six-week experimental period. The difference between the amount of compound feed administered and the amount of leftover feed was used to calculate the ADFI parameter. The production parameter for FCR was given as kg of feed eaten for every kilogram of eggs produced. The HDEP parameter was calculated dividing the number of laid eggs divided by the number of hens and multiplied by 100.

Table 2. Diet formulation and proximal analysis results.

Specifications	C	E
Corn, %	39.06	49.66
Wheat, %	20.00	-
White grape pomace, %	-	6.00
Soybean meal, 46 CP %	26.33	28.25
Methionine, %	0.23	0.26
L-Threonine, %	0.01	0.01
Calcium carbonate, 38%	9.20	9.16
Monocalcium phosphate, %	0.56	0.66
Salt, %	0.37	0.37
Vegetal oil, %	3.19	4.58
Choline 60%	0.05	0.05
Premix *, 1%	1.00	1.00
Total ingredients, %	100.00	100.00
Calculated composition		
Metabolizable energy, kcal/kg	2850.00	2850.00
Dry matter, %	89.29	90.10
Crude protein, %	18.00	18.00
Crude digestible protein, %	15.00	14.50
Ether extract, %	5.53	7.14
Crude ash, %	2.26	2.58
Crude fiber, %	4.71	5.65
Calcium, %	4.19	4.19
Available phosphorus, %	0.38	0.38

Table 2. *Cont.*

Specifications	C	E
Calcium/Phosphorus	11.03	11.03
Sodium, %	0.18	0.18
Chloride, %	0.27	0.25
Lysine, %	0.89	0.90
Digestible lysine, %	0.80	0.81
Methionine, %	0.50	0.52
Digestible methionine, %	0.48	0.50
Methyionine + cysteine, %	0.85	0.80
Methyionine + cystine, %	0.72	0.72
Threonine, %	0.64	0.66
Tryptophan, %	0.20	0.19
Arginine, %	1.05	1.04
Linoleic acid (C18:2)	2.86	3.69
Metabolizable energy/Crude protein	158.33	158.33
Antioxidant analyzed composition		
Total vitamin E (mg/kg)	83.23	96.40
DPPH (mM echiv Trolox)	1.19	2.41
Total polyphenols, mg/g GAE	2.34	3.71

Note: * 1 kg Premix contains 1,100,000 IU/kg Vit. A; 200,000 IU/kg Vit. D3; 2700 IU/kg vit. E; 300 mg/kg Vit. K; 200 mg/kg Vit. B1; 400 mg/kg Vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg Vit. B6; 4 mg/kg Vit. B7; 100 mg/kg Vit. B9; 1.8 mg/kg Vit. B12; 2000 mg/kg Vit. C; 8000 mg/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium.

2.4. Egg Collection and Their Internal and External Quality Assessment

During the entire experimental period (2, 4, and 6 weeks, respectively), a total number of 324 eggs were collected randomly (18 eggs/group/period collection) to assess the internal and external quality parameters of the eggs. The following measurements were realized: the egg weight and its components (albumen, yolk, and shell) were measured using a Kern EW6000-1M Electronic Balance, with a precision of 0.001 (Kern & Sohn GmbH, D-72336 Balingen, Germany), the egg freshness (albumen height and Haugh unit calculation), and eggshell breaking strength, measured using a Digital Egg Tester DET-6500 (NABEL Co., Ltd., Kyoto, Japan). The eggshell thickness was measured within a range of 0.10–0.60 mm, with an accuracy of $\pm$ 0.02 mm, with the concave part of the eggshell side down, using a digital micrometer with a range of 0–10 mm, with a measuring force of 1.5 N or less (Mitutoyo 547-360 ABSOLUTE Digimatic Thickness Gauge). The pH of the albumen and yolk was measured using a portable pH meter (Five Go F2-Food kit with LE 427IP67, Sensor Metler Tolledo, Greifensee, Switzerland). To measure the yolk color, the DET6500 uses natural light (artificial light varies greatly between locations depending on its light source), being estimated based on the DSM's YolkFan^{TM}. The CIE-Lab system (Commission Internationale de l'Eclaraige) with a customized aperture (8 mm/4 mm/1 $\times$ 3 mm), 2.6 s measuring time, high accuracy of 0.04, and an observer angle of 2°/10° was used to measure egg yolk color parameters (L*, a*, and b*) using a portable colorimeter 3nh YS3020 (Shenzhen Threenh Technology Co., Ltd., Beijing, China). The lightness (L*) scale, ranging from 0 (perfect black) to 50 (mid-gray) and up to 100 (perfect white), along with the saturation index a* (indicating green with negative values, red with positive values, and neutral at 0) and the saturation index b* (negative values representing blue, positive values representing yellow, and neutral at 0) were assessed using CIE—L* a* b* color reflectance coordinates.

2.5. Chemical Analyses of Samples

2.5.1. Proximate Analyses

The nutrient concentration of the samples (raw materials, compound feed, and eggs) was analyzed using standardized methods: the gravimetric method (BMT model ECOCELL Blueline Comfort, Nuremberg, Germany) was used to determine the DM concentration;

the Kjeldahl method (Kjeltek auto 1030—Tecator (FOSS Tecator AB, Höganäs, Sweden)) was used to determine CP concentration; extraction in organic solvents (FOSS Tecator AB, Höganäs, Sweden) was used to determine the EE concentration; a method with intermediary filtration (Fibertec 2010 System—Foss Tecator, Sweden) was used for the CF concentration; the procedures described in Regulation (CE) No. 152/2009 (Nabertherm Labotherm L15/11/P320 Comfort (Bremen, Germany)) were used for crude ash determination.

2.5.2. Minerals Assessment

The minerals' concentration assessment, for zinc (Zn), iron (Fe), copper (Cu), and manganese (Mn) in the raw materials, compound feed, and eggs, was conducted using flame atomic absorption spectrometry [Thermo Electron—SOLAAR M6 Dual Zeeman Comfort [19]. The results were expressed as μg/g (ppm) of the dried sample.

2.5.3. Vitamin E Assessment

For vitamin E, determination was accomplished using an RP HPLC analytical method described by Vărzaru et al. [20]. A high-performance liquid chromatograph (HPLC Finnigan Surveyor Plus, Thermo-Electron Corporation, Waltham, MA, USA) and a PDA-UV (292 nm) with a Hypersil BDS C18 column, with silica gel and dimensions of 250 × 4.6 mm and a particle size of 5 μm (Thermo-Electron Corporation, Waltham, MA, USA), were used. Chromatographic parameters were as follows: flow rate of 1.5 mL/min and a mobile phase of 4% water, using 96% methanol.

2.5.4. Determination of Total Polyphenols Content

The total polyphenol content of the plants was spectrometrically determined using the Folin–Ciocalteu method, as described by Untea et al. [21]. The reading of absorbance was performed at 732 nm, and gallic acid was used for the calibration curve, with the results being expressed as mg of gallic acid equivalent per gram of sample.

2.5.5. Determination of 2,2-Diphenyl-1-picrylhydrazyl for Measuring Antioxidant Capacity

The antioxidant activity of raw materials, feed, and egg samples was measured using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity as described by Untea et al. [22]. The absorbance was read at 517 nm using a spectrophotometer (Jasco V-530, Japan Servo Co., Ltd., Tokyo, Japan). The results were expressed as mM eq Trolox after a standard calibration curve was constructed by plotting percentage inhibition against different Trolox concentrations.

2.6. Statistical Analysis

All data were subjected to analysis of variance using the GLM procedure of the Minitab software (version 17, Minitab® Statistical Software). The data obtained were analyzed by two-way ANOVA (Analysis of Variance) following the following statistical model:

$$Yijk = \mu + \alpha i + \beta j + \alpha i\beta j + eijk$$

where Yijk = the variable measured for the kth observation of the ith treatment and jth temperature; μ is the sample mean, and αi is the effect of the ith treatment; βj is the effect of temperature; αiβj = the interaction of ith treatment and jth temperature, and εijk is the effect of error. The differences were highly significant when $p < 0.001$ and significant if $p < 0.05$.

3. Results

3.1. Productive Performance Parameters

Table 3 shows the body weight measured at the beginning and the end of the experiment, under different thermal stress conditions (NT, HST, and HST) and two different diets (C and WGP).

Table 3. Effect of dietary WGP supplementation on laying hens' body weight at the beginning and the end of the experiment.

Specifications	NT		HST		LST		SEM	*p*-Value
	C	WGP	C	WGP	C	WGP		
Initial body weight (g/layer)	1610.28	1678.33	1648.33	1658.15	1660	1653.71	15.14	0.9386
Final body weight (g/layer)	1787.22 ^{cd}	1822.78 ^{cd}	1599.40 ^{abef}	1592.59 ^{abef}	1734.17 ^{cdf}	1849.38 ^{cde}	15.789	<0.0001

Note: C—control diet; WGP—control diet supplemented with 6% white grape pomace; SEM, standard error of the mean; $^{a–f}$ Mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$; NT (normal temperature); HST (high-stress temperature); LST (low-stress temperature).

The initial body weight of laying hens under NT, HST, and LST conditions registered no significant differences ($p = 0.9386$) between groups or thermal stress conditions. Significant differences ($p < 0.0001$) are observed in final body weights after 6 experimental weeks, indicating a statistically significant effect of the diets and temperature conditions. Under NT conditions, the laying hens from the C and WGP groups showed an increase in body weight (1787.22 g and 1822.78 g, respectively) compared to the initial weight (1610.28 g and 1678.33, respectively). In HST conditions, there was a significant decrease in final body weight for both the C (1599.40 g) and WGP (1592.59 g) groups, indicating that high stress negatively affected the weight gain of laying hens. Under LST conditions, the WGP group showed the highest final body weight (1849.38 g), suggesting that WGP might have a positive effect on weight gain under low-stress conditions compared to the C group.

The results obtained suggest that both temperature and group significantly influenced the final body weight of laying hens, especially HST which caused weight loss and WGP potentially enhancing the weight gain under LST conditions.

Table 4 presents the hens' production parameters data under different temperature conditions (NT, HST, and LST). At 2 weeks, under NT conditions, the ADFI parameter's values are highest ($p = 0.0001$) in the C group compared to the WGP group. Under HST or LST conditions, no significant differences ($p = 0.251$) can be observed between the two groups. The D×T interaction, evaluated at 2 weeks, registered highly significant differences ($p = 0.0001$) for the ADFI parameter.

Generally, a decrease in ADFI across all temperature conditions was observed, but hens under NT conditions with the WGP diet (121.23 g) still had a relatively high intake.

The ADFI evaluated at 4 and 6 weeks continues with the same trend, with hens in the NT condition with the C diet consistently exhibiting the highest ADFI, while hens under HST and LST conditions had significantly lower feed intakes. WGP supplementation slightly mitigated the reduction in feed intake under LST compared to HST conditions. The D×T interaction, evaluated at 4 weeks, registered significant differences ($p = 0.001$), while at 6 weeks a highly significant difference ($p = 0.0001$) was noticed, for ADFI parameter. It is important to note that, according to the Lohmann Tierzucht Management Guide [23], the average feed consumption for Lohmann Brown Classic hens ranges between 115 and 125 g/hen/day.

The hen-day egg production (HDEP) parameter at 2 weeks registered the highest value for the NT condition in the C group (93.25%) and was lower under HST, particularly in the C group. Under LST conditions, the WGP supplementation registered a higher percentage closer to the NT level. The D×T interaction, evaluated at 2 weeks, registered significant differences ($p = 0.010$) for the HDEP parameter.

At 4 weeks, the HDEP parameter was significantly higher ($p = 0.0001$) under NT and LST conditions compared to HST, with the highest values registered for the WGP groups compared to the C groups.

Table 4. Effect of dietary WGP supplementation on different thermal stress conditions on laying hens' productive performances.

Parameter	Period	Diet	NT	HST	LST	SEM	D	T	D×T
							p-Value		
ADFI (g/hen/day)	2 weeks	C	130.00 ^{a}	87.07 ^{d}	111.94 ^{c}	1.090	0.251	0.0001	0.0001
		WGP	121.23 ^{b}	93.64 ^{d}	119.49 ^{bc}				
	4 weeks	C	124.78 ^{a}	93.25 ^{d}	115.13 ^{c}	0.516	0.261	0.0001	0.001
		WGP	120.81 ^{b}	91.82 ^{d}	118.04 ^{bc}				
	6 weeks	C	122.70 ^{a}	94.73 ^{d}	109.41 ^{c}	0.608	0.052	0.0001	0.0001
		WGP	117.22 ^{b}	89.96 ^{e}	114.58 ^{b}				
HDEP (%)	2 weeks	C	93.25 ^{a}	88.48 ^{ab}	83.53 ^{b}	1.060	0.859	0.010	0.010
		WGP	90.48 ^{ab}	85.18 ^{b}	90.41 ^{ab}				
	4 weeks	C	95.63 ^{a}	86.27 ^{bc}	90.67 ^{ab}	0.877	0.170	0.0001	0.029
		WGP	96.43 ^{a}	84.39 ^{c}	96.91 ^{a}				
	6 weeks	C	94.84 ^{ab}	87.14 ^{c}	90.87 ^{bc}	0.832	0.083	0.0001	0.108
		WGP	97.22 ^{a}	85.98 ^{c}	95.85 ^{ab}				
AEW (g)	2 weeks	C	61.99 ^{b}	61.97 ^{b}	63.31 ^{b}	0.194	0.545	0.0001	0.0001
		WGP	62.28 ^{b}	59.97 ^{c}	64.93 ^{a}				
	4 weeks	C	64.27 ^{b}	59.75 ^{d}	62.94 ^{c}	0.139	0.0001	0.0001	0.0001
		WGP	64.89 ^{ab}	59.08 ^{d}	65.73 ^{a}				
	6 weeks	C	64.96 ^{a}	59.95 ^{c}	63.21 ^{b}	0.152	0.650	0.0001	0.0001
		WGP	64.89 ^{a}	58.12 ^{d}	65.40 ^{a}				
FCR (feed intake (kg)/egg production (kg))	2 weeks	C	2.36 ^{a}	1.64 ^{d}	2.14 ^{abc}	0.038	0.981	0.0001	0.011
		WGP	2.21 ^{ab}	1.87 ^{cd}	2.06 ^{bc}				
	4 weeks	C	2.15 ^{a}	1.89 ^{b}	2.08 ^{a}	0.024	0.0001	0.0001	0.054
		WGP	1.98 ^{ab}	1.87 ^{b}	1.87 ^{b}				
	6 weeks	C	2.15 ^{a}	1.85 ^{b}	1.95 ^{b}	0.022	0.0001	0.0001	0.057
		WGP	1.94 ^{b}	1.82 ^{b}	1.86 ^{b}				

Note: C—control diet; WGP—control diet supplemented with 6% white grape pomace; SEM, standard error of the mean; $^{a–e}$ Mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$; NT (normal temperature); HST (high-stress temperature); LST (low-stress temperature); D (diet); T (temperature); ADFI (average daily feed intake); HDEP (hen-day egg production); AEW (average egg weight); FCR (feed conversion rate).

The D×T interaction, evaluated at 4 weeks, registered significant differences ($p = 0.029$) for the HDEP parameter. At 6 weeks, the situation was similar to 4 weeks; under the NT and LST conditions, the WGP group registered the highest values compared to the C and WPG groups under HST. However, WGP supplementation under LST conditions (95.85%) registered values reaching those of the C and WGP groups for NT conditions. At 6 weeks, no significant differences ($p = 0.108$) were detected for the D×T interaction in the HDEP parameter.

The AEW at 2 weeks under LST conditions with WGP had the highest value (64.93 g), while those under HST conditions, particularly with the control diet (59.97 g), had the lowest AEW values. At 4 weeks of evaluation, a similar pattern is observed, with the LST condition with WGP hens producing heavier eggs compared to their NT and HST counterparts.

At 6 weeks, in LST conditions the WGP hens still show the highest AEW, indicating that WGP supplementation can positively affect egg weight under low-stress conditions.

For the AEW parameter, highly significant differences ($p = 0.0001$) were observed in the D×T interaction at 2 weeks, 4 weeks, and 6 weeks.

The lowest FCR was registered at 2 weeks under HST conditions with the control diet (1.64), indicating more efficient feed conversion. However, under NT conditions, FCR was highest compared to the C diet (2.36). At 4 and 6 weeks, the FCR generally improves (decreases) under WGP supplementation across all temperature conditions, particularly under heat stress, suggesting that WGP may help hens utilize feed more efficiently in stressful environments. A significant statistical difference for FCR concerning the D×T interaction was observed only at 2 weeks ($p = 0.011$). At 4 weeks ($p = 0.054$) and 6 weeks ($p = 0.057$), only a tendency was noted.

Table 5 presents the egg percentage distribution of different size categories (small, middle, large, and extra large) during the 6 experimental weeks. The percentage of small eggs (<53 g) remained consistently low across all experimental weeks, with a peak production of 7.96% registered in the 6th week. The middle-sized eggs (53–63 g) registered the highest percentages, with a peak of 69.75% registered in the 2nd week for the WGP group. There was a noticeable decrease in the percentage of middle-sized eggs as the weeks progressed, especially on the 6th week (35.82% for the C group in the NT condition and 35.82% for the WGP group in the LST condition in week 6).

Table 5. The egg size percentage distribution (small, middle, large, and extra large) during the experimental period.

Egg Size Classification	Period	NT		HST		LST	
		C	WGP	C	WGP	C	WGP
Small (<53 g), %	2 weeks	0.88	1.33	1.27	4.46	0.72	0.45
	4 weeks	1.75	-	2.70	7.37	1.80	0.22
	6 weeks	-	-	4.31	7.96	0.84	0.43
Middle (53–63 g), %	2 weeks	61.23	62.67	57.46	69.75	46.17	35.23
	4 weeks	39.30	41.42	72.64	66.67	52.93	31.25
	6 weeks	35.17	41.11	71.38	69.32	51.46	35.82
Large (63–73 g), %	2 weeks	37.00	36.00	40.63	25.80	50.96	60.00
	4 weeks	52.40	54.81	24.32	25.32	42.79	62.07
	6 weeks	58.05	54.55	23.38	22.12	44.56	57.14
Extra large (>73 g), %	2 weeks	0.88	-	0.63	-	2.15	4.32
	4 weeks	6.55	3.77	0.34	0.64	2.48	6.47
	6 weeks	6.78	4.35	0.92	0.59	3.14	6.61

Note: C—control diet; WGP—control diet supplemented with 6% white grape pomace; NT (normal temperature); HST (high-stress temperature); LST (low-stress temperature); G (group); T (temperature).

The proportion of large eggs (63–73 g) increased throughout the experiment from the 2nd week, with the percentage ranging from 25.80% (WGP group—HST) to 62.07% (WGP group—LST), increasing by the 6th week, with values between 22.12% (WGP group—HST) and 58.05% (C group—NT). The extra-large eggs (>73 g) registered a low percentage but increased gradually over time, with the highest peak production registered on the 4th week for the C group in NT conditions and on the 6th week for the C group at 6.78%, and also a high percentage of extra-large eggs was noticed for the WGP group with LST conditions.

3.2. External and Internal Egg Quality Parameter

Table 6 presents the results for the external egg traits of the two groups and thermal stress conditions. The shape index values at 2 weeks registered no significant differences between groups or temperature ($p > 0.05$); at 4 weeks, no significant effects ($p > 0.05$) were noticed, and at 6 weeks, a significant interaction effect ($p = 0.013$) suggests that the combined effect of group and temperature significantly influences the shape index. Concerning the whole egg weight parameter at 2 weeks, significant differences were registered between groups ($p = 0.031$), temperatures ($p < 0.0001$), and their interaction

($p = 0.006$), with significantly higher egg weight values for the C and WPG groups under NT and WGP with LST conditions compared to both groups for the HST condition and the C group with LST conditions.

Table 6. Effect of dietary WGP supplementation on different thermal stress conditions (NT, HST, and LST) on external egg quality parameters.

Parameter	Period	NT		HST		LST		SEM	*p*-Value		
		C	WGP	C	WGP	C	WGP		D	T	D×T
Shape index	2 weeks	88.98	88.42	88.38	88.78	87.68	87.53	0.321	0.823	0.104	0.689
	4 weeks	88.42	88.55	88.80	87.75	88.12	87.79	0.247	0.239	0.464	0.386
	6 weeks	86.58	87.69	87.80	86.32	87.72	87.85	0.251	0.824	0.193	0.013
Whole egg weight (g)	2 weeks	63.48 ^{ab}	62.42 ^{abc}	58.68 ^{d}	59.95 ^{cd}	61.07 ^{bcd}	64.60 ^{a}	0.398	0.031	0.0001	0.006
	4 weeks	62.41	62.46	62.04	61.30	62.73	63.41	0.489	0.996	0.262	0.704
	6 weeks	62.89 ^{ab}	63.58 ^{a}	60.88 ^{bc}	60.25 ^{c}	63.37 ^{ab}	64.13 ^{a}	0.357	0.586	0.0001	0.452
Eggshell weight (g)	2 weeks	8.47 ^{a}	8.54 ^{a}	7.15 ^{c}	7.35 ^{bc}	8.20 ^{ab}	8.63 ^{a}	0.120	0.173	0.0001	0.681
	4 weeks	7.73 ^{abc}	7.97 ^{ab}	7.26 ^{bc}	6.94 ^{c}	8.35 ^{a}	7.88 ^{ab}	0.116	0.270	0.0001	0.188
	6 weeks	7.99 ^{a}	8.30 ^{a}	7.16 ^{c}	7.36 ^{bc}	7.71 ^{abc}	7.84 ^{ab}	0.083	0.074	0.0001	0.824
Breaking strengths (kgF)	2 weeks	4.73	4.06	4.02	4.07	4.58	4.21	0.227	0.309	0.589	0.654
	4 weeks	4.06 ^{ab}	4.46 ^{ab}	4.50 ^{ab}	3.25 ^{b}	5.14 ^{a}	4.75 ^{a}	0.177	0.108	0.004	0.037
	6 weeks	4.25	4.39	4.20	3.90	4.61	4.63	0.152	0.825	0.102	0.694
Shell thickness (mm)	2 weeks	0.41	0.41	0.39	0.39	0.41	0.40	0.008	0.661	0.429	0.982
	4 weeks	0.39 ^{ab}	0.41 ^{a}	0.37 ^{b}	0.38 ^{ab}	0.39 ^{ab}	0.38 ^{ab}	0.006	0.176	0.069	0.147
	6 weeks	0.41	0.41	0.38	0.38	0.38	0.40	0.004	0.364	0.002	0.570

Note: C—control diet; WGP—control diet supplemented with 6% white grape pomace; SEM, standard error of the mean; $^{a–d}$ Mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$; NT (normal temperature); HST (high-stress temperature); LST (low-stress temperature); D (diet); T (temperature).

At 4 and 6 weeks, significant temperature effects ($p < 0.0001$) were noticed but with no significant interaction effects, suggesting temperature has a consistent effect on egg weight regardless of the group. For the eggshell weight parameter, it was observed that temperature resulted in significant effects at all periods ($p < 0.0001$), indicating a strong influence of temperature on eggshell weight, with no significant group or interaction effects. The breaking strength parameter at 4 weeks registered significant temperature and interaction effects ($p < 0.05$), suggesting that specific conditions impact eggshell strength. The shell thickness parameter at 6 weeks showed a significant temperature effect ($p = 0.002$), indicating that temperature influences shell thickness over time.

Table 7 presents the results for internal egg traits for the C and WGP groups under varying thermal stress conditions. For the albumen weight parameter at 2 weeks, a significant interaction effect of group and temperature ($p = 0.033$) was registered. At 4 and 6 weeks, a significant temperature effect at 6 weeks ($p = 0.017$) was registered with the highest values for the WPG group in NT and LTS conditions. The yolk weight registered significant differences across all weeks across groups ($p = 0.0001$ at 2 weeks; $p = 0.035$ at 4 weeks; $p = 0.028$ at 6 weeks) and temperatures ($p < 0.002$ for HST and $p < 0.0001$ for NT and LTS), indicating that both factors consistently affect yolk weight. The pH levels for the albumen show some significant temperature effects at 4 and 6 weeks ($p < 0.05$), suggesting that changes in albumen pH are temperature-dependent over time. The pH yolk showed significant temperature effects at 2 weeks and 6 weeks ($p \leq 0.025$), with an interaction effect at 2 weeks ($p = 0.016$), indicating that yolk pH is affected by both temperature and the combination of group and temperature. The albumen height registered no significant differences across groups, temperatures, or interactions, suggesting this parameter remains stable. At 2 and 4 weeks, the yolk fan color registered significant group differences ($p \leq 0.015$) and temperature differences ($p < 0.05$), indicating that color is influenced by both factors. At 6 weeks, a strong temperature effect ($p = 0.010$) and group effect ($p < 0.0001$) showed the continued influence of these factors over time. The Haugh unit parameter had no significant differences, indicating that the overall quality index remains unaffected by

the variables measured. The yolk height differences were noticed at various periods, with an interaction effect at 2 weeks ($p = 0.017$), indicating that a specific combination of factors affects yolk height. The yolk diameter registered significant differences observed mainly as temperature and group effects ($p < 0.05$), showing that these factors impact yolk size. The yolk index presented significant effects in the interaction at 2 weeks ($p = 0.015$) and at the group level at 6 weeks ($p = 0.0001$), suggesting that combined effects of group and temperature alter the yolk index. For albumen and yolk temperature, both parameters exhibited significant differences across all weeks (p-values < 0.05 for group, temperature, and interaction in some cases), indicating that the temperature of the egg components is highly influenced by the experimental conditions.

Table 7. Effect of dietary WGP supplementation on different thermal stress conditions (NT, HST, and LST) on internal egg quality parameters.

Parameter	Period	NT		HST		LST		SEM	*p*-Value		
		C	WGP	C	WGP	C	WGP		D	T	D×T
Albumen weight (g)	2 weeks	39.36	37.43	37.14	37.15	37.30	38.86	0.373	0.821	0.143	0.033
	4 weeks	39.37	38.08	40.03	39.22	38.27	38.88	0.469	0.457	0.383	0.482
	6 weeks	38.71	38.52	38.41	37.61	39.68	39.36	0.302	0.305	0.017	0.826
Yolk weight (g)	2 weeks	15.65 ^{bc}	16.45 ^{ab}	14.39 ^{c}	15.45 ^{bc}	15.57 ^{bc}	17.11 ^{a}	0.198	0.0001	0.0001	0.553
	4 weeks	15.31 ^{ab}	16.41 ^{a}	14.75 ^{b}	15.14 ^{ab}	16.11 ^{ab}	16.65 ^{a}	0.221	0.035	0.002	0.622
	6 weeks	16.19 ^{ab}	16.77 ^{a}	15.31 ^{b}	15.28 ^{b}	15.97 ^{ab}	16.93 ^{a}	0.160	0.028	0.0001	0.207
pH albumen	2 weeks	8.66	8.70	8.79	8.73	8.57	8.60	0.050	0.974	0.145	0.823
	4 weeks	8.81 ^{ab}	8.79 ^{ab}	8.81 ^{ab}	8.84 ^{a}	8.49 ^{b}	8.74 ^{ab}	0.047	0.211	0.025	0.220
	6 weeks	8.59 ^{b}	8.62 ^{ab}	8.64 ^{ab}	8.68 ^{ab}	8.14 ^{ab}	8.86 ^{a}	0.035	0.366	0.0001	0.987
pH yolk	2 weeks	6.48 ^{ab}	6.37 ^{ab}	6.53 ^{a}	6.50 ^{ab}	6.36 ^{b}	6.48 ^{ab}	0.022	0.873	0.025	0.016
	4 weeks	6.48	6.49	6.75	6.57	6.51	6.71	0.052	0.875	0.137	0.128
	6 weeks	6.42	6.40	6.67	6.57	6.53	6.44	0.043	0.244	0.019	0.826
Ht (mm)	2 weeks	7.13	6.50	6.73	6.06	6.78	6.76	1.270	0.453	0.326	0.295
	4 weeks	7.05	6.87	6.98	6.66	6.61	6.93	0.165	0.793	0.794	0.494
	6 weeks	7.01	6.54	6.26	6.47	6.83	6.68	0.145	0.511	0.202	0.417
Yolk fan color	2 weeks	4.90 ^{b}	6.00 ^{a}	5.00 ^{b}	5.60 ^{ab}	5.50 ^{ab}	6.10 ^{a}	0.102	0.0001	0.019	0.269
	4 weeks	5.00 ^{ab}	5.40 ^{ab}	4.50 ^{b}	5.10 ^{ab}	5.30 ^{ab}	5.70 ^{a}	0.131	0.015	0.013	0.880
	6 weeks	5.17 ^{b}	6.50 ^{a}	4.76 ^{b}	6.06 ^{a}	4.83 ^{b}	6.33 ^{a}	0.078	0.0001	0.010	0.721
Haugh unit	2 weeks	82.40	77.88	81.66	77.07	81.74	80.26	1.590	0.122	0.838	0.812
	4 weeks	83.01	82.10	82.59	80.82	79.62	81.86	1.090	0.924	0.630	0.530
	6 weeks	82.56	78.87	78.28	79.34	80.18	79.96	1.090	0.544	0.601	0.438
Yolk height (mm)	2 weeks	19.14 ^{ab}	18.93 ^{ab}	18.37 ^{ab}	17.99 ^{b}	17.93 ^{b}	20.18 ^{a}	0.287	0.178	1.142	0.017
	4 weeks	19.30 ^{a}	18.52 ^{ab}	18.70 ^{ab}	18.49 ^{ab}	18.04 ^{ab}	17.68 ^{b}	0.210	0.132	0.016	0.721
	6 weeks	18.06	18.36	17.70	18.30	18.85	18.96	0.221	0.288	0.053	0.812
Yolk diameter (mm)	2 weeks	39.51	39.28	37.17	39.68	39.82	39.58	0.433	0.273	0.216	0.117
	4 weeks	39.23 ^{ab}	41.81 ^{a}	38.60 ^{ab}	38.33 ^{b}	39.63 ^{ab}	40.46 ^{ab}	0.446	0.099	0.025	0.184
	6 weeks	39.72 ^{ab}	40.45 ^{ab}	42.89 ^{a}	34.71 ^{b}	38.81 ^{ab}	40.43 ^{ab}	1.02	0.181	0.763	0.012
Yolk index	2 weeks	0.48	0.48	0.49	0.45	0.46	0.51	0.009	0.811	0.764	0.015
	4 weeks	0.49	0.45	0.48	0.48	0.46	0.44	0.008	0.050	0.026	0.211
	6 weeks	0.45 ^{b}	0.46 ^{b}	0.44 ^{b}	0.55 ^{a}	0.50 ^{ab}	0.47 ^{b}	0.010	0.055	0.059	0.0001
Albumen temperature	2 weeks	11.86 ^{ab}	12.40 ^{a}	11.06 ^{ab}	10.58 ^{b}	12.15 ^{a}	7.77 ^{c}	0.195	0.0001	0.0001	0.0001
	4 weeks	9.88 ^{d}	10.96 ^{c}	9.29 ^{d}	9.70 ^{d}	16.90 ^{a}	14.19 ^{b}	0.148	0.057	0.0001	0.0001
	6 weeks	15.48 ^{a}	14.47 ^{ab}	13.30 ^{bc}	14.15 ^{ab}	10.98 ^{d}	11.86 ^{cd}	0.215	0.433	0.0001	0.017
Yolk temperature	2 weeks	11.11 ^{ab}	11.33 ^{ab}	10.09 ^{b}	9.82 ^{b}	12.55 ^{a}	7.32 ^{c}	0.256	0.0001	0.007	0.0001
	4 weeks	10.36 ^{c}	12.35 ^{b}	9.92 ^{c}	9.96 ^{c}	16.46 ^{a}	14.98 ^{a}	0.206	0.533	0.0001	0.0001
	6 weeks	14.21 ^{a}	14.38 ^{a}	12.13 ^{b}	12.44 ^{b}	10.17 ^{c}	11.72 ^{b}	0.200	0.019	0.0001	0.098

Note: C—control diet; WGP—control diet supplemented with 6% white grape pomace; SEM, standard error of the mean; $^{a–d}$ Mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$; NT (normal temperature); HST (high-stress temperature); LST (low-stress temperature); D (diet); T (temperature).

3.3. Yolk Color

The effects of the apple and white grape pomaces dietary supplementation under different temperature stress are shown within Table 8. The L* values at 2 weeks significantly differ between groups ($p < 0.0001$) and temperatures ($p < 0.0001$). The NT-C and LST-C groups had the highest L* values, indicating a lighter color, while the HST-WGP group had the lowest, indicating a darker color. The interaction between diet and temperature D×T is not significant ($p = 0.281$). At 4 weeks, there are significant differences in L* values between groups ($p < 0.0001$) and temperatures ($p = 0.004$). The LST-C group shows the highest lightness value (L* = 46.18), meaning the lightest yolk color, while the HST-WGP group shows the lowest value (L* = 43.39), which indicates the darkest yolk color. There was no significant interaction effect ($p = 0.235$) of diet and temperature. At 6 weeks, a significant group effect ($p = 0.025$) shows differences in L* values between groups, with the NT-C group recording the highest lightness values compared to the WGP-HST group which recorded the darkest yolk color. No significant differences for temperature ($p = 0.064$) and the D×T interaction ($p = 0.139$) were registered, suggesting that neither temperature nor the D×T interaction significantly affects L* at 6 weeks.

Table 8. Effect of dietary WGP supplementation under different thermal stress conditions (H, HST, and LST) on yolk color.

Parameter	Period	NT		HST		LST		SEM	*p*-Value		
		C	WGP	C	WGP	C	WGP		D	T	D×T
L*	2 weeks	44.59 ^{a}	43.68 ^{ab}	43.42 ^{ab}	41.59 ^{c}	44.79 ^{a}	42.78 ^{bc}	0.212	0.0001	0.0001	0.281
	4 weeks	44.53 ^{bc}	43.77 ^{bc}	44.93 ^{ab}	43.39 ^{c}	46.18 ^{a}	44.21 ^{bc}	0.206	0.0001	0.004	0.235
	6 weeks	45.17 ^{a}	44.39 ^{ab}	44.57 ^{ab}	43.70 ^{b}	44.45 ^{ab}	44.58 ^{ab}	0.160	0.025	0.064	0.139
a*	2 weeks	0.69 ^{b}	1.41 ^{a}	0.58 ^{b}	1.27 ^{a}	0.87 ^{b}	1.38 ^{a}	0.046	0.0001	0.048	0.369
	4 weeks	0.47 ^{b}	0.62 ^{ab}	0.09 ^{c}	0.57 ^{ab}	0.64 ^{ab}	0.87 ^{a}	0.046	0.0001	0.0001	0.101
	6 weeks	0.35 ^{b}	1.59 ^{a}	0.27 ^{b}	1.53 ^{a}	0.21 ^{b}	1.50 ^{a}	0.036	0.0001	0.155	0.934
b*	2 weeks	14.84 ^{b}	17.96 ^{a}	14.47 ^{b}	15.29 ^{b}	15.11 ^{b}	17.63 ^{a}	0.210	0.0001	0.0001	0.005
	4 weeks	15.89 ^{a}	15.36 ^{a}	13.42 ^{b}	15.49 a	15.18 ^{a}	16.21 ^{a}	0.190	0.002	0.0001	0.0001
	6 weeks	14.41 ^{d}	17.09 ^{a}	14.48 ^{d}	16.44 ^{ab}	14.98 ^{cd}	15.63 ^{bc}	0.161	0.0001	0.274	0.001

Note: C—control diet; WGP—control diet supplemented with 6% white grape pomace; SEM, standard error of the mean; $^{a–d}$ Mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$; NT (normal temperature); HST (high-stress temperature); LST (low-stress temperature); D (diet); T (temperature). L* lightness scale, ranges from 0 (perfect black) to 50 (mid-gray) and 100 (perfect white); the saturation index a* (indicates green for negative values, red for positive values, and neutral at 0); the saturation index b* (negative values represents blue, positive values represent yellow, and neutral at 0).

The a* parameter at 2 weeks registered significant differences between groups ($p < 0.0001$) and temperatures ($p = 0.048$). Groups like NT-WGP and HST-WGP had higher a* values (a redness intensification of yolk color), compared to the NT-C and HST-C groups which registered lower values. No significant interaction ($p = 0.369$) was registered, indicating that the D×T interaction does not significantly affect the a* parameter at the 2-week period. At 4 weeks, both group ($p < 0.0001$) and temperature ($p < 0.0001$) have significant effects on a* parameter values. The LST-WGP group has the highest redness (a* = 0.87), while the HST-C group has the lowest (a* = 0.09). There was no significant interaction effect ($p = 0.101$). At 6 weeks, for the a* parameter, a significant group effect ($p < 0.0001$) shows statistical differences among groups, with the NT-WGP, HST-WGP, and LST-WGP groups showing higher redness yolk intensification. Temperature ($p = 0.155$) and the D×T interaction ($p = 0.934$) registered no significant effects.

For the b* parameter, at 2 weeks, it is noticed that all effects are significant: group ($p < 0.0001$), temperature ($p < 0.0001$), and D×T interaction ($p = 0.005$). This indicates that yellowness is highly influenced by group, temperature, and the D×T combination. The NT-WGP and LST-WGP groups showed the highest yellowness values, while the NT-C and HST-C groups showed lower values. At 4 weeks, the group ($p = 0.002$), temperature

($p < 0.0001$), and D×T interaction ($p < 0.0001$) all exhibited significant effects on b* values. The HST-WGP group registered the highest yellowness value compared to group HST-C. At 6 weeks, the group ($p < 0.0001$) and D×T interaction ($p = 0.001$) registered high statistical differences in yellowness yolk color, with the NT-WGP group showing the highest b* value.

3.4. Proximate Composition and Oxidative Stability of Yolk Under Different Thermal Stress Conditions

As presented in Table 9, no significant differences ($p > 0.05$) were found for DM%, with values ranging from 51.50% in the HST-WGP group to 52.15% in the LST-WGP group, regardless of diet or temperature effects. The highest CP content, which was statistically significant ($p = 0.0001$), was observed under LST conditions in both the C and WGP groups compared to the WGP group under HST conditions. For ash concentration, highly significant differences ($p = 0.0001$ for temperature; $p = 0.015$ for the D×T interaction) were observed in the LST-WGP group compared to the NT-C, NT-WGP, and HST-WGP groups.

Table 9. Effect of dietary WGP supplementation under different thermal stress conditions (NT, HST, and LST) on yolk proximate composition and its oxidative stability.

Parameter	NT		HST		LST		SEM	*p*-Value		
	C	WGP	C	WGP	C	WGP		D	T	D×T
DM%	51.88	51.80	51.51	51.50	51.57	52.15	0.106	0.297	0.103	0.161
CP%	17.09 ^{ab}	17.05 ^{ab}	16.85 ^{ab}	16.80 ^{b}	17.26 ^{a}	17.27 ^{a}	0.0566	0.745	0.0001	0.937
EE%	27.98	28.03	27.85	27.89	27.87	28.29	0.0885	0.178	0.408	0.376
Ash%	1.56 ^{bc}	1.54 ^{bc}	1.63 ^{ab}	1.52 ^{c}	1.64 ^{ab}	1.68 ^{a}	0.0135	0.132	0.0001	0.015

Note: C—control diet; WGP—control diet supplemented with 6% white grape pomace; SEM, standard error of the mean; $^{a–c}$ Mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$; NT (normal temperature); HST (high-stress temperature); LST (low-stress temperature); D (diet); T (temperature).

Figure 1 presents the total polyphenol concentrations which registered highly significant differences ($p = 0.0001$), with the highest polyphenol content being recorded in the NT-C and WGP groups with values of 1.63 and 1.59, respectively, significantly higher values compared to HST-C group (1.36) and LST-WGP group (1.34). No significant effects of diet ($p = 0.833$) or the D×T interaction ($p = 0.244$) were observed.

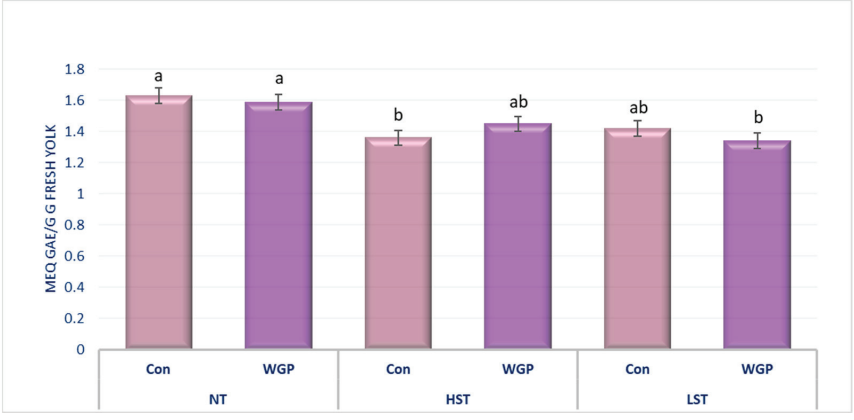

Figure 1. Total polyphenol concentration determined in yolk samples (mEq GAE/g g fresh yolk); ^{a,b} Mean values not sharing the same superscripts are significantly different at $p < 0.05$.

Figure 2 displays the DPPH results, with highly significant D×T interaction effects ($p = 0.0001$), where the highest antioxidant capacity was observed in the NT-WGP group

(3.47). The HST-WGP group recorded lower DPPH values (3.22) compared with the NT-WGP group, while the LST-C group recorded the lowest antioxidant capacity (2.02). Neither diet (p = 0.059) nor temperature (p = 0.058) independently influenced DPPH concentration.

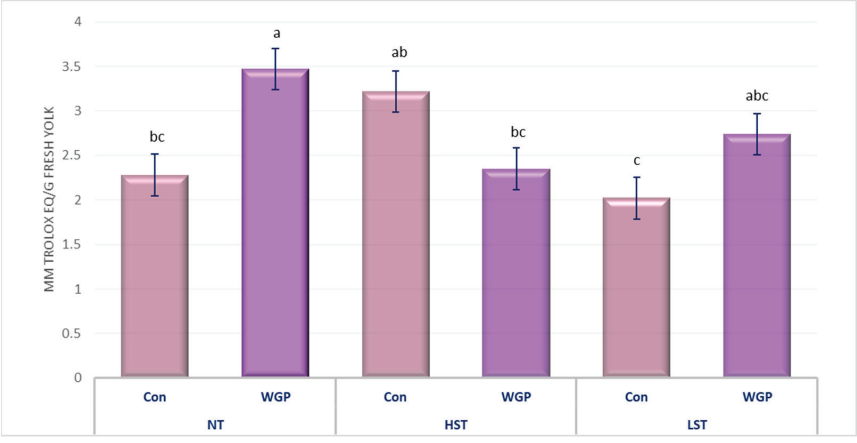

Figure 2. DPPH concentration determined in yolk samples (mM Trolox Eq/g fresh yolk), [a–c] Mean values not sharing the same superscripts are significantly different at $p < 0.05$.

4. Discussions

4.1. Productive Performance Parameters

Under HST conditions, both the C and WGP groups registered a significant decreasing in final body weight compared to all other groups under NT and LST conditions, highlighting the adverse impact of high temperatures on weight gain, likely due to reduced feed intake and increased energy expenditure for heat dissipation as an adaptive method [24]. Mashaly et al. [25] found similar findings in heat-stressed laying hens, reporting a significant reduction in both body weight (19%) and feed consumption between C and E groups. A recent study [26] noticed that high temperature–humidity index levels (>81) significantly reduced laying hens' body weight. Interestingly, in our study, under LST conditions, the WGP group outperformed the C group, suggesting a beneficial effect of WGP supplementation in colder environments. Based on these results, we assumed that WGP supplementation in laying hens' diet may positively influence metabolic efficiency, which contributed to the observed weight gain. Our hypothesis is supported by the antioxidants' and fibers' beneficial effects present in WGP, which could potentially help the body's adaptation to low temperatures. Zhou et al. [27] reported that a cold tolerance test (4 °C metabolism chamber) performed on mice indicated that dietary high concentrations of grape seed flour (54.04 mg/g) could be beneficial in maintaining body temperature suggesting that an increased energy expenditure positively correlated with more heat production. Also, Gollucke PB [28] stated that grape juice provides high energy and fructose intake that may lead to body weight gain.

Regardless of the thermal stress conditions, Goni et al. [29] reported that incorporating grape pomace into laying hen diets improved antioxidant status but had minimal effects on body weight. Brenes et al. [30] found that grape seed extract did not significantly affect body weight in broilers but improved antioxidant capacity. Previous studies [31] suggested that antioxidant supplementation can alleviate some negative effects of heat stress on poultry performance. Reis et al. [32] declared that a 1–3% addition of grape pomace flour in the diet of laying hens can be beneficial for their health and performance, particularly in enhancing egg production and quality, while also providing antioxidant benefits. Dietary inclusion of 4% and 6% grape pomace in laying hen diets had no significant effects on weight, feed intake, or egg production [33]. Similar findings were indicated by Dae Kim et al. [34],

who observed that low temperatures in laying hens significantly affect final body weight, feed intake, and FCR. Hafeez et al. [35] reported that incorporating grape seed extract at levels of 250–750 g/kg into laying hens' diets under normal temperature conditions did not significantly influence feed intake, body weight gain, or FCR. In contrast, Sayago-Ayerdi et al. [36] found that including grape seed byproducts above 6% in laying hens' diets under similar conditions negatively impacted feed intake. In our study, under NT conditions, the WGP group exhibited significantly lower ADFI, likely due to the increased dietary CF content. In contrast, under HST and LST conditions, ADFI did not differ significantly between the C and WGP groups across all periods, except at the 6th week under LST, where the WGP group showed a significantly higher ADFI. Overall, temperature and the D×T interaction had a highly significant effect on ADFI across all periods (2nd, 4th, and 6th weeks). Our findings align with previous research demonstrating that heat stress adversely affects feed intake in poultry. De Souza et al. [37] observed that broilers exposed to continuous or cyclical heat stress at 32 °C experienced a reduction in feed intake ranging from 25% to 45%. Similarly, Mitchell and Carlisle [38] reported a 29% decrease in feed intake and a 20% reduction in body weight in domestic fowl subjected to 35 °C for 14 days. These studies corroborate our results, highlighting the significant impact of elevated temperatures on poultry feed consumption and growth performance. The HDEP parameter was highly influenced by HST conditions on both C and WGP groups (especially at the 4th and 6th weeks) compared to NT and LST conditions. While no significant differences were observed between the C and WGP groups under LST conditions, the WGP group exhibited a slight increase in HDEP across all three periods suggesting a potential positive influence of WGP supplementation under LST conditions. This improvement may be attributed to enhanced nutrient absorption, as reported by Hosseini Vashan et al. [39], who found that including grape pomace at levels of 20, 40, and 60 g/kg in the diets of heat-stressed broilers (exposed to 37 °C for 6 h daily from days 25 to 42) improved nutrient absorption.

Similarly, Selim et al. [40] reported that incorporating grape pomace at 3%, 6%, and 9% into laying hens' diets under normal temperature conditions led to increased egg production rates and egg weights. The FCR value under NT conditions was higher, indicating less efficient feed utilization compared to HST or LST conditions. On the other hand, a lower FCR value for HST conditions is most probably due to reduced feed intake and egg production in both groups.

4.2. External and Internal Egg Quality Parameters

The external and internal egg quality parameters data showed that some egg quality traits, including weight, pH, strength, and temperature, were significantly influenced by both group and temperature. The interaction between these factors also plays a role, especially for parameters like yolk weight, pH, and shell thickness, indicating complex interactions affecting egg quality.

Our study's results indicate that HST conditions adversely affected whole egg weight, eggshell weight, and shell thickness compared to NT and LHS conditions. Similar results were reported in heat stress conditions by [25] which registered a significant decline in egg quality, including reductions in egg weight, shell weight, shell thickness, and specific gravity.

Kim et al. [4], under low temperature conditions of 12 ± 4.5 °C, found no significant ($p > 0.05$) differences concerning the egg weight, mass, and production.

Sahin et al. [41] found that dietary antioxidants improved egg production and quality under heat stress. Kara et al. [33] observed that incorporating 4% grape pomace into the diets of laying hens under normal temperature conditions led to a significant egg weight increase, while other egg quality parameters remained unchanged. According to Herranz et al. [13], a supplementation of 50 g/kg of grape pomace in laying hens' diet significantly enriched the egg yolks with gallic acid, improved albumen Haugh units and yolk color scores, and reduced the eggshell thickness. Kim et al. [26] observed a reduced eggshell thickness and strength and Haugh units under severe heat stress (33 °C). Additionally,

Attallah et al. [42] stated that the antioxidant properties of grape pomace (0.5%, 1%) may help mitigate oxidative stress, which can be exacerbated by heat, potentially leading to improved yolk quality.

Our results showed that WGP supplementation can enhance some productive performance metrics, especially under LST conditions.

4.3. Yolk Color

The data obtained in our study showed that all color parameters (L*, a*, and b*) were influenced by diet and temperature effects to varying extents, with significant group effects during all periods for each parameter. The D×T interaction effects are less significant, compared to both group and temperature. The L* parameter generally shows significant changes due to group and temperature, and the a* parameter is more influenced within groups, with temperature also exerting an influence at certain periods, whereas the b* parameter is significantly influenced by group, temperature, and their interaction, particularly noticeable in earlier weeks. The addition of WGP to the diet of laying hens has been found to improve the yolk color score, a benefit that is especially significant under heat-stress conditions. Research indicates that diets supplemented with grape pomace significantly increased yolk color scores, with one study reporting a 9.36% increase when 50 g/kg of grape pomace was included in the diet [13]. While specific studies on heat stress were not detailed, the overall benefits of grape pomace in enhancing yolk color and reducing lipid peroxidation suggest a positive impact on egg quality during stressful conditions [32,33]. Dae-Kim [34] reported an intensification of eggshell color under low thermal stress compared to normal temperatures, whereas Kim et al. [26] found that heat stress (33 °C) significantly diminished yolk color. Ronceros et al. [43] found that a 5% grape pomace inclusion produced darker, redder yolks, while higher inclusions (7% and above) resulted in lighter yolks with diminished yellow and red tones.

4.4. Antioxidant Capacity of Eggs Obtained Under Different Thermal Stress Conditions

Numerous studies have shown that polyphenols from grape pomace possess strong antioxidant properties that help alleviate the oxidative stress caused by extreme thermal stress conditions in poultry, reducing lipid peroxidation and enhancing overall antioxidant capacity [44]. In our study, the highest polyphenol concentrations were observed under normal temperature (NT) conditions. Both the HST and LST conditions registered lower polyphenol concentrations. Neither the type of WGP supplementation diet nor the D×T interaction significantly influenced the polyphenol content. The highest DPPH value recorded in the NT-WGP group suggested that NT conditions combined with the WGP diet (rich in polyphenols) promoted a higher antioxidant capacity in the yolk. The DPPH value observed for LST conditions was higher in the WPC group compared to the C group but without significant statistical differences. Reis et al. [32] found that a 3% dietary inclusion of grape pomace prevented the reduction in monounsaturated fatty acids in the yolk of stored eggs compared to the control group. Additionally, laying hens fed grape pomace (at 1%, 2%, or 3% inclusion levels) exhibited higher glutathione peroxidase and superoxide dismutase activities, along with greater total antioxidant capacity against peroxyl radicals in their serum, while showing the lowest levels of lipid peroxidation compared to the C group. Also, red grape pomace has been linked to increased antioxidant levels in eggs, which may mitigate oxidative stress associated with high temperatures. Studies indicate that dietary GP can increase the deposition of phenolic compounds in eggs, suggesting a transfer of these antioxidants from the diet to the egg [13]. Additionally, the antioxidant properties of grape pomace may help maintain the integrity of egg lipids during storage, thereby enhancing the nutritional value of eggs produced under heat stress [40]. Affordable and country-specific feed ingredients with unique properties, bioactive properties, and adequate inclusion levels can help alleviate thermal stress in laying hens [45,46].

5. Conclusions

This study aimed to explore the potential of locally available white grape pomace, a waste byproduct rich in antioxidants and polyphenols, as a dietary inclusion for laying hens under normal, heat, and cold thermal conditions. While the inclusion of 6% white grape pomace did not significantly impact productive performance or egg quality parameters overall, it demonstrated promising benefits under low thermal stress conditions for the following: average egg weight, hen-day egg production, eggshell weight, breaking strength, yolk weight, a* color parameter, and DPPH value. These findings highlight the potential of white grape pomace as a functional feed ingredient, particularly in mitigating the challenges of low thermal stress in poultry production.

Author Contributions: Conceptualization, T.D.P. and G.M.C.; methodology, T.D.P., G.M.C., A.E.C., A.E.U., M.S. and I.V.; software, T.D.P.; validation, T.D.P.; formal analysis, A.E.C., A.E.U., M.S. and I.V.; investigation, G.M.C. and A.E.C.; resources, G.M.C.; data curation, T.D.P.; writing—original draft preparation, G.M.C.; writing—review and editing, T.D.P. and G.M.C.; visualization, T.D.P. and A.E.C.; supervision, T.D.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministry of Agriculture and Rural Development—Romania, Programme Project ADER 816.

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of NATIONAL RESEARCH AND DEVELOPMENT INSTITUTE FOR BIOLOGY AND ANIMAL NUTRITION (IBNA-Balotesti, Romania; Protocol no. 4096/23.09.2022 following the Romanian legislation (Law 43/11.04.2014, Directive 2010/63/EU) for the feeding, handling, and slaughtering procedures of this study.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Oluwagbenga, E.M.; Fraley, G.S. Heat stress and poultry production: A comprehensive review. *Poult. Sci.* **2023**, *102*, 103141. [CrossRef] [PubMed]
2. Wasti, S.; Sah, N.; Mishra, B. Impact of Heat Stress on Poultry Health and Performances, and Potential Mitigation Strategies. *Anim. Open Access* **2020**, *10*, 1266. [CrossRef] [PubMed]
3. Alves, F.M.S.; Felix, G.A.; Almeida Paz, I.C.L.; Nääs, I.A.; Souza, G.M.; Caldara, F.R.; Garcia, R.G. Impact of exposure to cold on layer production. *Braz. J. Poult. Sci.* **2012**, *14*, 223–226. [CrossRef]
4. Kim, D.H.; Song, J.Y.; Park, J.; Kwon, B.Y.; Lee, K.W. The Effect of Low Temperature on Laying Performance and Physiological Stress Responses in Laying Hens. *Anim. Open Access* **2023**, *13*, 3824. [CrossRef] [PubMed]
5. Sinha, S.; Singh, S.N.; Saha, M.; Kain, T.C.; Tyagi, A.K.; Ray, U.S. Antioxidant and oxidative stress responses of sojourners at high altitude in different climatic temperatures. *Int. J. Biometeorol.* **2010**, *54*, 85–92. [CrossRef] [PubMed]
6. Şahin, K.; Önderci, M. Optimal dietary concentrations of vitamin C and chromium for alleviating the effect of low ambient temperature on serum insulin, corticosterone, and some blood metabolites in laying hens. *J. Trace Elem. Exp. Med. Off. Publ. Int. Soc. Trace Elem. Res. Hum.* **2002**, *15*, 153–161. [CrossRef]
7. Siegel, H. Stress, strains and resistance. *Br. Poult. Sci.* **1995**, *36*, 3–22. [CrossRef]
8. OECD/FAO. *OECD-FAO Agricultural Outlook 2023–2032*; OECD Publishing: Paris, France, 2023. Available online: https://chooser.crossref.org/?doi=10.1787/08801ab7-en (accessed on 21 November 2024).
9. Sandström, V.; Chrysafi, A.; Lamminen, M.; Troell, M.; Jalava, M.; Piipponen, J.; Kummu, M. Food system by-products upcycled in livestock and aquaculture feeds can increase global food supply. *Nat. Food* **2022**, *3*, 729–740. [CrossRef]
10. Onagbesan, O.M.; Uyanga, V.A.; Oso, O.; Tona, K.; Oke, O.E. Alleviating heat stress effects in poultry: Updates on methods and mechanisms of actions. *Front. Vet. Sci.* **2023**, *10*, 1255520. [CrossRef]
11. Caponio, G.R.; Minervini, F.; Tamma, G.; Gambacorta, G.; De Angelis, M. Promising Application of Grape Pomace and Its Agri-Food Valorization: Source of Bioactive Molecules with Beneficial Effects. *Sustainability* **2023**, *15*, 9075. [CrossRef]
12. Chamorro, S.; Romero, C.; Brenes, A.; Sánchez-Patán, F.; Bartolomé, B.; Viveros, A.; Arija, I. Impact of a sustained consumption of grape extract on digestion, gut microbial metabolism and intestinal barrier in broiler chickens. *Food Funct.* **2019**, *10*, 1444–1454. [CrossRef] [PubMed]
13. Herranz, B.; Romero, C.; Sánchez-Román, I.; López-Torres, M.; Viveros, A.; Arija, I.; Álvarez, M.D.; de Pascual-Teresa, S.; Chamorro, S. Enriching Eggs with Bioactive Compounds through the Inclusion of Grape Pomace in Laying Hens Diet: Effect on Internal and External Egg Quality Parameters. *Foods* **2024**, *13*, 1553. [CrossRef] [PubMed]

14. Pomoni, D.I.; Koukou, M.K.; Vrachopoulos, M.G.; Vasiliadis, L. Circular economy: A multilevel approach for natural resources and wastes under an agri-food perspective. *Water-Energy Nexus* **2024**, *7*, 103–123. [CrossRef]
15. Bist, R.B.; Bist, K.; Poudel, S.; Subedi, D.; Yang, X.; Paneru, B.; Mani, S.; Wang, D.; Chai, L. Sustainable poultry farming practices: A critical review of current strategies and future prospects. *Poult. Sci.* **2024**, *103*, 104295. [CrossRef] [PubMed]
16. Ozgan, A. Use of Grape Seed Oil in Functional Egg Production. Master's Thesis, University of Cukurova, Adana, Turkey, 2008.
17. Deng, Q.; Penner, M.H.; Zhao, Y. Chemical composition of dietary fiber and polyphenols of five different varieties of wine grape pomace skins. *Food Res. Int.* **2011**, *44*, 2712–2720. [CrossRef]
18. National Research Council. *Nutrient Requirements of Poultry*, 9th ed.; National Academy Press: Washington, DC, USA, 1994.
19. Untea, A.; Criste, R.C.; Vladescu, L. Development and validation of a microwave digestion–FAAS procedure for Cu, Mn and Zn determination in the liver. *Rev. Chim.* **2012**, *63*, 341–346.
20. Varzaru, I.; Untea, A.E.; Saracila, M. In vitro antioxidant properties of berry leaves and their inhibitory effect on lipid peroxidation of thigh meat from broiler chickens. *Eur. J. Lipid Sci. Technol.* **2020**, *122*, 1900384. [CrossRef]
21. Untea, A.E.; Varzaru, I.; Panaite, T.D.; Gavris, T.; Lupu, A.; Ropotă, M. The effects of dietary inclusion of bilberry and walnut leaves in laying hens' diets on the antioxidant properties of eggs. *Animals* **2020**, *10*, 191. [CrossRef]
22. Untea, A.; Lupu, A.; Saracila, M.; Panaite, T. Comparison of ABTS, DPPH, phosphomolybdenum assays for estimating antioxidant activity and phenolic compounds in five different plant extracts. *Bull. UASVM Anim. Sci. Biotechnol.* **2018**, *75*, 110–114. [CrossRef]
23. Lohmann Tierzucht Management Guide: Management Systems for Successful Egg Production. 2021. Available online: https://lohmann-breeders.com/media/2021/03/LTZ_MG_management-systems_EN.pdf (accessed on 20 November 2024).
24. Bosy-Westphal, A.; Hägele, F.A.; Müller, M.J. What Is the Impact of Energy Expenditure on Energy Intake? *Nutrients* **2021**, *13*, 3508. [CrossRef]
25. Mashaly, M.M.; Hendricks, G.L., 3rd; Kalama, M.A.; Gehad, A.E.; Abbas, A.O.; Patterson, P.H. Effect of heat stress on production parameters and immune responses of commercial laying hens. *Poult. Sci.* **2004**, *83*, 889–894. [CrossRef]
26. Kim, H.R.; Ryu, C.; Lee, S.D.; Cho, J.H.; Kang, H. Effects of Heat Stress on the Laying Performance, Egg Quality, and Physiological Response of Laying Hens. *Animals* **2024**, *14*, 1076. [CrossRef]
27. Zhou, F.; Yin, M.; Liu, Y.; Han, X.; Guo, J.; Ren, C.; You, Y. Grape seed flour intake decreases adiposity gain in high-fat-diet-induced obese mice by activating thermogenesis. *J. Funct. Foods* **2019**, *62*, 103509. [CrossRef]
28. Gollucke, P.B. Recent applications of grape polyphenols in foods, beverages, and supplements. *Recent Pat. Food Nutr. Agric.* **2010**, *2*, 105–109. [CrossRef]
29. Goñi, I.; Brenes, A.; Centeno, C.; Viveros, A.; Saura-Calixto, F.; Rebolé, A.; Arija, I.; Estevez, R. Effect of dietary grape pomace and vitamin E on growth performance, nutrient digestibility, and susceptibility to meat lipid oxidation in chickens. *Poult. Sci.* **2007**, *86*, 508–516. [CrossRef]
30. Brenes, A.; Viveros, A.; Goñi, I.; Centeno, C.; Sáyago-Ayerdy, S.G.; Arija, I.; Saura-Calixto, F. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. *Poult. Sci.* **2008**, *87*, 307–316. [CrossRef]
31. Habibian, M.; Ghazi, S.; Moeini, M.M. Effects of dietary selenium and vitamin E on growth performance, meat yield, and selenium content and lipid oxidation of breast meat of broilers reared under heat stress. *Biol. Trace Elem. Res.* **2016**, *169*, 142–152. [CrossRef]
32. Reis, J.H.; Gebert, R.R.; Barreta, M.; Boiago, M.M.; Souza, C.F.; Baldissera, M.D.; Santos, I.D.; Wagner, R.; Laporta, L.V.; Stefani, L.M.; et al. Addition of grape pomace flour in the diet on laying hens in heat stress: Impacts on health and performance as well as the fatty acid profile and total antioxidant capacity in the egg. *J. Therm. Biol.* **2019**, *80*, 141–149. [CrossRef]
33. Kara, K.; Kocaoğlu Güçlü, B.; Baytok, E.; Şentürk, M. Effects of grape pomace supplementation to laying hen diet on performance, egg quality, egg lipid peroxidation and some biochemical parameters. *J. Appl. Anim. Res.* **2016**, *44*, 303–310. [CrossRef]
34. Kim, D.H.; Kim, Y.B.; Lee, S.H.; Lee, Y.K.; Lee, S.D.; Lee, K.W. Identical thermal stress coupled with different temperature and humidity combinations affects nutrient digestibility and gut metabolites of laying hens. *Rev. Bras. Zootec.* **2023**, *52*, e20220067. [CrossRef]
35. Hafeez, A.; Hassni, S.F.; Naz, S.; Alonaizan, R.; Al-Akeel, R.K.; Sifa, D.; Shamsi, S.; Ullah Khan, R. Impact of grape (*Vitis vinifera*) seed extract on egg production traits, nutrients digestability, lipid peroxidation and fertility of golden laying hens (*Gallus gallus*) during early stage of production. *Vet. Q.* **2023**, *43*, 1–7. [CrossRef]
36. Sáyago-Ayerdi, S.G.; Brenes, A.; Viveros, A.; Goñi, I. Antioxidative effect of dietary grape pomace concentrate on lipid oxidation of chilled and long-term frozen stored chicken patties. *Meat Sci.* **2009**, *83*, 528–533. [CrossRef]
37. de Souza, L.F.A.; Espinha, L.P.; de Almeida, E.A.; Lunedo, R.; Furlan, R.L.; Macari, M. How heat stress (continuous or cyclical) interferes with nutrient digestibility, energy and nitrogen balances, and performance in broilers. *Livest. Sci.* **2016**, *192*, 39–43. [CrossRef]
38. Mitchell, M.A.; Carlisle, A.J. The effects of chronic exposure to elevated environmental temperature on intestinal morphology and nutrient absorption in the domestic fowl (*Gallus domesticus*). *Comp. Biochem. Physiol. Part A Physiol.* **1992**, *101*, 137–142. [CrossRef]
39. Hosseini-Vashan, S.; Safdari-Rostamabad, M.; Piray, A.; Sarir, H. The growth performance, plasma biochemistry indices, immune system, antioxidant status, and intestinal morphology of heat-stressed broiler chickens fed grape (*Vitis vinifera*) pomace. *Anim. Feed. Sci. Technol.* **2020**, *259*, 114343. [CrossRef]
40. Selim, S.; Abdel-Megeid, N.S.; Alhotan, R.A.; Ebrahim, A.; Hussein, E. Grape Pomace: Agrifood By-Product with Potential to Enhance Performance, Yolk Quality, Antioxidant Capacity, and Eggshell Ultrastructure in Laying Hens. *Vet. Sci.* **2023**, *10*, 461. [CrossRef]

41. Sahin, K.; Akdemir FAT, İ.; Orhan, C.; Tuzcu, M.; Hayirli, A.; Sahin, N. Effects of dietary resveratrol supplementation on egg production and antioxidant status. *Poult. Sci.* **2010**, *89*, 1190–1198. [CrossRef]
42. Attallah, O.K.; Mohammed, T.T.; Al-Anbari, N.N. Effect of adding grape pomace and resveratrol on some physiological traits and gene expression to prevent hemorrhagic fatty liver syndrome in laying hens. In *IOP Conference Series: Earth and Environmental Science*; IOP Publishing: Bristol, UK, 2022; Volume 1060, p. 012076.
43. Ronceros, B.; Quevedo-León, R.; Jara-Quezada, J.; Soto-Maldonado, C.; Lespinard, A.; Uquiche, E. Afianzamiento del color comercial y de la capacidad antioxidante en yema de huevos de gallinas ponedoras por suplementación dietética con harina de bagazo de uva. *AgroScience Res.* **2024**, *2*, 29–35. [CrossRef]
44. Thema, K.K.; Mlambo, V.; Egbu, C.F.; Mnisi, C.M. Use of red grape pomace and Aloe vera gel as nutraceuticals to ameliorate stocking density-induced stress in commercial male broilers. *Trop. Anim. Health Prod.* **2024**, *56*, 107. [CrossRef]
45. Cornescu, G.M.; Panaite, T.D.; Untea, A.E.; Varzaru, I.; Saracila, M.; Dumitru, M.; Vlaicu, P.A.; Gavris, T. Mitigation of heat stress effects on laying hens' performances, egg quality, and some blood parameters by adding dietary zinc-enriched yeasts, parsley, and their combination. *Front. Vet. Sci.* **2023**, *10*, 1202058. [CrossRef]
46. Govoni, C.; D'Odorico, P.; Pinotti, L.; Rulli, M.C. Usage of By-Products and Residues of the Food System in Livestock Diets Leads to Savings in Global Land and Water Resources. In Proceedings of the EGU General Assembly Conference Abstracts 2023, EGU-15056, Vienna, Austria, 23–28 April 2023.

agriculture

MDPI

Article

Effects of Marigold and Paprika Extracts as Natural Pigments on Laying Hen Productive Performances, Egg Quality and Oxidative Stability

Cristina-Camelia Matache [1,2], Gabriela Maria Cornescu [1,*], Dumitru Drăgotoiu [2], Ana Elena Cișmileanu [1], Arabela Elena Untea [3], Mihaela Sărăcilă [3] and Tatiana Dumitra Panaite [1]

[1] Nutrition Physiology Department, National Research and Development Institute for Biology and Animal Nutrition, 077015 Balotesti, Ilfov, Romania; camelia.matache@ibna.ro (C.-C.M.); ana_cismileanu@yahoo.com (A.E.C.); tatiana.panaite@ibna.ro (T.D.P.)

[2] Faculty of Animal Productions Engineering and Management, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 B-dul Marasti, District 1, 011464 Bucharest, Romania; dumitrudragotoiu@yahoo.com

[3] Food and Feed Quality Department, National Research and Development Institute for Biology and Animal Nutrition, 077015 Balotesti, Ilfov, Romania; arabela.untea@ibna.ro (A.E.U.); mihaela.saracila@ibna.ro (M.S.)

* Correspondence: gabriela_cornescu@yahoo.com; Tel.: +40-21-351-2082

Abstract: Enhancing the quality of eggs by using natural food sources has become a very important topic in the last decade. The objective of this study was to determine the influence of natural (marigold and paprika extracts) pigments on the shelf life of eggs from laying hens. This research was carried out for a 6-week period on 168 Lohmann Brown laying hens (45 weeks age) divided into four groups (C, E1, E2 and E3) to assess the performances, external and internal egg quality parameters, egg yolk color, and antioxidant profile. The control group (C) was fed a standard diet (16.39% PB, 2750 kcal EM/kg compound feed) and the experimental diets were supplemented with 0.07% marigold extract (E1), 0.07% paprika extract (E2), and a mixture containing 0.07% of both extracts (E3). In summary, the study demonstrated that adding natural pigments from marigold and paprika extract with highly antioxidant lipid capacity into the diets of laying hens improved egg quality when eggs were stored at 28 days, under both storage temperature conditions (4 °C and 20 °C).

Keywords: antioxidant capacity; eggs; lutein; marigold extract; natural pigments; paprika extract

Citation: Matache, C.-C.; Cornescu, G.M.; Drăgotoiu, D.; Cișmileanu, A.E.; Untea, A.E.; Sărăcilă, M.; Panaite, T.D. Effects of Marigold and Paprika Extracts as Natural Pigments on Laying Hen Productive Performances, Egg Quality and Oxidative Stability. *Agriculture* **2024**, *14*, 1464. https://doi.org/10.3390/agriculture14091464

Academic Editor: Lin Zhang

Received: 14 May 2024
Revised: 21 August 2024
Accepted: 22 August 2024
Published: 28 August 2024

1. Introduction

Eggs are a highly nutritious food that provides the consumer with necessary elements such as fatty acids, vitamins, minerals, and proteins [1]. Therefore, the egg has perishable qualities, much like any other food derived from animals, losing its interior quality from the time the hen lays it until it is consumed [2]. Conservation techniques become crucial to prevent premature perishability, with a focus on refrigeration, which tends to maintain the internal quality of eggs by delaying their degradation [1]. Temperature and relative humidity have the biggest effects on the internal quality of eggs. High temperatures during storage are associated with decreased albumen quality, which can be attributed to water and carbon dioxide loss that causes the albumen to become fluidified and the yolk membrane easier to crack when breaking eggs. The egg will lose water more quickly and lose weight as a result of the air chamber expanding if the relative humidity of the atmosphere around it is less than 99.6% [3].

The color of the egg yolk is another attribute associated with internal egg quality, and a factor contributing to customer decision-making, because it is typically linked to the nutritional value and quality of the egg. Corn is a notable source of xanthophylls, which are responsible for the yolk's yellowish color. However, the amount of these pigments

in corn varies throughout the year, primarily from harvest to harvest, which means that laying hens must use pigments in their diet to maintain the yolk's color given that the birds cannot synthesize xanthophylls; they must only get them from their dietary ingredients [4].

Carotenoids are a group of pigments found in plants, algae, and photosynthetic bacteria, responsible for the vibrant red, orange, and yellow hues in various fruits and vegetables [5]. Due to their importance to nutrition, health, and well-being, carotenoids have recently attracted much attention in the food and feeding industries. Carotenoids also serve as essential precursors for synthesizing vitamin A in animals, contributing to various physiological functions such as vision and immune system support [6]. Additionally, their antioxidant properties make carotenoids valuable in promoting human health by neutralizing harmful free radicals, potentially reducing the risk of chronic diseases [7]. Including natural pigments in poultry feed enhances these antioxidant benefits in the eggs, maintaining their flavor, color, and nutritional profile. This is crucial since eggs are a significant source of essential nutrients like vitamins A and E [8].

As Darvin et al. [9] stated, antioxidant combinations can significantly enhance antioxidant protection through a synergistic complex effect when the composition and concentration of antioxidants are optimally balanced to neutralize free radicals and reduce their harmful effects. Being seen as a strategy in laying hen diets, the plant/herbal supplementation had a positive impact on poultry physiological, productive, reproductive, and immunological performances [10]. Therefore, natural pigment extract utilization in animal feed can provide a triple benefit, as observed in various studies: enhanced oxidative stability of food lipids, increased carotenoid concentration, and a more intense egg yolk color [11].

The marigold extract, derived from *Tagetes erecta L.* (Asteraceae family), is well-known as an ornamental plant (yellow or orange flowers grouped in solitary inflorescences), but also for the rich content of carotenoids, is easy to purchase and available to anyone. The total carotenoid content of marigold extract is 4200 mg/kg [12]. Additionally, it is utilized in the production of dietary supplements intended to stop age-related macular degeneration and other eye disorders from affecting visual acuity. The content of the plant in lutein means it has many uses, such as commercial purposes as a natural pigment for poultry feed [13].

Paprika extract (*Capsicum annuum*) is one of the most well-known and often-used natural food pigments [14]. The bright orange-red pigment known as capsanthin, which gives paprika its unique red color, belongs to the xanthophylls class of oxygen-containing carotenoids. The strong antioxidant activity of capsanthin results in its shown chemopreventive, antitumor, skin photoprotective, anti-inflammatory, and antidiabetic properties. Capsanthin provides a lot of health benefits, is naturally occurring, and could be developed into a pharmaceutical, nutraceutical, or cosmeceutical product [15]. Carotenoids are becoming feed additives preferred as human food natural pigments due to their positive and healthy effects on animal products when added to their diets [16]. The specific carotenoids, lutein and zeaxanthin, have demonstrated antioxidant, anti-inflammatory, light absorbing, and blue filtering effects. It has been suggested that they may help prevent immune-mediated macular degeneration and the development of age-related cataracts [17–19].

The trial aimed to assess the nutritional feeding qualities of the two natural pigments from plant extracts, marigold and red pepper, in the diets of laying hens, based on the premise that the aforementioned plants can be considered phyto-additives with beneficial effects on laying hens' health, productivity and egg quality.

2. Materials and Methods

2.1. Ethical Statement

The feeding trial was carried out according to the protocol (No. 4096/23 September 2022) approved by the institute's Commission of Ethics in the experimental halls of the National Research Development Institute of Animal Biology and Nutrition (IBNA-Balotesti, Romania) following the Romanian legislation (Law 206/2004, Ordinance 28/31 August

2011, Law 43/11 April 2014, Directive 2010/63/EU) for the feeding, handling, and slaughtering procedures of a study.

2.2. Carotenoids Purchasing and Their Proximal Chemical Analyses

The natural carotenoid powders (marigold and red pepper extracts) were provided by Animal Feed Consulting (Ilfov, Romania). The natural pigments were produced by Kaesler Nutrition GmbH (Cuxhaven, Germany) under EC Regulation no. 1831/2003, labelled accordingly and packed in aluminium plus polymer bags of 25 kg. Samples of marigold and red pepper powders, of 500 g each, were analyzed to determine the proximate qualities of dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash, the minerals copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn), and the factors contributing to antioxidant activity, including polyphenols, antioxidant capacity, tocopherols, and carotenoids. The natural carotenoids and feed samples were analyzed using standardized methods to determine the nutrient concentration. The DM concentration was measured using the gravimetric method (BMT model ECOCELL Blueline Comfort, Nuremberg, Germany), the CP concentration was assessed using the Kjeldahl method [Kjeltek auto 1030–Tecator (FOSS Tecator AB, Höganäs, Sweden)], the EE concentration was determined by extraction in organic solvents (FOSS Tecator AB, Höganäs, Sweden), the CF concentration was assessed using intermediary filtration, (Fibertec 2010 System-Foss Tecator, Sweden), and crude ash [Nabertherm Labotherm L15/11/P320 Comfort, (Bremen, Germany)] was determined according to the procedures described in Regulation (CE) No. 152/2009.

2.3. Birds Performances, Housing and Experimental Diets

The 6-week experiment used 168 Lohmann Brown layers (45 weeks old), assigned into four groups: control (C), experimental group 1 (E1), experimental group 2 (E2) and experimental group 3 (E3). They were housed in a hall under controlled microclimate conditions, where the temperature was recorded twice a day, at 8:00 a.m. and 3:00 p.m.

The registered daily average temperature was 22.44 °C ± 1.85, the humidity was 63.88% ± 6.02, and the ventilation was 27.4% ± 10.86. The hens were weighed individually (initial average weight of 1.760 ± 53.46 g) and housed in three-tier batteries, Big Dutchman digestibility cages, with 42 birds/group, 21 cages/group and 2 birds/cage, respectively, with a 16 h/24 h light regimen. Each cage (50 cm width × 40 cm height × 50 cm length) was considered an experimental unit and performance parameters were evaluated per pen. Feed and water were offered *ad libitum* throughout the experiment. Each of the four groups had a similar basal diet formulation (Table 1). Compared to the C diet, the E1 group included 0.07% marigold extract, the E2 group included 0.07% red pepper extract, and the E3 group included a combination of marigold and red pepper extracts. The structure of the experimental diets was developed using dedicated software for the formulation of compound feed (Brill® Formulation 2.5, AGRIFOOD, Lleida, Spain), in agreement with the feeding requirements of laying hens [20]. The diets were isoenergetic and isonitrogenous, containing 17% CP and 11.51 MJ metabolizable energy (ME) per kg diet (Table 1). Average body weight (BW, g/hen) was measured at the beginning and at the end of the experimental period. Daily production parameters were monitored: average daily feed intake (ADFI; g/hen/day); feed conversion rate (FCR; kg feed/kg egg); laying rate intensity (LRI; %); average egg weight (AEW; g) and viability (%). No medical treatment was applied to the hens throughout the six-week experimental period. The difference between the amount of feed administered and the amount of leftover feed that was not consumed each day was used to calculate the ADFI parameter. The production parameter for FCR was given as kg of feed eaten for every kilogram of eggs produced. By dividing the total number of eggs laid by the total number of laying hens, the LRI parameter was determined.

Table 1. Diet formulation and proximal analysis results.

Specifications	C	E1	E2	E3
Red pepper extract, %	-	-	0.07	0.05
Marigold extract, %	-	0.07	-	0.02
Corn, %	55.07	54.93	54.93	54.93
Soybean meal, %	13.97	14.00	14.00	14.00
Sunflower meal, %	16.00	16.00	16.00	16.00
Lysine, %	0.26	0.26	0.26	0.26
Methionine, %	0.23	0.23	0.23	0.23
Calcium carbonate, %	8.77	8.77	8.77	8.77
Monocalcium phosphate, %	1.01	1.01	1.01	1.01
Salt, %	0.36	0.36	0.36	0.36
Soybean oil, %	3.26	3.31	3.31	3.31
Choline 60%	0.05	0.05	0.05	0.05
Phytase	0.01	0.01	0.01	0.01
Premix *, 1%	1.00	1.00	1.00	1.00
Total ingredients, %	100.00	100.00	100.00	100.00
Calculated Analysis				
Metabolizable energy, kcal/kg	2.750	2.750	2.750	2.750
Crude protein, %	17.00	17.00	17.00	17.00
Calcium, %	3.90	3.90	3.90	3.90
Phosphorus, %	0.38	0.38	0.38	0.38
Lysine, %	0.94	0.94	0.94	0.94
Met + cist, %	0.85	0.85	0.85	0.85
Threonine, %	0.64	0.64	0.64	0.64
Chemical Analysis				
Gamma tocopherol (mg/kg)	7.31	8.79	8.13	8.06
Alpha-tocopherol (mg/kg)	34.61	36.07	28.25	31.69
Lutein + zeaxanthin (ppm)	9.90	22.19	9.06	14.77
Antioxidant capacity (μM Trolox)	22.64	26.89	30.07	28.28
Total polyphenols, mg/g GAE	4.31	4.89	4.54	4.74

* 1 kg premix contains 1,100,000 IU/kg vitamin A; 200,000 IU/kg vitamin D3; 2700 IU/kg vitamin E; 300 mg/kg vitamin K; 200 mg/kg vitamin B1; 400 mg/kg vitamin. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg vitamin B6; 4 mg/kg vitamin B7; 100 mg/kg vitamin B9; 1.8 mg/kg vitamin B12; 2000 mg/kg vitamin C; 8000 mg/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium.

2.4. Egg Collection and Their Quality Measurement

After 3 and 6 experimental weeks, a total of 144 eggs were collected randomly (18 eggs/group/period) to assess the internal and external quality parameters of the eggs: egg weight and its components (albumen, yolk, shell) with a Kern EW6000-1M Electronic Balance, precision 0.001 (Kern & Sohn GmbH, D-72336 Balingen, Germany), egg freshness, Haugh unit, and eggshell breaking strength, using the Digital Egg Tester DET-6500 (NABEL Co., Ltd., Kyoto, Japan). The eggshell thickness was measured within a range of 0.10–0.60 mm, with an accuracy of ± 0.02 mm, with the concave part of the eggshell side down, using a digital micrometer in the range of 0–10 mm, with a measuring force of 1.5 N or less (Mitutoyo 547-360 ABSOLUTE Digimatic Thickness Gauge, NABEL Co., Ltd., Kyoto, Japan). The pH of the albumen and egg yolk was measured using a portable pH meter (Five Go F2-Food kit with LE 427IP67, Sensor Metler Tolledo, Greifensee, Switzerland). To assess accurately the yolk color, DET6500 uses natural light, as artificial light varies greatly between locations depending on its light source. The yolk color was estimated using DSM YolkFan^{TM} based on daylight, without artificial light. Furthermore, utilizing the CIE-Lab system (Commission Internationale de l'Eclaraige) with a customized aperture (8 mm/4 mm/1 × 3 mm), 2.6 s measuring time, high accuracy of 0.04 and an observer angle of 2°/10°, the egg yolk color (parameters L*,

a*, and b*) was measured using the portable colorimeter 3nh YS3020 (Shenzhen Threenh Technology Co., Ltd., Beijing, China).

The oxidative stability of the fat yolk was measured at the end of the experiment and after 28 days when 144 eggs were collected randomly and kept at different storage temperatures: 18 eggs/group were kept at room temperature (20 °C ± 1) and another 18 eggs/group were kept in a refrigerator (4 °C) for 28 days and analyzed to assess the yolks' oxidative stability.

After measuring the internal and external quality parameters of the eggs, 6 yolk samples (3 yolks/sample) per group were formed and assayed for the antioxidant profile (lutein content, alpha and gamma tocopherols, vitamins A and E, antioxidant capacity and total polyphenols) and oxidative status of the yolk, represented by thiobarbituric acid reactive substances (TBARS) assessment. Yolk samples were stored at −20 °C until analysis. Before analysis, the samples were allowed to reach room temperature. Chemical analysis, represented by vitamins, polyphenols and antioxidant capacity, was performed on the dry yolk samples, while the oxidative stability of eggs and color measurement were determined on the fresh yolk samples.

2.5. Minerals Assessment

Flame atomic absorption spectrometry [Thermo Electron—SOLAAR M6 Dual Zeeman Comfort (Cambridge, UK)] was utilized for the concentration assessment of zinc (Zn), iron (Fe), copper (Cu), and manganese (Mn) in marigold and red pepper powder extracts [21]. The results have been expressed as µg/g (ppm) of dried sample.

2.6. Determination of Total Polyphenols Content

The total polyphenol content of plants was spectrometrically determined using the Folin–Ciocalteu method, as described by [22]. The reading of absorbance was performed at 732 nm, and gallic acid was used for the calibration curve, the results being expressed as mg gallic acid equivalents per gram sample.

2.7. Total Antioxidant Capacity Assessment

The total antioxidant capacity of samples was evaluated by the DPPH method described by [22]. The antioxidant activity was expressed as Trolox equivalents. The total antioxidant capacity of the extracts was based on the reaction between the sample solution and DPPH reagent prepared in methanol and the absorbance recorded at 517 nm using a V-530 Jasco (Japan Servo Co., Ltd., Tokyo, Japan) spectrophotometer, as described elsewhere.

2.8. Determination of Vitamin E, Lutein and Zeaxanthin

Vitamin E determination for plants, compound feed and eggs were performed using high-performance liquid chromatography (HPLC Finningan Surveyor Plus, ThermoElectron Corporation, Waltham, MA, USA) and a PDA-UV detector at a wavelength of 292 nm, as described by Vărzaru et al. [23]. The results have been expressed as mg/kg.

Lutein and zeaxanthin content were analyzed using a high-performance liquid chromatograph (Perkin Elmer 200 series, Shelton, CT, USA) with a UV detector (445 nm), and a Nucleodur C18 column (Macherey-Nagel, Dueren, Germany), as described by Vărzaru et al. [23]. The results have been expressed as mg/kg.

2.9. Determination of Thiobarbituric Acid Reactive Substances (TBARS)

The oxidative stability of yolk was indicated by the levels of thiobarbituric acid reactive substances (TBARS), according to the methods described by Untea et al. [22]. The TBARS values were calculated from a standard curve of malondialdehyde and expressed as milligrams of malondialdehyde (MDA) per kg of sample (mg MDA/kg). The absorbance of the prepared sample was read at 532 nm.

2.10. Statistical Analysis

All data were subjected to an analysis of variance using the GLM procedure of the Minitab software (version 17, Minitab® Statistical Software). The level of significance was set at $p < 0.05$. The experimental results (production performances and antioxidant profile) were analyzed according to the following linear model:

$$Yij = \mu + Aj + eij$$

where Yij means the value of the trait (the dependent variable); μ, overall mean; Aj, the treatment effect; and eij, random observation error.

The effect of feeding time on external and internal egg quality parameters and yolk color was analyzed according to a 2 × 2 factorial arrangement to determine whether the factors studied (treatment and feeding time) influenced the egg parameters for different periods. The data obtained were analyzed by two-way ANOVA using the Tukey test following the statistical model:

$$Yijk = \mu + \alpha i + \beta j + \alpha i\beta j + eijk$$

where Yijk = variable measured for the kth observation of the ith treatment and jth feeding or storage time; μ is the sample mean; αi is the effect of the ith treatment; βj is the effect of the jth feeding or storage time; αiβj is the interaction of ith treatment and jth feeding or storage time, and eijk is the effect of error. The differences were highly significant when $p < 0.001$ and significant if $p < 0.05$.

The graphs for the oxidative stability parameters (TBARS) were statistically constructed using GraphPad Prism 9.1.2 software (GraphPad Software, La Jolla, CA, USA). Values were determined to be significant when * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, with different letters indicating significant differences between groups ($p < 0.05$).

3. Results

3.1. Nutritional Profile of Marigold and Red Pepper Powder Extracts

The nutritional compositions of natural pigments are presented in Table 2. The marigold extract powder showed higher CP (5.15%) and CF (1.17%) contents, compared to red pepper extract powder (0.25% CP and 0.21 CF %). The red pepper extract registered a higher content of EE (4.21%) compared to marigold extract (0.23%). Also, the trace mineral content (Cu, Fe, Mn, Zn) was higher in marigold extract compared to red pepper extract powder. However, Cu and Zn could not be detected in red pepper extract powder samples.

Table 2. The nutritional profile of marigold and red pepper powder extracts.

Parameters	Red Pepper Extract Powder	Marigold Extract Powder
Proximate Composition		
Dry matter (DM), %	92.79	94.84
Crude protein (CP), %	0.25	5.15
Ether extract (EE), %	4.21	0.23
Crude fiber (CF), %	0.21	1.17
Ash, %	55.54	50.74
Mineral Content		
Cooper (Cu), %	n.d.	3.78
Iron (Fe), %	284.26	1296.77
Manganese (Mn), %	20.87	158.94
Zinc (Zn), %	n.d.	33.71

Table 2. *Cont.*

Parameters	Red Pepper Extract Powder	Marigold Extract Powder
Antioxidant Activity		
Total polyphenols, mg/g GAE	5.32	3.64
Antioxidant capacity (μM Trolox/kg)	22.80	52.82
Delta tocopherol (mg/kg)	n.d.	n.d.
Gamma tocopherol (mg/kg)	560.32	68.22
Alpha-tocopherol (mg/kg)	199.43	150.11
Lutein + zeaxanthin (ppm)	956.26	3786.66
Astaxanthin, (mg/kg)	660.05	n.d.
Canthaxanthin (mg/kg)	31.72	n.d.

where: n.d., not detectable.

Regarding the antioxidant profile, the marigold extract powder was the richest in lutein (3786.66 ppm) and antioxidant capacity (52.82 μM Trolox). The red pepper extract presented the highest values for total polyphenols content (5.32%) and the concentrations of astaxanthin (660.05 ppm) and canthaxanthin (31.72 ppm), not detectable in the red pepper extract's case. The richest source of gamma-tocopherol (mg/kg) was red pepper extract powder, its content being 8.21 times higher compared to the concentration encountered in marigold extract powder.

3.2. Productive Performance Parameters

Table 3 presents the results related to the production performances. There were no significant differences ($p > 0.05$) between the groups C, E2 and E3 groups in terms of ADFI parameter, except for the E1 group, which recorded a significantly lower consumption ($p < 0.0001$). At the same time, the lowest FCR values were highly significant ($p < 0.0001$) for the C and E3 groups compared to the E1 and E2 groups. Regarding the LRI (%) parameter and total number of eggs laid, no significant differences ($p < 0.110$; $p < 0.081$) were observed between experimental groups. There were highly significant ($p < 0.0001$) differences recorded for the AEW parameter in the E2 and E3 groups compared to the C and E1 groups.

Table 3. Effect of marigold and red pepper extract powder in laying hens' diets on the productive performances of layers.

Specifications	C	E1	E2	E3	SEM	*p*-Value
Initial body weight (g/layer)	1667.60	1624.60	1684.00	1657.00	18.500	0.71
Final body weight (g/layer)	1830.52	1825.00	1894.20	1830.20	17.080	0.43
Average daily feed intake (g/hen/day)	118.92 ^{a}	116.21 ^{b}	120.76 ^{a}	119.96 ^{a}	0.469	<0.0001
Feed conversion rate (kg feed/kg egg)	2.15 ^{bc}	2.21 ^{b}	2.22 ^{b}	2.11 ^{c}	0.017	<0.0001
Laying rate intensity (%)	89.02	89.21	89.02	90.33	0.531	<0.110
Average egg weight (g)	63.46 ^{b}	63.17 ^{b}	64.39 ^{a}	64.37 ^{a}	0.095	<0.0001
Total egg weight (g)	1393.65	1326	1406.95	1429.91	10.561	<0.081

C—control diet; E1—control diet supplemented with 0.07% marigold extract powder; E2—control diet supplemented with 0.07% red pepper extract powder; E3—control diet supplemented with a mix of 0.05% red pepper extract powder and 0.02% marigold extract powder; SEM, standard error of the mean; $^{a–c}$ mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$.

3.3. External and Internal Egg Quality Parameters

Table 4 data presents the internal and external egg parameters over the two periods (3 weeks and 6 weeks) and groups (C, E1, E2, E3).

Table 4. Effect of marigold and red pepper extract powder in laying hens' diets on external and internal egg quality parameters.

Specification		Egg Weight and Components						Albumen			Yolk			
		Whole Egg Weight (g)	Albumen (g)	Yolk (g)	Eggshell (g)	Shell Thickness (mm)	Breaking Strengths (kgF)	pH Albumen	Albumen Height (mm)	Haugh Units	pH Yolk	Yolk Height (mm)	Yolk Diameter (mm)	Yolk Index
3 weeks	C	60.95	38.47	14.91	7.57	0.38	3.54 ^{b}	8.29 ^{d}	7.23 ^{ab}	84.42 ^{ab}	6.15	18.23 ^{ab}	40.83	0.45
	E1	62.25	40.44	14.38	7.44	0.38	4.08 ^{ab}	8.44 ^{cd}	8.23 ^{a}	90.07 ^{a}	6.37	17.78 ^{ab}	39.73	0.45
	E2	61.57	38.48	15.27	7.82	0.39	4.85 ^{a}	8.30 ^{d}	7.63 ^{ab}	86.68 ^{ab}	6.43	17.12 ^{ab}	41.78	0.41
	E3	61.57	38.67	15.32	7.58	0.37	4.57 ^{ab}	8.36 ^{d}	7.85 ^{ab}	88.23 ^{ab}	6.29	17.25 ^{ab}	40.98	0.42
6 weeks	C	61.29	38.95	15.07	7.27	0.39	4.51 ^{ab}	8.85 ^{a}	7.16 ^{ab}	83.60 ^{ab}	6.33	17.92 ^{ab}	39.91	0.45
	E1	62.14	39.40	15.40	7.34	0.38	4.23 ^{ab}	8.62 ^{bc}	7.76 ^{ab}	87.18 ^{ab}	6.25	18.32 ^{a}	40.67	0.45
	E2	61.32	38.97	15.22	7.13	0.39	4.42 ^{ab}	8.67 ^{ab}	6.68 ^{b}	80.67 ^{b}	6.22	17.51 ^{ab}	41.21	0.43
	E3	60.87	38.07	15.64	7.17	0.38	4.42 ^{ab}	8.69 ^{ab}	7.18 ^{ab}	83.90 ^{ab}	6.16	17.16 ^{ab}	41.63	0.41
Main effects														
Group	C	61.12	38.71	14.99	7.42	0.38	4.02	8.57	7.19	84.01	6.24	17.83 ^{a}	40.37	0.45 ^{a}
	E1	62.19	39.92	14.89	7.38	0.37	4.15	8.53	8.00	88.62	6.31	17.81 ^{a}	40.20	0.45 ^{a}
	E2	61.44	38.73	15.24	7.47	0.38	4.64	8.49	7.16	83.68	6.33	17.32 ^{ab}	41.50	0.42 ^{b}
	E3	61.22	38.37	15.48	7.38	0.37	4.50	8.52	7.51	86.07	6.23	17.25 ^{b}	41.31	0.42 ^{b}
	SEM $_{group}$	0.412	0.435	0.258	0.141	0.004	0.199	0.030	0.225	1.34	0.064	0.183	0.554	0.008
Period	3 weeks	61.58	39.01	14.97	7.60 ^{a}	0.38	4.26	8.35 ^{b}	7.74 ^{a}	87.35 ^{a}	6.31	17.60	40.83	0.43
	6 weeks	61.41	38.85	15.33	7.23 ^{b}	0.39	4.40	8.70 ^{a}	7.19 ^{b}	83.84 ^{b}	6.24	17.72	40.85	0.44
	SEM $_{period}$	0.292	0.307	0.182	0.099	0.003	0.140	0.021	0.159	0.951	0.045	0.130	0.392	0.005
Interaction (p-Value)														
group		0.260	0.077	0.378	0.961	0.081	0.115	0.279	0.042	0.048	0.602	0.001	0.259	0.003
period		0.669	0.701	0.168	0.012	0.114	0.493	0.000	0.020	0.013	0.259	0.490	0.970	0.736
period × group		0.850	0.504	0.493	0.520	0.945	0.09	0.001	0.575	0.574	0.158	0.326	0.574	0.782

C—control diet; E1—control diet supplemented with 0.07% marigold extract powder; E2—control diet supplemented with 0.07% red pepper extract powder; E3—control diet supplemented with a mix of 0.05% red pepper extract powder and 0.02% marigold extract powder; SEM, standard error of the mean; $^{a–d}$ mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$.

There were no significant differences registered ($p > 0.05$) concerning the whole egg weight, albumen and yolk weight, breaking strength, eggshell thickness, pH yolk, and yolk diameter across groups, periods, or their interaction. Significant differences were found regarding eggshell weight between periods ($p < 0.012$). Highly significant differences in values for pH albumen were found between periods ($p < 0.0001$) and between period*groups interactions ($p = 0.001$). For the albumen height parameter, significant differences ($p < 0.05$) were observed between groups ($p = 0.042$) and between periods ($p = 0.02$); significantly lower albumen heights were registered at 6 weeks compared to 3 weeks. Consequently, the same trend was noticed for the Haugh unit parameter, where significant differences were registered between groups ($p < 0.048$) and between periods ($p = 0.013$). Other significant differences between groups were observed for yolk height ($p < 0.001$) and yolk index ($p < 0.003$). For most of the analyzed parameters, there were statistically significant differences between groups, periods and their interaction, indicating that the observed changes were primarily due to periods rather than different treatment groups. Indeed, as time increased, lower values but without statistical significance were noticed for whole egg albumen, albumen and eggshell weight, and yolk pH.

3.4. Yolk Color

Table 5 presents the effects of marigold and red pepper extracts' inclusion in the diet on yolk color. The presence of these two carotenoids exhibited a significant effect on the L*, a*, and b* color parameters. Concerning the yolk fan color, highly significant differences ($p < 0.0001$) were noted between groups, with the darkest colors in E3 and E2 groups compared to C and E1 groups. Also, highly significant differences ($p < 0.0001$) regarding the darkest hue were measured at 3 weeks compared to 6 weeks. No significant differences ($p = 0.139$) were registered for the period and group interaction.

Table 5. Egg yolk color assessment at 3 weeks and 6 weeks (average values/group).

Specifications		Yolk Fan Color (DSM)	L*	a*	b*
3 weeks	C	4.83 ^{d}	42.84 ^{a}	0.49 ^{d}	20.54 ^{bc}
	E1	7.33 ^{c}	42.88 ^{a}	2.80 ^{bc}	27.51 ^{a}
	E2	9.00 ^{ab}	40.30 ^{bc}	4.44 ^{a}	20.62 ^{bc}
	E3	9.67 ^{a}	42.88 ^{a}	4.42 ^{a}	21.60 ^{bc}
6 weeks	C	4.50 ^{d}	42.06 ^{ab}	0.12 ^{d}	21.52 ^{bc}
	E1	6.89 ^{c}	39.79 ^{c}	2.21 ^{c}	28.18 ^{a}
	E2	8.78 ^{ab}	40.13 ^{bc}	3.52 ^{ab}	18.99 ^{c}
	E3	8.11 ^{bc}	39.74 ^{c}	3.00 ^{bc}	22.67 ^{b}
Main effects					
Group	C	4.67 ^{c}	42.45 ^{a}	0.30 ^{c}	21.03 ^{bc}
	E1	7.11 ^{b}	41.34 ^{ab}	2.50 ^{b}	27.84 ^{a}
	E2	8.89 ^{a}	40.21 ^{b}	3.98 ^{a}	19.81 ^{c}
	E3	8.89 ^{a}	41.31 ^{ab}	3.71 ^{a}	22.14 ^{b}
	SEM $_{group}$	0.222	0.331	0.169	0.458
Period	3 weeks	7.71 ^{a}	42.23 ^{a}	3.04 ^{a}	22.57 ^{a}
	6 weeks	7.07 ^{b}	40.43 ^{b}	2.21 ^{b}	22.84 ^{a}
	SEM $_{period}$	0.157	0.234	0.120	0.324
Interaction (p-Value)					
group		0.000	0.000	0.000	0.000
period		0.006	0.000	0.000	0.549
period × group		0.139	0.001	0.149	0.123

C—control diet; E1—control diet supplemented with 0.07% marigold extract powder; E2—control diet supplemented with 0.07% red pepper extract powder; E3—control diet supplemented with powder a mix of 0.05% red pepper extract powder and 0.02% marigold extract powder; L* (lightness), a* (redness), b* (yellowness); SEM, standard error of the mean; $^{a–d}$ mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$.

For the L* parameter, highly significant differences ($p < 0.000$) were noticed between the C and E2 groups, whereas a highly significant statistical difference with the lowest values of the L* parameter was registered on the 6 weeks. Concerning group and period interaction ($p = 0.001$), highly significant statistical differences were recorded. Highly significant differences ($p < 0.000$) were found in the a* parameter between the groups, with values of 3.98 for E2 and 3.77 for E3, compared to 2.50 for E1 and 0.30 for the control (C). These differences were also found to be significant in the period evaluation, with values of 3.04 at 3 weeks compared to 2.21 at 6 weeks. For the b* parameter, the only highly significant difference ($p < 0.000$) was observed in the group analysis, where the E1 group showed higher yellow values compared to the control (C), E2, and E3 groups.

3.5. Antioxidant Profile

Table 6 presents the effects of the two dietary extracts on the antioxidant profile of egg yolks. Concerning the lutein and zeaxanthin concentrations, diet E1 showed the highest concentration ($p < 0.0001$), whihc was significantly higher that that of the C, E2 and E3 groups. Group E3 had a higher concentration compared to the E2 group, but this was lower than in the E1 group. The antioxidant capacity of group E2 exhibited the highest antioxidant capacity ($p < 0.0001$), which was significantly higher compared to the C group. For the total polyphenols content, the E2 group registered significantly higher values ($p < 0.002$) compared to the C and E1 groups. For other parameters, such as alpha-tocopherol, gamma-tocopherol, vitamin E, and vitamin A, there were no significant differences among the experimental diets. Nevertheless, it is noteworthy that a trend towards significance was observed for gamma-tocopherol on experimenatl groups compared to the C group ($p = 0.052$).

Table 6. Effects of dietary marigold and red pepper extract powder on antioxidant profile (average values/group).

Specifications	Experimental Diets				SEM	*p*-Value
	C	E1	E2	E3		
Lutein + zeaxanthin (ppm)	7.77 ^{c}	28.67 ^{a}	8.03 ^{c}	12.35 ^{b}	0.436	0.0001
Alfa tocopherol (ppm)	158.48	179.34	160.43	163.74	6.01	0.167
Gama tocopherol (ppm)	15.83	18.21	17.92	17.65	0.533	0.052
Vitamin E (ppm)	174.31	197.55	178.35	181.39	6.44	0.166
Vitamin A (ppm)	19.84	22.76	19.09	21.26	1.07	0.195
Antioxidant capacity (μM Trolox)	0.54 ^{b}	0.60 ^{ab}	0.69 ^{a}	0.60 ^{ab}	0.033	0.067
Total polyphenols, mg/g GAE	0.15 ^{c}	0.19 ^{bc}	0.29 ^{a}	0.24 ^{ab}	0.020	0.002

C—control diet; E1—control diet supplemented with 0.07% marigold extract powder; E2—control diet supplemented with 0.07% red pepper extract powder; E3—control diet supplemented with a mix of 0.05% red pepper extract powder and 0.02% marigold extract powder; SEM, standard error of the mean; $^{a–c}$ mean values within a row not sharing the same superscripts are significantly different at $p < 0.05$.

3.6. Oxidative Stability

In Figure 1 are presented the differences registered between experimental groups regarding the TBARS values. Common practices in the scientific evaluation of egg stability and quality involve monitoring them at intervals of 7, 14, 21, or 28 days to understand the qualitative transformations that occur during the product's shelf life under various storage conditions and temperatures (https://agriculture.ec.europa.eu/farming/animal-products/eggs_en (accessed on 17 June 2024). In our study, after 28 days, under both storage conditions (room temperature and refrigeration), the TBARS values were highly significant ($p < 0.0001$) when compared to the C group. The TBARS values decreased significantly ($p < 0.0001$), which indicates that the natural extracts of marigold and paprika demonstrated a high lipid oxidative capacity, delaying the degradation of yolk fat. There were no statistically significant differences ($p > 0.05$) between the experimental groups for the 4 °C versus the 20 °C conditions.

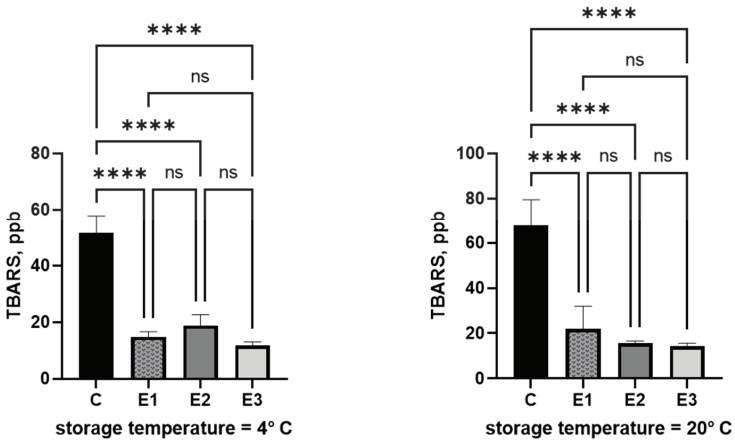

Figure 1. Effect of dietary marigold and red pepper extract powders on egg yolk TBARS concentration evolution in time (after 28 days storage period), with **** $p < 0.0001$ indicating a significant difference between groups ($p < 0.05$). ns: not significant.

4. Discussion

4.1. Nutritional Composition of Powder Extracts of Marigold and Red Pepper

Both marigold and red pepper extracts provide a natural yellow/red hue to egg yolks, with a high efficiency and rate of transfer to eggs. Marigold extract contains a high content of polyphenols, lutein and zeaxanthin compared to red pepper extract. This makes it a potent source of antioxidants, valuable for enhancing immune function. On the other hand, red pepper extract registered a higher antioxidant capacity, higher concentrations of gamma-tocopherol, alpha-tocopherol, astaxanthin and canthaxanthin, and crucial trace minerals such as copper, iron, manganese, and zinc, compared to marigold extract. Other studies on marigolds [24] registered a concentration of 12.16% for Cu and 1262.54 mg for Fe, while the lutein + zeaxanthin was 11.221 ppm and the total polyphenols were 13.55 mg GAE/g. Varying results were found in the literature when assessing the antioxidant activities of plant extracts due to different factors such as extraction solvents, assay procedures, and sample processing methods used [25,26]. Also, within marigold fresh flowers, the lutein concentration can vary from 4 mg/g in greenish-yellow flowers to 800 mg/g in orange-brown flowers. Dark colors have roughly 200 times more lutein esters compared to light colors [27]. Panaite et al. [28] found in kappa pepper the highest concentrations of lutein at 5.631 mg/kg, zeaxanthin at 1.528 mg/kg, lycopene at 0.351 mg/kg, and β-carotene at 7.576 mg/kg, as well as a total carotenoid content of 16.719 mg/kg compared to sea buckthorn pomace and carrot as alternative carotenoid sources.

4.2. Dietary Effects of Powder Extracts of Marigold and Red Pepper on Productivity Parameters

The results of the study indicate that while the experimental groups were not significantly different concerning production indicators including total egg weight or laying rate index, they varied significantly in terms of feed conversion ratio, average egg weight, and average daily feed intake, respectively.

Similar favorable results were observed by Oliveira [29], who concluded that supplementing extracts of marigold (0.1%) and paprika (0.6%) showed no apparent effect on the production of laying hens ($p > 0.05$). Compared to the 0.6% inclusion and control groups, the authors reported that 0.7% red pepper extract enhanced average daily feed intake and average egg weight. Skrivan [30] examined the effects of 150, 250, and 350 mg/kg marigold extracts when included in laying hens' diets, finding no differences concerning the productive performance compared to a corn-based treatment. In egg-laying quails, Oliveira [29] included 0.06% paprika extract and 0.01% marigold extract, and registered an

increased nutritional digestibility of the small intestine, a lower average feed intake, and a higher feed/gain ratio. A slightly increased egg weight was noted as well by Jang et al. [31] in lutein-supplemented groups. However, Skřivan et al. [30] reported a decrease in egg weight in the group fed with marigold flower extract. Other researchers [32] concluded that lutein from marigold flower extract did not affect egg weight. Atai et al. [33] examined the effects of 100, 200, 400, and 800 ppm lutein on 70 Brown-Nick laying hens (39-week-old) over a 6-week trial period. They found no significant effects on final body weight, feed intake, or feed conversion ratio, but noted higher egg production in the 100, 200, and 400 ppm lutein groups compared to the control and 800 ppm groups. Wang et al. [34] observed that supplementation with 0.075%, 0.15%, 0.30%, and 0.60% of marigold extract did not affect feed intake, body weight gain, or feed conversion ratio. Other authors [35] also found improvements in egg mass, egg production and feed conversion rate in an experiment on 160 laying hens fed with a diet supplemented with 0.5, 1, and 1.5% red pepper extract.

4.3. Dietary Effects of Powder Extracts of Marigold and Red Pepper on External and Internal Egg Quality Parameters

Supplementation with the dietary marigold extract (E1), paprika (E2), and both extracts (E3) did not significantly affect whole egg weight, albumen and yolk weight, eggshell thickness, breaking strength, yolk pH, or yolk diameter. This suggests that the inclusion of these natural pigment extracts does not significantly impact the egg quality under the experimented conditions. Significant differences ($p < 0.05$) were observed in eggshell weight during the two different periods from 3 weeks to 6 weeks, indicating that time may influence the shell quality and the pH of the albumen, which might be related to changes in egg freshness or storage conditions. Significant differences in albumen height were observed between groups and periods, with a noted decrease at 6 weeks compared to 3 weeks and with a similar pattern reflected in the Haugh unit, suggesting a decline in freshness over time. Differences were also statistically significant for yolk height and index between groups, indicating that the diets might have some specific effects on yolk structure and quality. The changes observed during the two periods of time, with generally lower values in parameters such as albumen height and Haugh unit, emphasize that the observed variations were more influenced by the duration of the storage period rather than the type of dietary pigment. Grčević et al. [36], when using 1 g/kg of marigold extract (E1) and 2 g/kg of marigold extract supplementation (E2), noticed that the greatest values of albumen height and HU were measured in the E2 group, both in fresh and in stored eggs, but the authors could not explain the marigold extract's influence on these egg quality parameters. Maia et al. [37] tested four levels of marigold flower extract (2.10; 2.40; 2.70; 3.00 ppm) and observed a linear reduction in albumen percentage. Chowdhury et al. [38] used 40 g of marigold flower in laying pullets, observing no statistical differences compared to the C group regarding the albumen index, weight and Haugh unit measured at 8 and 12 experimental weeks. Mixed results have been found concerning the effects of marigold extract on egg quality parameters, with some studies showing improvements and others not finding significant effects. The mixed results indicate that adding these plant extracts might have some potential benefits for productive performance, or no significant effect. In another similar study, Spasevski et al. [39] conducted a trial using marigold (1.5%, 1%, 0.5%) and paprika (1.5%, 1%, 0.5%), and a combination of the two plants (marigold 1% + paprika 0.5%), and found no significant differences ($p > 0.05$) in egg weight, shell, yolk, or albumen. Oliveira et al. [29] stated that none of the experimental groups (paprika vs. marigold or the mix of the two) produced changes in egg weight or Haugh unit. The inclusion of paprika extract improved egg quality, lowering egg pH values and increasing yolk height. A significant difference in laying rate and egg mass ($p < 0.02$) was registered in the experimental groups vs. the control group. Also, Moraleco et al. [40] included marigold flower (0.8%) and paprika (0.8%) extracts in the diets of 90 Black Avifran hens (60 weeks old) with no significant effects ($p > 0.05$) concerning egg/shell weight or shell percentage.

Further, when 4% paprika extract was used in the diets of Lohmann Brown laying hens aged 30 weeks, the authors declared there were no significant differences either in the feed consumption of the hens or in the quality parameters of the eggs [41]. Hussain et al. [42] supplemented the diets of layer hens (27-week-old) with 4% marigold powder for 60 days, noticing that egg weight was significantly ($p < 0.05$) impacted by the dietary inclusion of marigold. Maia et al. [37] included four levels of marigold flower extract (2.10; 2.40; 2.70; 3.00 ppm) and noticed that the yolk and Haugh unit linearly increased concomitantly with levels of marigold, whereas the percentage of albumen decreased linearly. Also, Skřivan et al. [30] stated that the addition of marigold extract in the diets of laying hens increased hen egg production and egg weight.

4.4. Dietary Effects of Powder Extracts of Marigold and Red Pepper on Yolk Color

Although the yolk color does not significantly influence the egg's nutritional value, consumers often associate a darker/intense yellow or golden-orange hue with a healthier egg, rich in natural carotenoids. Worldwide, preferences for yolk color can differ significantly across various countries and regions. The highest amounts of lutein and zeaxanthin, which provide a yellow hue, were registered in the E1 group for marigold extract, and the highest value for the a* redness parameter coincided with the E2 group, red paprika extract. Overall, dietary supplementation in the E1, E2, and E3 groups effectively enhanced yolk pigmentation compared to the C group. Authors Belyavin and Marangos [43] suggested that, for optimal yolk coloration, the hens' diets should be supplemented with both yellow and red xanthophylls. The marigold flower contains 12 g/kg of total xanthophylls (80 to 90% lutein). Similarly, paprika meal contains 4 to 8 g/kg of total xanthophylls (50 to 70% capsanthin). Our results are similar to those of Skřivan M. et al. [30], who stated that the level of lutein and zeaxanthin increased yolk color parameters. Lokaewmanee et al. [32] noted that incorporating marigold extract into the diet of laying hens at a rate of 0.4% increased yolk redness. The other authors Englmaierová and Skrivan [44] and Skrivan et al. [30] stated that including marigold extract in hens' diets led to an increased yolk coloration compared to diets based only on maize. Niu et al. [45] noticed a dose-dependent carotenoid concentration in the yolk ranging from 3.43 mg/g to 16.83 mg/g as paprika extract inclusion rates varied from 0.1% to 0.8%. Lokaewmanee et al. [46] found no combined effects of paprika and marigold on yolk coloration, although Moura et al. [47] reported an increase in coloration effects when the two plant extracts were combined.

Oliveira et al. [29] reported that a 0.6% red pepper inclusion rate significantly enhanced yolk color compared to groups uisng only marigold extract or a mixture of paprika and marigold. Furthermore, Lokaewmanee [46,48] considered that red pepper powder could be used as a potent natural colorant for poultry, enhancing yolk coloration. Santos-Bocanegra et al. [49] observed that hens fed with dietary red xanthophylls from capsicum (7.5 ppm) or yellow xanthophylls from *Tagetes* (4.0 ppm) exhibited intense yolk pigmentation, classified as 11.7, compared to synthetic carotenoids (citranaxanthin, canthaxanthin pigments) at various concentrations, resulting in yolk color ranging from 13 to 14 at the highest concentration. Panaite et al. [28] provided 2% kapia pepper, in a 4-week experiment, to Lohmann Brown layers (43 weeks of age), and registered the highest reddish yolk pigmentation. Furthermore, Grčević et al. [36] tested 0.2% and 0.4% dietary additions of marigold powder extract on laying hens at 31 weeks of age, observing a color intensification due to the positive correlation between the inclusion rate and the increased lutein concentration. Yolk color was evaluated using the RocheFan, rating the color from 1 (very light) to 15 (very dark orange), and the yolk color was found to be 12.66. Maia et al. [37] observed a quadratic effect concerning marigold inclusion (2.73 and 2.80 ppm/kg) when assessed using YolkFan DSM®, especially regarding redness/yellowness.

4.5. Dietary Effects of Powder Extracts of Marigold and Red Pepper on Antioxidant Profile

In our study, we observed that while the yolks from the group receiving marigold extract exhibited the highest concentrations of lutein and zeaxanthin, the highest values for antioxidant capacity and total polyphenols were found in group E2 compared to group C.

The lutein concentration in red pepper was around 4 times higher compared to marigold, and similar results were found for the antioxidant capacity values, which were approximately 2.3 times higher compared to marigold. According to Biacs et al. [50], the level of antioxidant compound, and the content and composition of carotenoids, are linked to the quality and stability of paprika color. As Daood et al. [51] stated that the yellow color of paprika is attributed to β-carotene and zeaxanthin ester concentrations, while capsorubin, cryptocapsin esters, and capsanthin contribute to the red coloration of carotenoids in red peppers. Wang et al. [52] used 24 kg of dried marigold flower (170.2 g free lutein, 80% purity) and showed via PCL assay that lutein exhibited higher antioxidant activity (0.266 mM Trolox equivalent) compared to β-carotene (0.027 mM) and lycopene (0.018 mM). Additionally, using the b-CLAMS assay, only lutein displayed the inhibition of peroxidation.

4.6. Dietary Effects of Powder Extracts of Marigold and Red Pepper on Oxidative Stability

Our experiment highlighted the effects of marigold and red pepper extract supplementation on lipid oxidation in eggs, as measured by TBARS values, an indicator of lipid peroxidation. The significant TBARS values reduced after 28 days, for all experimental groups (E1, E2, and E3), compared to the C group, and this suggests that the two natural extracts have antioxidant properties, effectively delaying the degradation of yolk fat. No significant differences were found among the experimental groups (E1, E2, and E3), neither at 4 °C nor at 20 °C, after 28 days of storage, and this indicates that both marigold and red pepper extracts, whether used individually or in combination, presented similar effectiveness in reducing lipid oxidation. Other researchers found that natural sources of essential polyunsaturated fatty acid (flaxseed) can be successfully combined with dietary natural antioxidants such as kapia pepper, dried carrot, sea buckthorn pomace [28], thyme [53], pine wood [54] and grape pomace [55,56] to reduce lipid peroxidation in the yolk. The study conducted by Romero et al. [55] concluded that grape pomace demonstrated a higher antioxidant capacity compared to grape extract in yolk. Also, Panaite et al. [28] observed a significant decrease in TBARS values in the groups supplemented with 2% kapia pepper and 2% linseed meal (0.14 mg MDA/kg). Grčević et al. [36] noticed that dietary supplementation with 400 mg/kg of marigold extract led to a slightly reduced oxidation value compared to the control group, but no statistical differences were observed regarding the lipid oxidation levels among fresh egg yolks. Meanwhile, Englmaierová et al. [44] established a significant influence ($p < 0.001$) of lutein supplementation (250 mg/kg) on the enhancement of the oxidative stability of yolk lipids during a 28-day storage period at 18 °C. Rezaei et al. [57] observed a significant improvement in the oxidative stability of yolks in hens fed marigold pigments after 3 weeks ($p < 0.05$). Skřivan et al. [30] reported that the dietary inclusion of 950 mg marigold extract/kg reduced lipid peroxidation levels. Additionally, all supplementary levels of marigold flower extract (150, 350, 550 and 750 mg marigold extract/kg diet) notably enhanced the lipid oxidative stability in eggs stored for 28 days at 18 °C. According to Cadun [58], food that is intended for human consumption should have lipid oxidation levels below 3 mg MDA/kg of sample, with a maximum limit of 7–8 mg MDA/kg sample.

5. Conclusions

Marigold and red pepper represent natural sources of carotenoids with the capacity to improve yolk color pigmentation, which is an important criterion of consumer preference, without affecting the health and the productive performances of laying hens. In our study, we obtained decreased TBARS values after 28 days of storage at both 4 and 20 °C, and observed significantly increased yolk coloration in all experimental groups. Therefore,

these two natural extracts could enhance egg quality during different storage periods and under different temperature conditions as an alternative to synthetic colorants, with potential applications in animal nutrition, and further benefits for food preservation and human health.

Author Contributions: Conceptualization, T.D.P. and C.-C.M.; methodology, T.D.P., C.-C.M., G.M.C., A.E.C., A.E.U. and M.S.; software, T.D.P.; validation, T.D.P.; formal analysis, T.D.P. and C.-C.M.; investigation, C.-C.M., G.M.C. and A.E.C.; resources, T.D.P.; data curation, T.D.P.; writing—original draft preparation, C.-C.M.; writing—review and editing, T.D.P. and G.M.C.; visualization, T.D.P. and C.-C.M.; supervision, T.D.P. and D.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Research, Innovation and Digitization, Romania—Project PN 23 20 0101.

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of NATIONAL RESEARCH AND DEVELOPMENT INSTITUTE FOR BIOLOGY AND ANIMAL NUTRITION (IBNA-Balotesti, Romania; Protocol no. 4096/23 September 2022) following the Romanian legislation (Law 43/11 April 2014, Directive 2010/63/EU) for the feeding, handling, and slaughtering procedures of the study.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Paulino, M.T.F.; de Oliveira Grieser, D.; Gasparino, E.; Maia, K.M.; Toledo, J.B.; Ton, A.P.S.; Budel, E.C.; Marcato, S.M. Influence of pigments on the shelf life of eggs from layers hens in the final phase of production. *Res. Soc. Dev.* **2022**, *11*, e155111133484. [CrossRef]
2. Honorato, C.A.; Seabra, B.S.; Siqueira, M.S.; Melgarejo, M.R.; Fraga, T.L. Qualidade e características físicas de ovos comerciais. *Nucl. Anim.* **2016**, *8*, 29–36. [CrossRef]
3. Silva, R.C.; Nascimento, D.J.W.; Oliveira, D.L.D.; Furtado, D.A. Termohigrometria no transporte e na qualidade de ovos destinados ao consumo humano. *Rev. Bras. Eng. Agríc. Ambient.* **2015**, *19*, 668–673. [CrossRef]
4. Volp, A.C.P.; Renhe, I.R.T.; Stringueta, P.C. Pigmentos naturais bioativos. *Alim. Nutr. Araraquara* **2009**, *20*, 157–166.
5. Maoka, T. Carotenoids as natural functional pigments. *J. Nat. Med.* **2020**, *74*, 1–16. [CrossRef]
6. Elvira-Torales, L.I.; García-Alonso, J.; Periago-Castón, M.J. Nutritional importance of carotenoids and their effect on liver health: A review. *Antioxidants* **2019**, *8*, 229. [CrossRef] [PubMed]
7. Surai, P.F. Polyphenol compounds in the chicken/animal diet: From the past to the future. *J. Anim. Physiol. Anim. Nutr.* **2014**, *98*, 19–31. [CrossRef]
8. Detofol, D.F.; Rauta, J.; Winck, C.A. Logística aplicada no processo de produção de ovos comerciais. *Rev. Visão Gestão Organ.* **2018**, *7*, 52–69. [CrossRef]
9. Darvin, M.E.; Lademann, J.; von Hagen, J.; Lohan, S.B.; Kolmar, H.; Meinke, M.C.; Jung, S. Carotenoids in human skin in vivo: Antioxidant and photo-protectant role against external and internal stressors. *Antioxidants* **2023**, *11*, 1451. [CrossRef]
10. El-Sabrout, K.; Aggag, S.; Mishra, B. Advanced Practical Strategies to Enhance Table Egg Production. *Scientifica* **2022**, *2022*, 1393392. [CrossRef]
11. Dansou, D.M.; Zhang, H.; Yu, Y.; Wang, H.; Tang, C.; Zhao, Q.; Qin, Y.; Zhang, J. Carotenoid enrichment in eggs: From bio-chemistry perspective. *Anim. Nutr.* **2023**, *14*, 315–333. [CrossRef]
12. Altuntaş, A.; Aydin, R. Fatty acid composition of egg yolk from chickens fed a diet including marigold (*Tagetes erecta* L.). *J. Lipids* **2014**, *2014*, 564851. [CrossRef] [PubMed]
13. Burlec, A.F.; Pecio, Ł.; Kozachok, S.; Mircea, C.; Corciovă, A.; Vereștiuc, L.; Cioancă, O.; Oleszek, W.; Hăncianu, M. Phytochemical profile, antioxidant activity, and cytotoxicity assessment of *Tagetes erecta* L. flowers. *Molecules* **2021**, *26*, 1201. [CrossRef] [PubMed]
14. Kodama, T.; Watanabe, E.; Masuyama, T.; Tsubuku, S.; Otabe, A.; Katsumata, Y.; Bernard, B.K. Studies of the Toxicological Potential of Capsinoids: III. A Two-Generation Reproduction Study of CH-19 Sweet Extract in Rats. *Int. J. Toxicol.* **2008**, *27 (Suppl. S3)*, 29–39. [CrossRef] [PubMed]
15. Kennedy, L.E.; Abraham, A.; Kulkarni, G.; Shettigar, N.; Dave, T.; Kulkarni, M. Capsanthin, a plant-derived xanthophyll: A review of pharmacology and delivery strategies. *AAPS PharmSciTech* **2021**, *22*, 203. [CrossRef]
16. Lourenço-Lopes, C.; Carreira-Casais, A.; Fraga-Corral, M.; Garcia-Oliveira, P.; Soria, A.; Jarboui, A.; Barral, M.; Otero, P.; Simal-Gandara, J.; Prieto, M.A. Carotenoids as natural colorful additives for the food industry. In *Natural Food Additives*; IntechOpen: London, UK, 2021. [CrossRef]

17. Rakonjac, S.; Bogosavljevic-Boskovic, S.; Pavlovski, Z.; Skrbic, Z.; Doskovic, V.; Petrovic, M.D.; Petricevic, V. Laying hen rearing Systems: A review of Chemicals composition and hygienic conditions of eggs. *J. World's Poult. Sci.* **2014**, *70*, 151–163. [CrossRef]
18. Kelly, E.R.; Plat, J.; Haenen, G.R.M.M.; Kijlstra, A.; Berendschot, T.T.J.M. The effect of modified eggs and egg-yolk based bevegare on serum lutein and zeaxanthin concentrations and macular pigment optical density: Results from a randomized trial. *PLoS ONE* **2014**, *9*, e92659. [CrossRef]
19. Bovier, E.R.; Renzi, L.M.; Hammond, B.R. A doucle-blind, placebo-controlled study on the effects of lutein and zeaxanthin on neural processing speed and efficiency. *PLoS ONE* **2014**, *9*, e108178. [CrossRef]
20. National Research Council. *Nutrient Requirements of Poultry*, 9th ed.; National Academy Press: Washington, DC, USA, 1994.
21. Untea, A.; Criste, R.C.; Vladescu, L. Development and validation of a microwave digestion–FAAS procedure for Cu, Mn and Zn determination in liver. *Rev. Chim.* **2012**, *63*, 341–346.
22. Untea, A.E.; Varzaru, I.; Panaite, T.D.; Gavris, T.; Lupu, A.; Ropotă, M. The effects of dietary inclusion of bilberry and walnut leaves in laying hens' diets on the antioxidant properties of eggs. *Animals* **2020**, *10*, 191. [CrossRef]
23. Varzaru, I.; Panaite, T.D.; Untea, A.E.; Olteanu, M.; Bordei, N.; Van, I. Composition of some botanical mixtures as potential feed additives for laying hens. *Food Feed Res.* **2015**, *42*, 59–66. [CrossRef]
24. Panaite, T.D.; Olteanu, M.; Untea, A.E.; Ropota, M.; Varzaru, I.; Lupu, A. Feeding value of local phyto-additives, potential ingredients in poultry diets. *Sci. Papers Ser. D Anim. Sci.* **2019**, *62*, 122.
25. Rivas-García, L.; Crespo-Antolín, L.; Forbes-Hernández, T.Y.; Romero-Márquez, J.M.; Navarro-Hortal, M.D.; Arredondo, M.; Llopis, J.; Quiles, J.L.; Sánchez-González, C. Bioactive properties of *Tagetes erecta* edible flowers: Polyphenol and antioxidant characterization and therapeutic activity against ovarian tumoral cells and caenorhabditis elegans tauopathy. *Int. J. Mol. Sci.* **2024**, *25*, 280. [CrossRef] [PubMed]
26. Toliba, A.O.; Egorov, M.A.; Sukhenko, L.T.; Akmaev, E.P. Physicochemical properties and food application of marigold flower extracts prepared by conventional and supercritical CO_2 methods. *Int. J. Adv. Res.* **2018**, *6*, 876–885. [CrossRef]
27. Gregory, G.K.; Chen, T.S.; Philip, T. Quantitative analysis of lutein esters in marigold flowers (*Tagetes erecta*) by high performance liquid chromatography. *J. Food Sci.* **1986**, *51*, 1093–1094. [CrossRef]
28. Panaite, T.D.; Nour, V.; Saracila, M.; Turcu, R.P.; Untea, A.E.; Vlaicu, P.A. Effects of linseed meal and carotenoids from different sources on egg characteristics, yolk fatty acid and carotenoid profile and lipid peroxidation. *Food* **2021**, *10*, 1246. [CrossRef] [PubMed] [PubMed Central]
29. Oliveira, M.C.D.; Silva, W.D.D.; Oliveira, H.C.; Moreira, E.D.Q.B.; Ferreira, L.D.O.; Gomes, Y.D.S.; Souza, M.A.P.D. Paprika and/or marigold extracts in diets for laying hens. *Rev. Bras. Saude Prod. Anim.* **2017**, *18*, 293–302. [CrossRef]
30. Skrivan, M.; Englmaierová, M.; Skrivanová, E.; Bubancová, I. Increase in lutein and zeaxanthin content in the eggs of hens fed marigold flower extract. *Czech J. Anim. Sci.* **2015**, *60*, 89–96. [CrossRef]
31. Jang, I.; Ko, Y.; Kang, S.; Kim, S.; Song, M.; Cho, K.; Ham, J.; Sohn, S. Effects of dietary lutein sources on lutein-enriched egg production and hepatic antioxidant system in laying hens. *Poult. Sci. J.* **2014**, *51*, 58–65. [CrossRef]
32. Lokaewmanee, K.; Yamauchi, K.; Komori, T.; Saito, K. Enhancement of yolk color in raw and boiled egg yolk with lutein from marigold flower meal and marigold flower extract. *Jpn. Poult. Sci.* **2011**, *48*, 25–32. [CrossRef]
33. Atay, A. Effect of different levels of lutein on laying performance and egg quality in laying hens. *Indian J. Anim. Sci.* **2022**, *92*, 1102–1106. [CrossRef]
34. Wang, S.; Zhang, L.; Li, J.; Cong, J.; Gao, F.; Zhou, G. Effects of dietary marigold extract supplementation on growth performance, pigmentation, antioxidant capacity and meat quality in broiler chickens. *Asian-Australas J. Anim. Sci.* **2017**, *30*, 71. [CrossRef] [PubMed]
35. Sözcü, A. Effects of supplementing layer hen diet with red pepper (*Capsicum annuum* L.) powder as natural yolk colourant on laying performance, pigmentation of yolk, egg quality and serum immunoglobulin levels. *J. Poult. Res.* **2019**, *16*, 80–85. [CrossRef]
36. Grčević, M.; Kralik, Z.; Kralik, G.; Galović, O. Effects of dietary marigold extract on lutein content, yolk color and fatty acid profile of omega-3 eggs. *J. Sci. Food Agric.* **2019**, *99*, 2292–2299. [CrossRef]
37. Maia, K.M.; Grieser, D.O.; Ton, A.P.S.; Aquino, D.R.; Paulino, M.T.F.; Toledo, J.B.; Marcato, S.M. Perfor-mance and egg quality of light laying hens fed with canthaxanthin and marigold flower extract. *S. Afr. J. Anim. Sci.* **2022**, *52*, 433–443. [CrossRef]
38. Chowdhury, S.D.; Hassin, B.M.; Das, S.C.; Rashid, M.H.; Ferdaus, A.J. Evaluation of marigold flower and orange skin as sources of xanthophyll pigment for the improvement of egg yolk color. *J. Poult. Sci.* **2008**, *45*, 265–272. [CrossRef]
39. Spasevski, N.; Tasić, T.; Vukmirović, Đ.; Banjac, V.; Rakita, S.; Lević, J.; Đuragić, O. Effect of different levels of marigold and paprika on egg production and yolk colour. *Arch. Zootech.* **2017**, *20*, 51–57.
40. Moraleco, D.D.; Valentim, J.K.; Silva, L.G.; Lima, H.J.D.Á.; Bitencourtt, T.M.; Dallago, G.M. Egg quality of lay-ing hens fed diets with plant extracts. *Acta Sci. Anim. Sci.* **2019**, *41*, e43801. [CrossRef]
41. Aktaran Bala, D.; Matur, E.; Ergul Ekiz, E.; Akyazi, I.; Ergen, E.; Erek, M.; Atmaca, G.; Eseceli, H.; Keten, M. Can dried tomato and red pepper powder be used as a dietary supplement to strengthen defence systems and production performance in laying hens. *EPS/Arch. für Geflügelkunde* **2020**, *84*, 1–15. [CrossRef]
42. Hussain, S.; Gulfreen, E.; Abid, S.; Khalil, S.; Rizwan, M.; Batool, N.; Aziz, A.; Abid, H.M.U.; Mahmood, N. Investigating the Impact of Marigold Supplementation on Egg Yolk Color Intensity: A Study on Dietary Additives. *J. Health Rehabil. Res.* **2024**, *4*, 1744–1751. [CrossRef]

43. Belyavin, C.G.; Marangos, A.G. Natural products for egg yolk pigmentation. In *Recent Advances in Animal Nutrition*; Haresign, W., Cole, D.J.A., Eds.; Butterworths: London, UK, 1987.
44. Englmaierová, M.; Skrivan, M. Effect of synthetic carotenoids, lutein, and mustard on the performance and egg quality. *Sci. Agric. Bohem.* **2013**, *44*, 138–143. [CrossRef]
45. Niu, Z.; Fu, J.; Gao, Y.; Liu, F. Influence of páprica extract supplement on egg quality of laying hens fed wheat-based diet. *Int. J. Poult. Sci.* **2008**, *7*, 887–889. [CrossRef]
46. Lokaewmanee, K.; Yamauchi, K.; Komori, T.; Saito, K. Enhancement of egg yolk color by paprika combined with a probiotic. *J. Appl. Poult. Res.* **2011**, *20*, 90–94. [CrossRef]
47. Moura, A.M.A.; Takata, F.N.; Nascimento, G.R.; Silva, A.F.; Melo, T.V.; Cecon, P.R. Pigmentantes naturais em rações à base de sorgo para codornas japonesas em postura. *Rev. Bras. Zootec.* **2011**, *40*, 2443–2449. [CrossRef]
48. Lokaewmanee, K.; Yamauchi, K.; Okuda, N. Effects of dietary red pepper on egg yolk colour and histological intestinal morphology in laying hens. *J. Anim. Physiol. Anim. Nutr.* **2013**, *97*, 986–995. [CrossRef]
49. Santos-Bocanegra, E.; Ospina-Osorio, X.; Oviedo-Rondon, E.O. Evaluation of xanthophylls extracted from *Tagetes erectus* (Marigold flower) and *Capsicum* sp. (Red pepper paprika) as a pigment for egg-yolks compare with Syn-thetic pigments. *Int. J. Poult. Sci.* **2004**, *3*, 685–689.
50. Biacs, P.; Czinkotai, B.; Hoschke, A. Factors affecting stability of coloured substances in paprika. *J. Agric. Food Chem.* **1992**, *40*, 363–367. [CrossRef]
51. Daood, H.G.; Palotás, G.; Palotás, G.; Somogyi, G.; Pék, Z.; Helyes, L. Carotenoid and antioxidant content of ground paprika from indoor-cultivated traditional varieties and new hybrids of spice red peppers. *Int. Food Res. J.* **2014**, *65*, 231–237. [CrossRef]
52. Wang, M.; Tsao, R.; Zhang, S.; Dong, Z.; Yang, R.; Gong, J.; Pei, Y. Antioxidant activity, mutagenici-ty/anti-mutagenicity, and clastogenicity/anti-clastogenicity of lutein from marigold flowers. *Food Chem. Toxicol.* **2006**, *44*, 1522–1529. [CrossRef]
53. Tadesse, D.; Retta, N.; Girma, M.; Ndiwa, N.; Dessie, T.; Hanotte, O.; Getachew, P.; Dannenberger, D.; Maak, S. Yolk Fatty Acid Content, Lipid Health Indices, and Oxidative Stability in Eggs of Slow-Growing Sasso Chickens Fed on Flaxseed Supplemented with Plant Polyphenol Extracts. *Foods* **2023**, *12*, 1819. [CrossRef]
54. Shahid, M.S.; Zhou, S.; Nie, W.; Wang, L.; Lv, H.; Yuan, J. Phytogenic antioxidants prolong n-3 fatty acid-enriched eggs' shelf life by activating the Nrf-2 pathway through phosphorylation of MAPK. *Foods* **2022**, *11*, 3158. [CrossRef] [PubMed]
55. Romero, C.; Arija, I.; Viveros, A.; Chamorro, S. Productive performance, egg quality and yolk lipid oxidation in laying hens fed diets including grape pomace or grape extract. *Animals* **2022**, *12*, 1076. [CrossRef]
56. Kara, K.; Kocaoğlu Güçlü, B.; Baytok, E.; Şentürk, M. Effects of grape pomace supplementation to laying hen diet on performance, egg quality, egg lipid peroxidation and some biochemical parameters. *J. Appl. Anim. Res.* **2016**, *44*, 303–310. [CrossRef]
57. Rezaei, M.; Zakizadeh, S.; Eila, N. Effects of pigments extracted from the marigold flower on egg quality and oxidative stability of the egg yolk lipids in laying hens. *Iran. J. Appl. Anim. Sci.* **2019**, *9*, 541–547.
58. Cadun, A.; Įakli, Ş.; Kişla, D.; Dinįer, T.; Erdem, Ö.A. Effects of fibers on the quality of fish patties stored at (0–4 °C). *J. Food Health Sci.* **2015**, *1*, 211–219. [CrossRef]

agriculture

MDPI

Article

Obtaining Goats' Dairy Products Enriched in Healthy Fatty Acids by Valuing Linseed or Hempseed as Dietary Ingredients

Ana Elena Cismileanu [1], Smaranda Mariana Toma [2,*], Mariana Ropota [3], Costin Petru Dragomir [4], Gabriela Maria Cornescu [1] and Catalin Dragomir [1]

[1] Laboratory of Physiology of Animal Nutrition, National Research—Development Institute for Animal Biology and Nutrition, 1 Calea Bucuresti, 077015 Balotesti, Romania; ana_cismileanu@yahoo.com (A.E.C.); gabriela_cornescu@yahoo.com (G.M.C.); catalin.dragomir@ibna.ro (C.D.)
[2] Laboratory of Animal Nutrition and Biotechnologies, National Research—Development Institute for Animal Biology and Nutrition, 1 Calea Bucuresti, 077015 Balotesti, Romania
[3] Laboratory of Quality of Feed and Food, National Research—Development Institute for Animal Biology and Nutrition, 1 Calea Bucuresti, 077015 Balotesti, Romania; m.ropota@yahoo.com
[4] Laboratory of Molecular Biology, National Research and Development Institute for Food Bioresources, 5th Ancuta Baneasa Str., 2nd District, 020323 Bucharest, Romania; costin9991@gmail.com
* Correspondence: smaranda.pop@ibna.ro; Tel.: +40-0213512084

Abstract: The study aimed to assess the effects of including linseeds or hempseeds in the diets of late lactation Murciano-Granadina dairy goats on the nutritional quality of the milk and cheese fat, expressed by the fatty acids profile and the healthy lipid indices. Thirty-six goats were randomly distributed in 3 groups of 12 animals each, according to a 3 × 3 Latin square design, and fed three different diets: group CON (control, with sunflower meal, 11.5% DM basis); group LIN, where sunflower meal was replaced by linseed; and group HMP, where sunflower meal was replaced by hempseeds. The replacement had no effects on the milk yields and the milk protein content as no significant differences were detected among groups. The significant increase of the fat content in the case of the LIN and HMP groups was accompanied by significant decreases in saturated fatty acids concentration and very significant increases in monounsaturated fatty acids. The content of n3 and n6-PUFAs (polyunsaturated fatty acids) increased, mainly due to a 4.1 times higher proportion of alpha-linolenic acid (ALA; C 18:3n-3) in LIN diet milk and a 1.3 times higher proportion of linoleic acid (LA; C 18:2n6c) in HMP diet milk. The conjugated linoleic acid (CLA; isomer *c*9, *t*11) was 1.9 times higher for the LIN diet and 5.05 times higher for the HMP diet. Feeding either linseed or hempseeds contributed to the reduction of the atherogenic and thrombogenic indices, increased the hypocholesterolemic: hypercholesterolemic ratio as well as the proportion of other desired fatty acids in the milk fat. The improved nutritional quality of milk, which has potentially far-reaching human health benefits, is maintained in cheese through the increase of the n3 and n6-PUFAs, especially for the LIN diet where the n6/n3 ratio decreased significantly, compared with the CON diet (3.62 vs. 6.88). The CLA concentration was significantly higher ($p < 0.001$) for the HMP cheese compared with the CON diet (1.89% vs. 0.78%). These effects highlight the opportunity of obtaining dairy products with improved nutritional quality using local feed resources.

Keywords: CLA; fatty acids; goat cheese; hempseed; linseed; milk

Citation: Cismileanu, A.E.; Toma, S.M.; Ropota, M.; Dragomir, C.P.; Cornescu, G.M.; Dragomir, C. Obtaining Goats' Dairy Products Enriched in Healthy Fatty Acids by Valuing Linseed or Hempseed as Dietary Ingredients. *Agriculture* **2024**, *14*, 1498. https://doi.org/10.3390/agriculture14091498

Academic Editor: Antonio Natalello

Received: 18 June 2024
Revised: 14 August 2024
Accepted: 20 August 2024
Published: 2 September 2024

1. Introduction

The existing knowledge regarding the potential effects of fats on human health allows food to be classified as "good" or "bad", depending on the nature of the fats they contain. From a human health perspective, the fat in the human diet should have an ideal fatty acids (FA) composition of 8% SFA (saturated fatty acids), 82% MUFA (monounsaturated fatty acids), and 10% PUFA (polyunsaturated fatty acids) [1]. Dairy products are a good source of fat in human nutrition but at the same time, dairy fat contains, on average, 70% SFA,

25% MUFA, and 5% PUFA and therefore may induce health problems like cardiovascular diseases. But the profile of FA can be healthier, especially by tailoring the feeding strategies for ruminants [2] to produce a shift of the of n6/n3 PUFAs ratio in the dairy products [3] or an improvement of the CLA content [4].

Goat milk and goats' dairy products are already associated by consumers with the image of healthy food; this offers the premises for introducing more valuable goat milk products to the market, by feeding the goats with dietary ingredients that are rich in PUFA, in quantities that allow detectable effects in milk. Such ingredients, rich in n3 or/and n6-PUFA, are oilseeds [5]; however, for some of them or their by-products, the effects on goats are less studied. For example, their high fat content may be a limitation of their use in goats' diets, as it may impair rumen processes such as cellulolysis. On the other hand, they should be included in proportions that are high enough to overcome rumen biohydrogenation of the unsaturated fatty acids.

The linseeds (*Linum usitatissimum* L.) contain oil with a high proportion (more than 50%) of ALA (alpha-linolenic acid; C 18:3n3), an important n3-PUFA acid, and they have been used successfully in the diets of ewes to produce n-3 enriched milk as mentioned by some authors [6–8]. More recently, Rapetti et al. [9] reported that after feeding linseeds and hempseeds (at a 9.3% proportion in the diet total DM), Alpine goat milk had higher levels of ALA; linseed induced the lowest n-6/n-3 ratio of the experimental groups, while the in case of LA, no differences were registered among diets.

The hempseeds (*Cannabis sativa* L.) were reconsidered for the use in dairy ruminant nutrition because of their high level of valuable PUFA–LA (linoleic acid; C 18:2n6) as the most predominant FA (53.4–60.0%) [10], followed by ALA (12.98–22.40%) [11], oleic, palmitic, and stearic acids. There are some examples of its inclusion in the nutrition of dairy small ruminants. The hempseeds were utilized successfully to manipulate the FA profile in the sheep milk [12], as well as in Carpathian goat milk with a diet with 4.7% hempseed oil on DM [13] or 9% hempseeds on DM [14]. Also, Cremonesi et al. included hempseeds in diets for Alpine lactating goats by 9.3% on DM [15] or 9.4% on DM [10], and their influence in the milk FA was noticeable. Also, an increased content of milk n3-PUFA and an improved n-6/n-3 ratio were obtained by Mierlita et al. [16] on Turcana dairy sheep after feeding with hempseeds and cake. Total CLA content increased by 2.0 times in the milk of the ewes that received hempseed and by 2.4 times with the hemp cake inclusion. The milk yield and milk fat content were increased but milk lactose decreased.

In this context, we intended to obtain enriched n3 and n6-PUFA milk from Murciano-Granadina goats, adapted to be raised in a farm from Romania using local linseeds or hempseeds in their diets. Therefore, the study aimed to assess the effects of the inclusion of 11.5% of linseeds or hempseeds (DM basis) in the hay-based diets on milk production, as well as on the fatty acid profiles of both milk and cheese.

2. Materials and Methods

2.1. Animals and Experiment Design

The study was carried out on a commercial farm in the southeast of Romania on Murciano-Granadina multiparous goats in late lactation, which have similar age, milk yield, and days in milk (Table 1). Thirty-six goats were randomly distributed in 3 groups of 12 animals each. According to a 3 × 3 Latin square design, each of the three groups was fed, in three successive periods, 3 experimental diets: CON (control group, based on sunflower meal), LIN (where sunflower meal was replaced by linseed), and HMP (where sunflower meal was replaced by hempseed) (see the Scheme 1 below). Each period of feeding lasted 28 days, of which 21 days were for adaptation to the diets and 7 days were for data recording and samplings.

The animals were kept indoors during the experiment, with free access to paddocks, and were group-fed. The animals were milked at 7:00 a.m. and 4:30 p.m., in a milking parlor allowing record of individual milk yields.

Table 1. Parameters of the goats' groups of at the beginning of the experiment.

	CON	LIN	HMP
Age, years	3.30	3.28	3.32
Days in milk	143.75	139.75	141.33
Average milk yield, kg/day	1.10	1.11	1.17

CON, control diet; LIN, linseed diet; HMP, hempseed diet.

	Group 1	Group 2	Group 3
Period 1	CON	LIN	HMP
Period 2	HMP	CON	LIN
Period 3	LIN	HMP	CON

CON, control diet; LIN, linseed diet; HMP, hempseed diet; Period 1-3, the three successive periods of feeding.

Scheme 1. The scheme of 3 × 3 Latin square experiment design.

2.2. Diets and Ingredients

The diets were formulated according to the French feeding system [17]; the control group was fed a diet that is typical for the indoor feeding of goats in Southeastern Europe: hay and a mixture of cereals and sunflower meal (3:1). In the experimental diets, the sunflower was totally replaced with linseed or hempseed in order to supply high amounts of PUFA-rich lipids. For practical reasons, no adjustments of the other dietary ingredients were made; therefore, the experimental diets had a higher energy supply and slightly lower protein supply. The goats were group-fed by limited amounts of compound feed (1.2 kg/head/day) and hay (1.5 kg/head/day) as presented in Table 2. The ingredients of the compound feeds were grinded including the oilseeds. Diets were fed twice daily in equal amounts (at 8.00 a.m. and 5.00 p.m.). Access to water and a trace-mineralized salt block was provided *ad libitum*.

Table 2. Consumption of diets and daily nutritive supplies.

Ingredients	CON	LIN	HMP
Diets' consumption, kg/day			
Grass hay	1.3	1.3	1.3
Alfalfa hay	0.2	0.2	0.2
Compound feed	1.2	1.2	1.2
Compound feeds' structure, %			
Maize (%)	33.4	33.4	33.4
Barley (%)	8.3	8.3	8.3
Oat (%)	12.5	12.5	12.5
Wheat (%)	8.3	8.3	8.3
Wheat bran (%)	8.3	8.3	8.3
Sunflower meal (%)	25.0	0.0	0.0
Linseeds (%)	0.0	25.0	0.0
Hempseeds (%)	0.0	0.0	25.0
Calcium carbonate (%)	2.2	2.2	2.2
Salt (%)	1.0	1.0	1.0
Vitamin-mineral premix for goat (%)	1.0	1.0	1.0
Nutritional and fatty acids supply from the consumed diets			
Dry matter, kg/day	2.356	2.356	2.358
MFU(UFL)/kg DM/day	0.787	0.865	0.863
PDIN, g/day	219.05	181.90	185.86

Table 2. *Cont.*

Ingredients	CON	LIN	HMP
PDIE, g/day	192.67	183.48	190.01
Ether extract, g/day	30.70	107.85	109.29
Calcium, g/day	20.74	20.93	20.10
Phosphorus, g/day	12.03	9.70	9.51
C 16:0, g/day	5.61	9.71	10.62
C 18:0, g/day	0.89	3.61	2.82
C 18:1, g/day	6.32	20.72	15.49
C 18:2n-6, g/day	10.11	21.23	48.52
C 18:3n-3, g/day	2.45	39.08	15.64
Others, g/day	1.27	2.06	4.98
Total FA, g/day	26.66	96.41	98.06

CON = control diet; LIN = linseed diet; HMP = hempseed diet; MFU = Milk Feed Units (UFL), according to INRA system, 2010; 1 UFL = 1700 kcal; PDIN = protein truly digested in the small intestine when the protein is the limiting factor, according to INRA system, 2010; PDIE = protein truly digested in the small intestine when the energy is the limiting factor, according to INRA system, 2010.

The proximate composition of every dietary ingredient was assessed using commonly accepted methods [18]: dry matter (DM) by the gravimetric method, crude protein (CP) by the Kjeldahl method, crude fiber (CF) by successive hydrolysis in alkali and acid environment, ether extractives (EE) by extraction in organic solvents, and ash determined by the gravimetric method.

The levels of CP and CF for both oilseeds were comparable (Table 3). As for the composition of fatty acids, the Romanian variety of linseeds used in the experiment contained 51.33% ALA, 20.83% oleic acid, and 16.42% LA. The hempseeds utilized in the experiment were also sourced from a Romanian variety, primarily cultivated for oil production and characterized by low concentrations of delta-9-tetrahydrocannabinol. The hempseeds contain a high concentration (over 80% of the oil) of long-chain essential PUFAs, as detailed in Table 3. Specifically, they consisted of 53.41% LA and 18.08% ALA. Oleic acid content was 13.19% whereas palmitic and stearic acids were also present.

Table 3. Chemical composition and summarized fatty acids profile of the local varieties of linseeds and hempseeds and of the sunflower meal.

	Linseeds	Hempseeds	Sunflower Meal
Chemical composition			
Dry matter, g/kg	904.06	910.62	902.88
Crude protein, g/kg DM	228.35	236.36	450.99
Crude fat, g/kg DM	291.12	293.60	6.67
Crude fibre, g/kg DM	290.43	303.11	158.88
Nitrogen-free extract, g/kg DM	148.77	126.54	298.98
Ash, g/kg DM	41.32	40.38	84.47
Fatty acids (g/100 g total fatty acids)			
C 16:0	6.09	7.19	14.97
C 18:0	4.00	2.82	7.72
C 18:1	20.83	13.19	28.57
C 18:2n-6	16.42	53.41	37.04
C 18:3n-3	51.33	18.08	0.58
Others	1.44	6.12	2.07

C 16:0, palmitic acid; C 18:0, stearic acid; C 18:1, oleic acid; C 18:2n-6, linoleic acid; C 18:3n-3, alpha-linolenic acid.

2.3. Milk Samples

Two sets of milk samples were collected from each animal at the end of the experimental periods. The first set of samples was used for proximate analyses and therefore preserved with bronopol and stored at 4 °C until further analysis by infra-red spectroscopy.

The second set of milk samples was frozen (−18 °C), without preservatives, for future assessment of the fatty acids' profile.

The proximate milk analysis for fat, protein, lactose, casein, urea, specific density, pH, and total solids was done by FTIR (Fourier Transform Infrared Spectrometer) rotation scanning with a CombiScope FTIR 200 device (Delta Instruments, Drachten, Holland) (ISO 9622:2013) [19]. The somatic cell count was determined according to the SR EN ISO 13366-2:2007 method [20].

The milk fatty acids composition was determined by the gas chromatography method after extracting the lipids by the EN ISO 661:2005/AC:2006 [21] method and then transmethylating the fatty acids with a mixture of concentrated H_2SO_4 (95%) and methanol. The resulting methyl esters of fatty acids (FAME) were separated using the gas chromatograph GC Perkin Elmer-Clarus 500, equipped with a capillary column of high-polarity stationary phase (Agilent BPX70; 60 m × 0.25 mm inner diameter × 0.25 μm thick film) and flame-ionization detector according to SR CEN ISO/TS 17764-2:2008 [22].

2.4. Cheese Samples

The cheese was manufactured from the milk distinctly collected from each experimental group. Ten pieces of cheese were manufactured for each group using a method that is common in the region. The raw milk, not standardized for fat content, was filtered, heated at 36 °C, treated with Rennet 8 g (Ideal Still Exim SRL, Chitila, Romania) as a coagulant, thoroughly mixed, stabilized, and left for 60 min to coagulate. After the milk had clotted, the curd was cut with a knife and left to express the whey for 15 min. After the elimination of the whey, the cheese mass was placed in cheese cube molds. The cheese cubes were pressed for 1.5 h at 20 °C, then cooled (6 °C). After being brined in a solution containing 18% NaCl for 16 h at a temperature of 10 °C, the cheese cubes were subsequently dried. They were then stored in a refrigerated environment at 5 °C until further assays were conducted.

The DM and the CF content (g/100 g) for each of the 30 manufactured cheese pieces were determined according to the same methods that were used for diet ingredients. The fatty acid profile was determined following fat extraction from dried cheese, conversion to fatty acid methyl esters (FAME), and GC analysis performed according to the method described for the milk samples.

The FAME peak areas were converted to fatty acid (FA) using the FAME-to-FA conversion factor for milk. FAs were expressed as a percentage of the total identified FA (% of total FA), or in gravimetric contents (mg/100 g cheese), using the conversion factor for milk and milk products (0.945) for the calculation of total FA from total lipids [23].

2.5. Health Lipid Indices of Milk and Cheese Fat

In addition to the profile of individual FA with each diet, the proportion of beneficial FA was also calculated using the following parameters: PUFA/SFA ratio values; proportion of desired fatty acids (DFA); HSF (hypercholesterolemic SFA) and hypocholesterolemic/hypercholesterolemic (h/H) ratio. The nutritional quality of milk and cheese fat was also assessed by the calculation of health indices: the atherogenic index (AI), calculated according to [24], and the thrombogenic index (TI), calculated following [25].

2.6. Statistical Analysis

The effects of dietary inclusion of linseed or hempseed on productive performances, milk and cheese quality, and health indices were analyzed using the general linear model procedure (Minitab version 17, SAS Institute Inc., Cary, NC, USA), according to the following model:

$$Y_{ijk} = \mu + A_i + B_j + C_k + e_{ijk}$$

where Y represents variable studied during the trial; μ represents the overall mean; A represents the effect due to the treatment/diet (CON, LIN and HMP); B represents the effect due to the period (blocking factor); C represents the effect due to the group (blocking factor); and e represents the error.

Significance was declared at $p < 0.05$, while values between 0.05 and 0.1 were considered as tendencies.

3. Results

3.1. Proximate Composition of Milk and Cheese

No significant differences of the milk yield were detected among the three groups (1.18 L/head/day for CON, 1.14 L for LIN and 1.19 L for HMP, $p = 0.686$). Consequently, as the diets were fed in limited amounts (1.2 kg compound feeds/head, 1.5 kg hay/head), the feed consumption: milk yield ratio was not significantly changed by the inclusion of either linseeds or hempseeds: 2.06 kg DM/L of milk (CON), 2.26 kg DM/L of milk (LIN) and 2.08 kg DM/L of milk (HMP).

Also, in the case of protein and casein content, there were no significant differences among groups (Table 4).

Table 4. The milk yield and the proximate composition of the milk.

Specification	CON	LIN	HMP	SEM	*p*-Value		
					Treatment	Period	Group
Milk yield, kg/day	1.18	1.14	1.19	0.043	0.686	0.190	0.063
Milk fat, %	5.58 ^{b}	6.18 ^{a}	6.10 ^{a}	0.113	0.0001	0.805	0.0001
Milk protein, %	4.03	3.99	3.96	0.032	0.570	0.0001	0.749
Lactose, %	4.75 ^{b}	4.83 ^{a}	4.83 ^{a}	0.017	0.001	0.0001	0.012
Casein, g/L	32.99	32.72	31.89	0.443	0.185	0.0001	0.563
Urea N, mg/100 mL	48.49 ^{a}	40.14 ^{b}	41.98 ^{b}	1.323	0.0001	0.0001	0.050
Specific density, g/L	1026.87	1026.84	1026.83	0.103	0.951	0.005	0.0001
pH	6.57	6.57	6.58	0.017	0.850	0.0001	0.037
Total solids, %	15.27 ^{b}	15.78 ^{a}	15.69 ^{ab}	0.150	0.040	0.002	0.001
Somatic cells count × 1000 per mL	1943	2403	2354	235	0.312	0.119	0.001

CON, control diet; LIN, linseed diet; HMP, hempseed diet; SEM, standard error of the mean; ^{a}, ^{b}—means in rows marked with different uppercase superscripts significantly differ at $p < 0.001$; $n = 36$, number of milk samples analyzed.

On the other hand, milk fat content was significantly higher ($p = 0.0001$) in the case of the LIN (6.18%) and HMP (6.10%) diets compared to the CON diet (5.58%). Also, milk lactose content was significantly higher ($p = 0.001$) in the case of the LIN and HMP diets compared to the CON diet. A highly significant ($p = 0.0001$) decrease in the urea-N percentage was recorded with the LIN and HMP diets. This parameter is a predictor of nitrogen excretion and is linearly correlated with dietary crude protein content.

The other parameters for milk, like pH, density, and the number of somatic cells, were not influenced by the diets, with similar values being recorded among the three experimental groups. Only total solids for the LIN diet slightly differed from CON diet ($p = 0.04$).

The fat and protein contents of the goat cheese were also assessed, as shown in Table 5, and no significant differences were observed among the diets.

Table 5. The proximate composition of the cheese.

Specification	CON	LIN	HMP	SEM	*p*-Value
Goat cheese fat, %	21.67	21.26	22.03	1.00	0.864
Goat cheese protein, %	15.32	14.28	14.10	0.55	0.258

CON, control diet; LIN, linseed diet; HMP, hempseed diet; SEM, standard error of the mean; $n = 30$, number of cheese samples analyzed.

3.2. Profile of Fatty Acids in Milk

The particular oil composition of the local varieties of linseeds and hemp is reflected by the results of analyses of the milk fatty acids profile, presented in Table 6.

Table 6. The effect of linseeds and hempseeds in goats' diets on fatty acids milk composition (g FAME/100 g total FAME).

Fatty Acids in Milk		CON	LIN	HMP	SEM	*p*-Value		
						Treatment	Period	Group
Butyric	C 4:0	0.04 ^{b}	0.05 ^{b}	0.06 ^{a}	0.006	0.013	0.194	0.897
Caproic	C 6:0	1.32	1.31	1.42	0.038	0.057	0.124	0.597
Caprylic	C 8:0	3.11 ^{ab}	3.07 ^{b}	3.20 ^{a}	0.041	0.044	0.147	0.0001
Capric	C 10:0	12.09 ^{a}	10.67 ^{c}	11.12 ^{b}	0.143	0.0001	0.006	0.0001
Undecanoic	C 11:0	0.44 ^{a}	0.35 ^{b}	0.38 ^{b}	0.013	0.0001	0.987	0.185
Lauric	C 12:0	7.47 ^{a}	5.56 ^{b}	5.71 ^{b}	0.167	0.0001	0.0001	0.459
Tridecanoic	C 13:0	0.17	0.15	0.15	0.008	0.250	0.700	0.640
Myristic	C 14:0	11.98 ^{a}	9.46 ^{b}	9.64 ^{b}	0.175	0.0001	0.010	0.953
Miristoleic	C 14:1	0.78 ^{a}	0.57 ^{b}	0.59 ^{b}	0.022	0.0001	0.002	0.025
Pentadecanoic	C 15:0	0.31 ^{a}	0.28 ^{b}	0.26 ^{b}	0.008	0.001	0.0001	0.179
Pentadecenoic	C 15:1	1.05 ^{a}	0.96 ^{b}	0.96 ^{b}	0.021	0.002	0.0001	0.001
Palmitic	C 16:0	26.23 ^{a}	21.31 ^{c}	22.64 ^{b}	0.360	0.0001	0.004	0.802
Palmitoleic	C 16:1	1.98 ^{b}	1.78 ^{c}	2.23 ^{a}	0.042	0.0001	0.366	0.328
Heptadecanoic	C 17:0	0.36	0.35	0.34	0.008	0.372	0.001	0.776
Heptadecenoic	C 17:1	0.44 ^{a}	0.39 ^{b}	0.41 ^{ab}	0.010	0.003	0.0001	0.649
Stearic	C 18:0	6.93 ^{c}	10.23 ^{a}	8.16 ^{b}	0.235	0.0001	0.007	0.718
Oleic cis	C 18:1n9c	19.98 ^{b}	24.57 ^{a}	24.21 ^{a}	0.370	0.0001	0.004	0.769
Linoleic trans	C 18:2n6t	0.67 ^{c}	1.90 ^{a}	1.22 ^{b}	0.071	0.0001	0.0001	0.002
Linoleic cis	C 18:2n6c	2.07 ^{b}	2.66 ^{a}	2.72 ^{a}	0.072	0.0001	0.247	0.061
Arachidic	C 20:0	0.05 ^{b}	0.11 ^{a}	0.06 ^{b}	0.008	0.0001	0.019	0.399
Linolenic gamma	C 18:3n6	0.06 ^{c}	0.13 ^{a}	0.09 ^{b}	0.007	0.0001	0.0001	0.966
Eicosenoic	C 20:1n9	0.06 ^{a}	0.06 ^{a}	0.04 ^{b}	0.004	0.0001	0.984	0.0001
Linolenic alfa	C 18:3n3	0.36 ^{b}	1.50 ^{a}	0.47 ^{b}	0.052	0.0001	0.0001	0.001
Conjugated Linoleic–rumenic acid	CLA (c9, t11)	0.36 ^{c}	0.69 ^{b}	1.82 ^{a}	0.081	0.0001	0.070	0.205
Eicosadienoic	C 20:2n6	0.12 ^{b}	0.14 ^{b}	0.17 ^{a}	0.008	0.0001	0.042	0.829
Eicosatrienoic Ω6	C 20:3n6	0.11 ^{b}	0.11 ^{b}	0.15 ^{a}	0.010	0.0001	0.101	0.001
Eicosatrienoic Ω3	C 20:3n3	0.08 ^{b}	0.10 ^{b}	0.14 ^{a}	0.011	0.0001	0.008	0.007
Arachidonic	C 20:4n6	0.15 ^{a}	0.11 ^{b}	0.12 ^{b}	0.005	0.0001	0.010	0.001
Total SFA [1]		70.46 ^{a}	62.92 ^{b}	63.31 ^{b}	0.539	0.0001	0.003	0.534
Total MUFA [2]		24.24 ^{b}	28.47 ^{a}	28.55 ^{a}	0.425	0.0001	0.021	0.359
Total PUFA [3]		3.74 ^{b}	7.29 ^{a}	6.97 ^{a}	0.208	0.0001	0.002	0.016
n-3 PUFA [4]		0.41 ^{b}	1.55 ^{a}	0.57 ^{b}	0.053	0.0001	0.0001	0.001
n-6 PUFA [5]		3.07 ^{c}	5.02 ^{a}	4.42 ^{b}	0.128	0.0001	0.008	0.001
n-6/n-3 PUFA [6]		7.67 ^{a}	3.50 ^{b}	7.32 ^{a}	0.237	0.0001	0.001	0.015
PUFA/SFA		0.06 ^{b}	0.12 ^{a}	0.11 ^{a}	0.004	0.0001	0.003	0.013
DFA		35.10 ^{c}	46.12 ^{a}	43.83 ^{b}	0.367	0.0001	0.0001	0.363
HSFA		45.68 ^{a}	36.48 ^{b}	37.90 ^{b}	0.535	0.0001	0.0001	0.763
h/H ratio		0.49 ^{b}	0.76 ^{a}	0.73 ^{a}	0.020	0.0001	0.001	0.619
AI		2.94 ^{a}	1.86 ^{b}	1.91 ^{b}	0.062	0.0001	0.002	0.683
TI		1.99 ^{a}	1.68 ^{b}	1.60 ^{b}	0.036	0.0001	0.024	0.081

CON, control diet; LIN, linseed diet; HMP, hempseed diet; SEM, standard error of the mean; [1] saturated fatty acids, [2] monounsaturated fatty acids, [3] polyunsaturated fatty acids, [4] omega-3 polyunsaturated fatty acids; [5] omega-6 polyunsaturated fatty acids, [6] omega 6/omega 3 ratio; DFA = MUFA + PUFA + C 18:0; HSFA = C 12:0 + C 14:0 + C 16:0; h/H ratio = (C 18:1c9 + C 18:2n6 + C 20:4n6 + C 20:5n3 + C 22:5n3)/(C 12:0 + C 14:0 + C 16:0); AI = (C 12:0 + 4 × C 14:0 + C 16:0)/(MUFA + PUFA); TI = (C 14:0 + C 16:0 + C18:0)/(0.5 × MUFA + 0.5 × n6 + 3 × n3 + n3/n6); ^{a}, ^{b}, ^{c}—means in rows marked with different uppercase superscripts significantly differ at $p < 0.001$.

The LIN and HMP diets were associated with a significant decrease ($p < 0.0001$) in the total SFAs (62.92 and 63.31 g/100 g total FAME) vs. the CON diet (70.46 g/100 g total FAME). The butyric (C:4) and caprylic (C:8) acids were slightly increased ($p < 0.05$) for the HMP diet, but the caproic acid (C 6:0) was not influenced by the diet. A decrease was registered for capric acid (C 10:0), an important and specific acid for goat milk, with the

LIN (10.67 g/100 g total FAME) and HMP (11.12 g/100 g total FAME) diets vs. the CON diet (12.09 g/100 g total FAME). The lauric acid (C 12:0), the myristic acid (C 14:0), and the palmitic acid (C 16:0) also registered significant decreases. Only the stearic acid (C 18:0), the most abundant long-chain FA in milk, was increased with both the LIN (10.23 g/100 g total FAME) and HMP (8.16 g/100 g total FAME) diets vs. the CON diet (6.93 g/100 g total FAME).

The LIN and the HMP groups had significantly ($p < 0.001$) reduced proportions of the minor MUFAs like miristoleic (C 14:1) and pentadecenoic (C 15:1); the palmitoleic acid (C 16:1) registered a decrease only with the LIN diet. The total MUFA percentage was increased for both the LIN and HMP groups vs. the CON group. The highest increase in the total MUFA was due to the increase of the cis-oleic acid (C 18:1n9c), a major MUFA acid, by values of 24.57 g/100 g total FAME with the LIN diet and 24.21 g/100 g total FAME with the HMP diet vs. 19.98 g/100 g total FAME with the CON diet.

The total PUFA concentration showed a highly significant increase ($p < 0.0001$) for both the LIN and HMP diets compared to the CON diet.

The major n-6 PUFA, cis-linoleic acid (C 18:2n6c), similarly increased for both experimental groups (2.66 for the LIN diet and 2.72 for the HMP diet) compared to the CON diet (2.07 g/100 g total FAME). The sum of the n6-PUFA acids with the LIN diet was 5.02 g/100 g total FAME, a higher value than with the HMP diet (4.42 g/100 g total FAME), but both were significantly higher compared to the CON diet (3.07 g/100 g total FAME). The major n-3 acid, the alpha-linolenic acid ALA (C 18:3n3), was highly significantly ($p < 0.0001$) increased only within the LIN diet (1.50 g/100 g total FAME) compared to the CON diet (0.36 g/100 g total FAME) and HMP diet (0.47 g/100 g total FAME).

The percentage of conjugated linoleic acid (CLA), namely the dominant isomer—rumenic acid, c9, t11-CLA (or C 18:2n cis911t)—registered a significant increase ($p < 0.0001$) with the HMP diet (1.82 g/100 g total FAME) compared to the CON diet (0.36 g/100 g total FAME) and LIN diet (0.69 g/100 g total FAME); also, significant differences were noticed between the LIN and CON diets. This confirms that the presence of dietary oil sources rich in C18:2 and C18:3 acids improves the milk quality.

Health-related indices such as n6/n3, PUFA/SFA, DFA, HSFA, h/H, AI, and TI were also influenced (Table 6). The n6/n3 ratio decreased significantly only for the LIN diet. The DFA index increased significantly for both the LIN (46.12) and HEM (43.83) diets vs. the CON (35.10) diet. Also, the h/H ratio increased for both the LIN and HMP diets, while the HSFA index had important decreases for both the LIN and HMP diets. The linseeds and hempseeds determined the significant decrease in both the AI and TI indices.

3.3. Profile of Fatty Acids in Cheese

The results for cheese (Table 7) were expressed as g FAME/100 g total FAME but also as mg fatty acid/100 g cheese, a parameter which is more relevant to the farmers and consumers.

The linolenic-alfa acid concentration increased in the LIN diet ($p < 0.0001$) compared to the CON and HMP diets, while for the CLA concentration, the HMP diet registered the highest value compared to the CON and LIN diets.

The FAME profile for the principal classes was relatively similar between cheese and milk. Consequently, the SFA decreased for both the LIN (63.60 g/100 g total FAME) and HMP (62.62 g/100 g total FAME) diets compared to the CON diet (67.80 g/100 g total FAME), the MUFA increased for both groups (27.48, and 28.80 g/100 g total FAME, respectively), and the PUFA increased (7.30, and 7.11 g/100 g total FAME, respectively).

The content of n3-PUFAs compared to the CON diet was highest in the case of the LIN diet, similarly as for milk content. The n6-PUFAs were increased by 1.29 times for the LIN diet and by 1.16 times for the HMP diet. Consequently, the n6/n3 ratio values, similar to those found in milk, exhibited a significant decrease within the LIN diet (3.62) compared with the CON diet (6.88) and the HMP diet (8.00).

Table 7. The FA composition in the goat cheese as g FAME/100 g total FAME (%) and as mg fatty acid/100 g cheese.

Fatty Acids in Cheese		(g FAME/100 g Total FAME)					mg Fatty Acid/100 g Cheese				
		CON	LIN	HMP	SEM	*p*-Value	CON	LIN	HMP	SEM	*p*-Value
Butyric	C 4:0	0.04	0.05	0.04	0.007	0.642	3.69	3.60	4.01	0.70	0.900
Caproic	C 6:0	1.36	1.35	1.30	0.074	0.841	120.44	125.27	119.99	9.96	0.912
Caprylic	C 8:0	3.08	3.07	3.05	0.092	0.967	264.65	266.92	260.42	15.0	0.953
Capric	C 10:0	11.50 ^{a}	10.56 ^{b}	10.78 ^{ab}	0.238	0.027	1099.81 ^{a}	963.63 ^{b}	1076.71 ^{ab}	77.2	0.049
Undecanoic	C 11:0	0.40	0.37	0.36	0.017	0.276	38.68	32.37	33.32	2.21	0.122
Lauric	C 12:0	6.31	5.48	5.32	0.293	0.055	555.08	474.48	518.30	39.2	0.341
Tridecanoic	C 13:0	0.18	0.15	0.15	0.015	0.388	15.31	13.02	13.47	1.20	0.363
Myristic	C 14:0	10.88 ^{a}	9.63 ^{b}	9.43 ^{b}	0.352	0.015	966.24 ^{a}	852.98 ^{b}	912.83 ^{b}	54.3	0.033
Myristoleic	C 14:1	0.64	0.56	0.58	0.037	0.300	61.17	48.73	52.15	3.52	0.061
Pentadecanoic	C 15:0	0.27	0.26	0.25	0.010	0.354	25.43	23.30	22.25	1.31	0.253
Pentadecenoic	C 15:1	0.93	0.93	0.93	0.037	0.998	85.57	80.51	79.38	4.81	0.654
Palmitic	C 16:0	25.41	22.55	23.03	0.825	0.047	2385.47	2010.23	2045.85	140	0.137
Palmitoleic	C 16:1	1.87 ^{ab}	1.80 ^{b}	2.13 ^{a}	0.090	0.036	176.90 ^{ab}	165.81 ^{b}	209.94 ^{a}	13.7	0.048
Heptadecanoic	C 17:0	0.34	0.33	0.31	0.012	0.272	32.40	30.39	28.80	1.69	0.354
Heptadecenoic	C 17:1	0.38	0.37	0.37	0.017	0.945	34.58 ^{a}	33.96 ^{b}	33.35 ^{b}	1.39	0.008
Stearic	C 18:0	7.97 ^{b}	9.66 ^{a}	8.53 ^{ab}	0.432	0.030	696.28 ^{b}	890.32 ^{a}	820.62 ^{ab}	53.5	0.046
Oleic cis	C 18:1n9c	21.69 ^{b}	23.78 ^{ab}	24.74 ^{a}	0.838	0.046	1939.63 ^{b}	2240.84 ^{a}	2232.33 ^{a}	117	0.050
Linoleic trans	C 18:2n6t	1.03 ^{b}	1.84 ^{a}	1.24 ^{ab}	0.199	0.027	82.35 ^{b}	212.14 ^{a}	131.10 ^{b}	16.6	0.0001
Linoleic cis	C 18:2n6c	2.46	2.79	2.85	0.120	0.063	216.26 ^{b}	257.29 ^{a}	244.01 ^{a}	15.2	0.047
Arachidic	C 20:0	0.06 ^{b}	0.14 ^{a}	0.07 ^{b}	0.016	0.001	6.57 ^{b}	14.91 ^{a}	7.61 ^{b}	1.54	0.0001
Linolenic gamma	C 18:3n6	0.12	0.15	0.10	0.020	0.213	11.59	14.05	10.95	1.93	0.514
Eicosenoic	C 20:1n9	0.05	0.04	0.05	0.009	0.815	5.31 ^{b}	4.03 ^{a}	3.73 ^{a}	0.504	0.048
Linolenic alfa	C 18:3n3	0.48 ^{b}	1.30 ^{a}	0.47 ^{b}	0.123	0.0001	43.35 ^{b}	121.98 ^{a}	63.04 ^{b}	11.2	0.0001
Conjugated Linoleic–rumenic acid	CLA (c9, t11)	0.78 ^{b}	0.74 ^{b}	1.89 ^{a}	0.177	0.001	80.50 ^{b}	68.16 ^{b}	157.21 ^{a}	23.7	0.037
Eicosadienoic	C 20:2n6	0.13	0.15	0.19	0.019	0.104	13.03 ^{b}	13.72 ^{b}	17.04 ^{a}	1.97	0.032
Eicosatrienoic-n6	C 20:3n6	0.12	0.10	0.13	0.0233	0.536	11.80 ^{b}	9.12 ^{b}	13.46 ^{a}	2.45	0.050
Eicosatrienoic-n3	C 20:3n3	0.10	0.12	0.11	0.025	0.819	8.72 ^{b}	10.48 ^{ab}	11.15 ^{a}	2.23	0.047
Arachidonic	C 20:4n6	0.13	0.11	0.13	0.009	0.065	11.82 ^{b}	9.70 ^{a}	12.07 ^{b}	0.943	0.003
Total SFA		67.80 ^{a}	63.60 ^{b}	62.62 ^{b}	1.230	0.087	6210.05 ^{a}	5701.42 ^{b}	5864.18 ^{b}	283	0.016
Total MUFA		25.56 ^{b}	27.48 ^{a}	28.80 ^{a}	0.771	0.021	2303.16 ^{b}	2573.88 ^{a}	2610.88 ^{a}	200	0.008
Total PUFA		5.35 ^{b}	7.30 ^{a}	7.11 ^{a}	0.563	0.024	479.42 ^{b}	716.64 ^{a}	660.03 ^{a}	85.3	0.009
n-3 PUFA		0.58 ^{b}	1.42 ^{a}	0.58 ^{b}	0.158	0.001	52.07 ^{b}	132.46 ^{a}	74.19 ^{b}	12.5	0.0001
n-6 PUFA		3.99 ^{b}	5.14 ^{a}	4.64 ^{ab}	0.337	0.039	346.85 ^{b}	516.02 ^{a}	428.63 ^{b}	53.8	0.031
n-6/n-3 PUFA		6.88 ^{a}	3.62 ^{b}	8.00 ^{a}	0.626	0.001	6.66 ^{a}	3.90 ^{b}	5.78 ^{a}	0.572	0.000
PUFA/SFA		0.08 ^{b}	0.11 ^{a}	0.11 ^{a}	0.010	0.022	0.08 ^{b}	0.13 ^{a}	0.11 ^{a}	0.009	0.0001
DFA		38.88	44.44	44.44	1.650	0.031	3478.86	4180.84	4091.53	251	0.005
HSFA		42.60 ^{a}	37.66 ^{b}	37.78 ^{b}	1.390	0.028	3906.79 ^{a}	3337.69 ^{b}	3476.98 ^{b}	216	0.040
h/H ratio		0.59 ^{b}	0.76 ^{a}	0.77 ^{a}	0.046	0.040	0.58 ^{b}	0.81 ^{a}	0.75 ^{a}	0.059	0.009
AI		2.43 ^{a}	1.91 ^{ab}	1.84 ^{b}	0.161	0.023	2.45 ^{a}	1.79 ^{b}	1.90 ^{b}	0.188	0.033
TI		2.66 ^{a}	2.01 ^{b}	2.21 ^{b}	0.073	0.024	2.73 ^{a}	1.93 ^{b}	2.17 ^{b}	0.167	0.050

CON, control diet; LIN, linseed diet; HMP, hempseed diet; SEM, standard error of the mean; [1] saturated fatty acids, [2] monounsaturated fatty acids, [3] polyunsaturated fatty acids, [4] omega-3 polyunsaturated fatty acids; [5] omega-6 polyunsaturated fatty acids, [6] omega 6/omega 3 ratio; DFA = MUFA + PUFA + C 18:0; HSFA = C 12:0 + C 14:0 + C 16:0; h/H ratio = (C 18:1c9 + C 18:2n6 + C 20:4n6 + C 20:5n3 + C 22:5n3)/(C 12:0 + C 14:0 + C 16:0); AI = (C 12:0 + 4 × C 14:0 + C 16:0)/(MUFA + PUFA); TI = (C 14:0 + C 16:0 + C 18:0)/(0.5 × MUFA + 0.5 × n6 + 3 × n3 + n3/n6); ^{a}, ^{b}—means in rows marked with different uppercase superscripts significantly differ at $p < 0.001$.

The PUFA/SFA ratio was significantly improved in both the LIN and HMP groups (0.11 and 0.11, vs. 0.08 for CON diet, respectively). The content of DFA increased but not significantly in cheese, in a similar way with the milk fat. The HSFA index registered a reduction of almost 12% with the LIN diet and nearly 11% with the HMP diet. The h/H ratio was increased between 1.28–1.30 times for each diet. The value of the AI and TI indices decreased with both LIN and HMP diets.

4. Discussions

Although the energy supply of LIN and HMP diets was higher than the CON diet (due to the replacement of a fat-extracted meal with oilseeds), the milk yield was not statistically different among groups. This might be related to the high proportion of dietary fat in LIN and HMP groups. This lack of effect is in line with the results of two previous studies [9,15], where the inclusion of 9.3% DM of linseeds or hempseeds in the diet for Alpine lactating goats also resulted in similar milk yields among groups.

The increased milk fat content is consistent with the fact that replacement of sunflower meal with linseeds and hempseeds represents a supplementation of total dietary lipids,

equivalent with 4.6% of DM intake. The effect of increasing milk fat after adding linseed to the diet was also reported in ewes by [7], in Cilentana grazing goats by [26], and by [9] in Alpine goats for both oilseeds. A similar effect of the hempseeds on the milk fat content was observed by [16], also on ewes, along with an increase of the milk yield. Even in iso-energetic diets, Sampelayo et al. [27] found that the replacement of starch with fat, as energy sources, has increased milk fat content and stimulated the presence of oleic, vaccenic, and rumenic acids, as well as linolenic acid and other trans fatty acids in goat milk.

Whereas the protein and casein contents were not modified by the LIN and HMP diets, the lactose content was higher in these diets. This increase was associated with the higher energy levels in these diets (0.865 UFL and 0.863 UFL compared to 0.787 UFL). The increased lactose levels could also be attributed to a higher glucose availability for lactose synthesis in the mammary gland as a result of feeding with these lipid-enriched diets. This effect was presented by other researchers [24] in goats fed starch-enriched or lipid-supplemented diets. Tudisco [8] also reported an increase in lactose content for goat fed linseed (4.61%) compared with pasture-fed goat (4.57%).

The milk urea-N registered a decrease for both LIN and HMP diets; this decrease is also in line with other authors [28] who showed that milk urea nitrogen is also negatively correlated with the increasing energy content of the diet, like in our study for LIN and HMP groups.

The levels of short SFAs in milk observed in our study were slightly decreasing. They enhance the milk's taste and flavor and, according to Chilliard [4], the SFAs serve as an energy source for the proper functioning of internal organs, the nervous system, and muscles in the human body.

The decrease of the levels of long SFAs was a natural consequence of dietary long-chain FA, as presented by [4].

Other researchers [13] reported similar results, such as a decrease in SFAs de novo synthesized (C 10:0-C 16:0) and an increase in C 4:0 and C 18:0 acids, as well as in PUFA amounts in goat milk, after the dietary inclusion of hempseed oil. Similar results were obtained by [10] concerning elevated stearic acid (C 18:0) levels in Alpine lactating goats fed with a diet comprising 9.4% hempseed in DM, wherein they also noted a reduction in the C 8:0-C 16:0 acids.

The magnitude of the increase of the concentration of total milk MUFA percentage was comparable in both the LIN and HMP groups. This increase also contributed to the significant increase in the DFA index (46.12 for the LIN diet and 43.83 for the HMP diet vs. 35.10 for the CON diet).

The increase in cis-linoleic acid in milk was due to its prevalence in hempseeds (53.41%) and its significant presence in linseeds (16.42%). This effect was presented by other authors [29] who observed that the sum of oleic, linoleic, and α-linolenic acids in the milk fat exhibited a linear increase corresponding to the dietary daily intake of unsaturated lipids. The increased presence of alpha-linolenic acid exclusively in the milk from the LIN diet emphasizes the effect of the linseeds on the milk composition by the presence of this major FA in the linseeds in a very high amount, 51.33%. A similar effect was presented by [9]. The difference in the major PUFA composition in these two oil seeds is summarized in the n6/n3 ratio, for milk which significantly decreased only with the LIN diet but remained unchanged within the HMP diet due to the absence of an increase in milk n3-PUFAs.

Our obtained values for the LIN and HMP diet on CLA isomers are in line with other studies on various diet types and oilseeds. Correddu et al. [30] found increased levels of vaccenic acid in milk of dairy sheep fed linseed. High levels of 0.7% for both CLA isomers (rumenic acid and *t*10 *c*12 CLA) and for oleic acid were reported by [7] for ewes fed extruded linseeds. Increased levels of CLA isomers were also found for diets with hempseed as presented by other authors [14] on Carpathian goats and by [10] on Alpine lactating goats supplemented with 9.4% hempseed in different types of diets (based on pasture hay/shrubs–grass rangeland/less fermentable or less degradable ingredients). According to Cozma et al. [13], a very high increase of CLA-rumenic acid concentration

in milk fat was observed when goats' diets were supplemented with hempseed oil. Our CLA values for HMP diet were similar to those from an extensive, grazing-based, milk production system presented by the author Slots [31], who found that cows fed natural pasture yielded a CLA content of 1.75%.

Both vaccenic and rumenic acids are known for anti-atherosclerotic effects by lowering blood levels of triglycerides and LDL cholesterol fractions, anti-cancer effects, and beneficial effects on the immune system [32,33].

The main fatty acid classes showed comparable values in both cheese and milk; however, only the Cis-linoleic acid registered a high increase in milk, whereas in cheese, the increase was not statistically significant. Also, the CLA-rumenic acid corresponding to the LIN diet was not increased in cheese compared to the CON diet (although significantly different values were observed for milk). However, the direction of changes between LIN or HMP comparing to the CON diet were similar to those registered in milk; therefore, the minor inconsistencies between milk and cheese were caused by the cumulated individual errors (sampling, analyses, etc.).

The health indices were similar for cheese and milk (as FAME), and after expressing FA in an absolute amount (mg FA/100 g cheese), the modifications of the indices were even better highlighted. These indices are consistent with the findings of [30], who recorded decreased values of AI and TI and an increased h/H ratio when dairy ewes were fed linseed. Similar improved health-promoting indices were registered by other authors [34] for semi-hard cheese made from milk of French Alpine goats fed a basal diet with 90 g/kg DM extruded linseed. The results for the n6/n3 ratio, for milk and for cheese, were around 5.0, which is recommended to be below 5.0 in the human diet in order to mitigate the risk of developing cardiovascular diseases and cancer [35].

5. Conclusions

The replacement of sunflower meal with equal quantities of linseeds or hempseeds in the diets of Murciano-Granadina goats, at a level of 11.5% proportion of the total dry matter intake, had no influence on raw milk yield or milk protein content but led to a significant increase in the milk fat content.

In the case of raw milk composition, the inclusion of either LIN of HMP led to the decreases in the SFA proportion and the increases in the MUFA proportion (mainly the oleic acid). Inclusion of LIN induced a significant decrease in the n3:n6-PUFA ratio.

Also, in the LIN diet milk, the ALA (C 18:3n-3) increased by 4.1 times, while the LA (C 18:2n6c) also increased by 1.3 times. In the HMP diet milk, there was no increase in ALA, and the increase of LA was similar with the LIN diet.

The increased contents of healthy FA, such as n3-PUFA, and CLA-rumenic led to higher health indices of the milk corresponding to the goats that were fed a linseed or hempseed diet.

The improved FA profile of the milk collected from the goats fed either LIN or HMP diets was found also in the cheese manufactured from this milk, which is a good base for obtaining premium-labelled dairy products through application of feeding strategies that covers also the non-grazing periods or production systems.

Author Contributions: Conceptualization, A.E.C., C.D. and S.M.T.; methodology, A.E.C., C.D. and S.M.T.; software, C.P.D.; formal analysis, A.E.C., M.R. and C.P.D.; writing—original draft preparation, A.E.C.; writing—review, C.D., S.M.T., G.M.C. and C.P.D.; project administration, C.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Ministry of Research, Innovation and Digitalization, Project PN 23-20 0101, and by The Romanian Agency UEFISCDI, Project 26PTE/2017.

Institutional Review Board Statement: All experimental procedures of the study were performed in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes and experimental procedures, according to an experimental protocol (No. 4244/12.08.2017) approved

by the Research Ethics Commission of the National Research and Development Institute for Animal Biology and Nutrition.

Data Availability Statement: All data are contained within article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Jenkins, T.C.; McGuire, M.A. Major advances in nutrition: Impact on milk composition. *J. Dairy Sci.* **2006**, *89*, 1302–1310. [CrossRef] [PubMed]
2. Nicolae, M.; Dragomir, C.; Pop, S. *Căile Modificării Conținutului Laptelui în Lipide la Rumegătoare*; Ars Academica Publisher House: București, Romania, 2008.
3. Oliveira, X.S.; Palma, A.S.V.; Reis, B.R.; Franco, C.S.R.; Marconi, A.P.; Shiozaki, F.A.; Netto, A.S. Inclusion of soybean and linseed oils in the diet of lactating dairy cows makes the milk fatty acid profile nutritionally healthier for the human diet. *PLoS ONE* **2021**, *16*, e0246357. [CrossRef]
4. Chilliard, Y.; Glasser, F.; Ferlay, A.; Bernard, L.; Rouel, J.; Doreau, M. Diet rumen biohydrogenation and nutritional quality of cow and goat milk fat. *Eur. J. Lipid. Sci. Tech.* **2007**, *109*, 828–855. [CrossRef]
5. Nudda, A.; Battacone, G.; Boaventura Neto, O.; Cannas, A.; Helena, A.; Francesconi, D.; Atzori, A.S.; Pulina, G. Feeding strategies to design the fatty acid profile of sheep milk and cheese. *R. Bras. Zootec.* **2014**, *43*, 445–456. [CrossRef]
6. Zhang, R.H.; Mustafa, A.F.; Zhao, X. Effects of feeding oilseeds rich in linoleic and linolenic fatty acids to lactating ewes on cheese yield and on fatty acid composition of milk and cheese. *Anim. Feed Sci. Technol.* **2006**, *127*, 220–233. [CrossRef]
7. Gomez-Cortes, P.; Bach, A.; Luna, P.; Juarez, M.; De La Fuente, M.A. Effects of extruded linseed supplementation on n-3 fatty acids and conjugated linoleic acid in milk and cheese from ewes. *J. Dairy Sci.* **2009**, *92*, 4122–4134. [CrossRef]
8. Tudisco, R.; Grossi, M.; Addi, L.; Musco, N.; Cutrignelli, M.I.; Calabrò, S.; Infascelli, F. Fatty Acid Profile and CLA Content of Goat Milk: Influence of Feeding System. *J. Food Res.* **2004**, *3*, 93–100. [CrossRef]
9. Rapetti, L.; Colombini, S.; Battelli, G.; Castiglioni, B.; Turri, F.; Galassi, G.; Battelli, M.; Crovetto, G.M. Effect of Linseeds and Hemp Seeds on Milk Production, Energy and Nitrogen Balance, and Methane Emissions in the Dairy Goat. *Animals* **2021**, *11*, 2717. [CrossRef]
10. Cremonesi, P.; Capra, E.; Turri, F.; Lazzari, B.; Chessa, S.; Battelli, G.; Colombini, S.; Rapetti, L.; Castiglioni, B. Effect of Diet Enriched with Hemp Seeds on Goat Milk Fatty Acids, Transcriptome, and mRNAs. *Front. Anim. Sci.* **2022**, *3*, 909271. [CrossRef]
11. Bailoni, L.; Bacchin, E.; Trocino, A.; Arango, S. Hemp (*Cannabis sativa* L.) Seed and Co-Products Inclusion in Diets for Dairy Ruminants: A Review. *Animals* **2021**, *11*, 856. [CrossRef]
12. Mierliță, D. Fatty acid profile and health lipid indices in the raw milk of ewes grazing part-time and hemp seed supplementation of lactating ewes. *S. Afr. J. Anim. Sci.* **2016**, *46*, 237–246. [CrossRef]
13. Cozma, A.; Andrei, S.; Pintea, A.; Miere, D.; Filip, L.; Loghin, F.; Ferlay, A. Effect of hemp seed oil supplementation on plasma lipid profile, liver function, milk fatty acid, cholesterol, and vitamin A concentrations in Carpathian goats. *Czech J. Anim. Sci.* **2015**, *60*, 289–301. [CrossRef]
14. Mierliță, D.; Mierliță, S.; Struti, D.I.; Mintas, O.S. Effects of Hemp Seed on the Production, Fatty Acid Profile, and Antioxidant Capacity of Milk from Goats Fed Hay or a Mixed Shrubs–Grass Rangeland. *Animals* **2023**, *13*, 3435. [CrossRef] [PubMed]
15. Cremonesi, P.; Conte, G.; Severgnini, M.; Turri, F.; Monni, A.; Capra, E.; Rapetti, L.; Colombini, S.; Chessa, S.; Battelli, G.; et al. Evaluation of the effects of different diets on microbiome diversity and fatty acid composition of rumen liquor in dairy goat. *Animal* **2018**, *12*, 1856–1866. [CrossRef]
16. Mierliță, D. Effects of diets containing hemp seeds or hemp cake on fatty acid composition and oxidative stability of sheep milk. *S. Afr. J. Anim. Sci.* **2018**, *48*, 504–514. [CrossRef]
17. INRA. *Alimentation des Bovins, Ovins et Caprins. Besoins des Animaux—Valeurs des Aliments—Tables INRA 2010*, Edition remaniée; Jarrige, R., Ed.; Institut National de Recherche Agronomique: Paris, France, 2010.
18. *EC 152/2009*; Commission Regulation (EC) No. 152/2009 Laying Down the Methods of Sampling and Analysis for the Official Control of Feed. European Union: Bruxelles, Belgium, 2009.
19. *ISO 9622:2013*; Milk and Liquid Milk Products — Guidelines for the Application of Mid-Infrared Spectrometry. ISO (International Organization for Standardization): Geneva, Switzerland, 2013.
20. *SR EN ISO 13366-2:2007*; Milk. Enumeration of Somatic Cells. Part 2: Guidelines for the Operation of Fluoro-Opto-Electronic Counters (Lapte. Enumerarea Celulelor Somatice. Partea 2: Linii Directoare Privind Modul de Operare a Numărătoarelor Fluoro-Opto-Electronice). ASRO (Romanian Standardization Association): Bucharest, Romania, 2007.
21. *EN ISO 661:2005/AC:2006*; Corrigendum—Animal and Vegetable Fats and Oils—Preparation of Test Sample (ISO 661:2003). ISO (International Organization for Standardization): Geneva, Switzerland, 2006.
22. *SR CEN ISO/TS 17764-2:2008*; Animal Feeding Stuffs—Determination of the Content of Fatty Acids—Part 2: Gas Chromatographic Method. (Nutreţuri. Determinarea ConţInutului de Acizi Graşi. Partea 2: Metoda Prin Cromatografie în Fază Gazoasă). ASRO (Romanian Standardization Association): Bucharest, Romania, 2008.
23. Weirauch, J.L.; Posati, L.; Anderson, B.A.; Exler, J. Lipid Conversion Factors for Calculating Fatty Acid Contents of Foods. *J. Am. Oil Chem. Soc.* **1977**, *54*, 36–40. [CrossRef]

24. Chilliard, Y.; Ferlay, A.; Rouel, J.; Lamberet, G. A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. *J. Dairy Sci.* **2003**, *86*, 1751–1770. [CrossRef]
25. Ulbricht, T.L.; Southgate, D.A. Coronary heart disease: Seven dietary factors. *Lancet* **1991**, *338*, 985–992. [CrossRef]
26. Musco, N.; Tudisco, R.; Esposito, G.; Iommelli, P.; Totakul, P.; D'Aniello, B.; Lombardi, P.; Amato, R.; Wanapat, M.; Infascelli, F. Effects of Linseed Supplementation on Milk Production, Composition, Odd- and Branched-Chain Fatty Acids, and on Serum Biochemistry in Cilentana Grazing Goats. *Animals* **2022**, *12*, 783. [CrossRef]
27. Sanz Sampelayo, M.R.; Chilliard, Y.; Schmidely, P.; Boza, J. Influence of type of diet on the fat constituents of goat and sheep milk. *Small Rumin. Res.* **2007**, *68*, 42–63. [CrossRef]
28. Giovanetti, V.; Boe, F.; Decandia, M.; Bomboi, G.C.; Atzori, A.S.; Cannas, A.; Molle, G. Milk Urea Concentration in Dairy Sheep: Accounting for Dietary Energy Concentration. *Animals* **2019**, *9*, 1118–1135. [CrossRef] [PubMed]
29. Martinez, M.A.L.; Núñez Sánchez, N.; Garzón Sigler, A.I.; Peña Blanco, F.; de la Fuente, M.A. Short communication. Relationships between the daily intake of unsaturated plant lipids and the contents of major milk fatty acids in dairy goats. *Span. J. Agri. Res.* **2015**, *13*, e06SC03. [CrossRef]
30. Correddu, F.; Gaspa, G.; Pulina, G.; Nudda, A. Grape seed and linseed, alone and in combination, enhance unsaturated fatty acids in the milk of Sarda dairy sheep. *J. Dairy Sci.* **2016**, *99*, 1725–1735. [CrossRef]
31. Slots, T.; Butler, G.; Leifert, C.; Kristensen, T.; Skibsted, L.H.; Nielsen, J.H. Potentials to differentiate milk composition by different feeding strategies. *J. Dairy Sci.* **2009**, *92*, 2057–2066. [CrossRef] [PubMed]
32. Addis, M.; Cabiddu, A.; Decandia, M.; Spada, S.; Acciaro, M.; Pirisi, A.; Sitzial, M.; Costa, E.; Cannas, A.; Molle, G. Effects of different fat-enriched concentrates on fatty acid profile of cheese from grazing dairy sheep. *Ital. J. Anim. Sci.* **2009**, *8*, 378–380. [CrossRef]
33. Mills, S.; Ross, R.P.; Hill, C.; Fitzgerald, G.F.; Stanton, C. Milk intelligence: Mining milk for bioactive substances associated with human health. *Int. Dairy J.* **2011**, *21*, 377–401. [CrossRef]
34. Klir Šalavardic, Ž.; Novoselec, J.; Ronta, M.; Colovic, D.; Šperanda, M.; Antunovic, Z. Fatty Acids of Semi-Hard Cheese Made from Milk of Goats Fed Diets Enriched with Extruded Linseed or Pumpkin Seed Cake. *Foods* **2022**, *11*, 6. [CrossRef]
35. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* **2002**, *56*, 365–379. [CrossRef]

agriculture

MDPI

Article

Blood Parameter Response in Growing Alpine Goat Kids Fed Diets Containing Extruded Flaxseed or Pumpkin Seed Cake

Željka Klir Šalavardić *, Josip Novoselec, Mislav Đidara and Zvonko Antunović

Department for Animal Production and Biotechnology, Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, V. Preloga 1, 31000 Osijek, Croatia; jnovoselec@fazos.hr (J.N.); mdidara@fazos.hr (M.Đ.); zantunovic@fazos.hr (Z.A.)

* Correspondence: zklir@fazos.hr

Abstract: Blood parameters can provide information on the nutritional status of goat kids, which is related to both health and performance. The present study aimed to research whether feeding extruded flaxseed (FS) and pumpkin seed cake (PC), as an alternative protein source in diets, has an effect on the hematological and serum biochemical parameters of goat kids during growth. In the small-scale goat farm, 31 French Alpine goat kids aged 32 days were used for the study. The goat kids were subjected to three different feeding treatments: a mixture containing soybean meal and extruded soybeans (CON), a mixture containing 16% PC (PC-16), and a mixture containing 9% FS (FS-9). They were monitored during the suckling, weaning, and post-weaning growth periods. PC-16 and FS-9 in goat kids' diets did not result in any changes regarding average daily weight gain. The WBC count was higher in goat kids fed FS-9 and PC-16 compared to CON (9.84 and 9.54 vs. 6.61 $\times$ 10^9 L) diets during the weaning period. GGT activity was lowest in the serum of goat kids fed PC-16 compared to CON post-weaning (38.65 vs. 48.40 U/L). In addition, FS-19 increased GPx compared to kids fed PC-16 post-weaning (809.7 vs. 600.8 U/L). Regarding blood parameters, PC-16 and FS-9 can be used in goat kids' nutrition as alternative sources of proteins on a small-scale goat farm without compromising goat kids' growth.

Keywords: goat kids; whole blood; serum; pumpkin seed cake; extruded flaxseed; alternative protein sources

Citation: Klir Šalavardić, Ž.; Novoselec, J.; Đidara, M.; Antunović, Z. Blood Parameter Response in Growing Alpine Goat Kids Fed Diets Containing Extruded Flaxseed or Pumpkin Seed Cake. *Agriculture* **2024**, *14*, 1667. https://doi.org/10.3390/agriculture14101667

Academic Editors: Tatiana Dumitra Panaite and Mihaela Hăbeanu

Received: 19 August 2024
Revised: 21 September 2024
Accepted: 22 September 2024
Published: 24 September 2024

1. Introduction

The performance of livestock is influenced by a variety of factors, including the type of production systems used, breed, age, sex, nutritional status, hormonal status, and environment [1]. The health and performance of animals are related to their nutritional status, which is reflected in metabolic products in the blood [2,3]. At the beginning of their lives, goat kids are monogastric animals or non-functional ruminants that become functional ruminants at around two months of age. Goat blood parameters can be influenced by the weaning processes and dynamics as well as the development of the liver, rumen, and immune system during the transition from pre-ruminant to ruminant [4]. It can also be a tool to check the quality of the feed, e.g., nutrient availability, mineral status, digestibility, and absorption by the animals [5]. Abdelsattar et al. [3] reported that the age of goats has a great influence on their blood profile, especially around the weaning. Thus, goat kids are very sensitive, especially when there are changes in diet, such as switching to a diet containing voluminous feeds and cereals with a gradual reduction in milk protein [6]. Goat kids are mostly known and valued for their meat, which is highly appreciated in Mediterranean Europe [7,8]. The meat of goat kids has a high nutritional value since it is rich in proteins and low in fat, with a significant proportion of beneficial fatty acids [9,10]. These attributes are what provide goat meat its potential for use in human diets and overall

health. Furthermore, goat farming requires less input, due to natural adaptation to free-range farming, and yields lean red meat that is a healthier alternative [11]. These factors make goat kids' meat a potentially sustainable supply of red meat.

Most of the livestock production systems worldwide are based on unsustainable feeding sources to maintain the need for proteins in animal nutrition, the most common of which is soybean meal [12]. Soybeans are an excellent source of protein and are often used in commercial feed rations to increase the crude protein content of the diet. As soybeans are in high demand, their increased cultivation and production as a crop are often associated with negative environmental impacts and increased use of natural resources, e.g., deforestation and soil depletion [13], a loss of biodiversity and natural habitats, long transport distances, high tillage, and fertilizer requirements [14]. Furthermore, using soybeans is costly in today's global economy. Due to current feed imports, food production is no longer sustainable in many countries as the cost of food production rises [15]. In order to maintain a lower-cost protein supply, it is necessary to maintain the use of various alternative protein sources for animal nutrition. As reported by Klir et al. [16], soybeans, both extruded and as meal, can be completely replaced by pumpkin seed cake (PC, *Cucurbita pepo* L.) in the diet of lactating dairy goats due to the very high content of crude proteins and crude fats. Boldea et al. [17] included PC in the diet for lactating dairy goats and improved the fatty acids in their milk. Antunović et al. [18] found that PC is a high-quality feed that can partially replace soybean meal and is practicable in terms of good energy and protein balance in lamb's serum, while lowering the serum NEFAs and BHB of lambs fed with 10% and 15% of PC in organic farming. In addition, Li et al. [19] completely replaced soybean meal with PC and dried distillers' grains with a soluble mixture in dairy cows and concluded that this diet promoted antioxidant functions in dairy cows. Pumpkin is considered an agro-industrial by-product and sometimes industrial waste which has a very high potential as a nutraceutical [20] and has medicinal and pharmacological properties [21]. In addition, pumpkin is cultivated according to organic principles [22], so that it can be used as high-quality feed in organic animal farming. Moreover, PC, as feed for ruminants, is one of the local and low-cost feeds that meets the requirements of both farmers and customers [17].

On the other hand, extruded flaxseed (FS, *Linum usitatissimum* L.) has been used for over twenty years to enrich animal products with n-3 polyunsaturated fatty acids (PUFAs) in ruminant diets. Colonna et al. [23] reported that the saturated fatty acids in meat decreased, while monounsaturated fatty acids, PUFAs, and conjugated linoleic acid increased when goat kids were fed diets containing 3% flaxseed. Hao et al. [24] indicated that soybean meal can be partially replaced by flaxseed meal in the diets of fattening lambs in an optimal proportion of 12%, and increased the average daily weight gain. Ababakri et al. [25] observed increased serum cholesterol levels in ewes fed 10% FS in feed mixtures, while in the research by Nudda et al. [26], kidney and liver function parameters did not differ in the serum of dairy goats fed 180 g/day of FS in the diet. Alves Dutra et al. [27] reported that flaxseed added to the diet of Alpine goats affected the metabolic profile of the blood, with values still within the physiological interval, except for triglycerides. However, the available literature lacks information on the use of FS and PC in the diet of goat kids during their growth. As far as we know, there is no study on the influence of PC in the diet of goat kids on metabolic status. The clarification of these questions is of considerable scientific interest in the search for feed called nutraceuticals that can reduce the soybean content in the diet and at the same time improve the health status of growing goat kids on a small-scale goat farm.

The hypothesis here was that FS and PC added in the feed mixture as alternative protein sources have no adverse effects on the blood parameters and goat kids' growth, which would be the novelty of the study. To test this hypothesis, the objective of this study was to evaluate two different feeding strategies for the inclusion of pumpkin seed cake and extruded flaxseed in the diet of Alpine goat kids and to research their effects on average

daily weight gain, hematological parameters, and biochemical serum parameters (energy, protein and mineral status, and enzyme activities) during goat kids' growth period.

2. Materials and Methods

This trial was carried out within the regulations of the Animal Protection Act of Croatia (NN 102/17, NN 32/19) and the Regulation on the Protection of Animals used for Scientific Purposes (NN 55/13, Declaration of Helsinki) and other relevant acts determining the welfare of farm animals as approved by the Bioethical Committee for the Animal Research of the Faculty of Agrobiotechnical Sciences Osijek (2158-94-02-24-18, 19 June 2024).

2.1. Animals and Management

At the small-scale goat farm in the Republic of Croatia, 31 French Alpine goat kids were used in the research. The experimental farm was located in the Slavonia part of Croatia (Marjančaci, Osijek-Baranja County, Croatia), characterized by a farm household system, using mainly family labor and using part of the products for family consumption and part for commercial uses (dairy and meat products). The goat kids were reared in a semi-intensive farming system, kept together with goats, until the age of two months, including a one-month weaning period. Each goat kid had given birth within seven days. The design of the experimental setup has been explained in Table 1. The experiment lasted for 55 days (from 32 to 87 days of kids' age), and measurements were taken during growth: I—at the end of the suckling period (32 ± 3 days old); II—at the end of the weaning period (60 ± 3 days old); and III—in the post-weaning period (87 ± 3 days old). The following average body weights of goat kids were determined: 7.9 ± 1.5 kg, 12.8 ± 1.5 kg, and 16.2 ± 1.6 kg in the suckling, weaning, and post-weaning periods, respectively. The average daily weight gain (ADWG) of goat kids was determined as the difference between two consecutive weights from the suckling to the weaning period (32nd–60th day) and from the weaning to the post-weaning period (60th–87th day).

Table 1. Experimental design and composition of experimental diets offered to goat kids on a small-scale goat farm.

Trait	Diets								
	Control			FS-9			PC-16		
Goat kids (n)	9			11			11		
Age of goat kids (days)	32	60	87	32	60	87	32	60	87
Growth period	Suckling	Weaning	Post-weaning	Suckling	Weaning	Post-weaning	Suckling	Weaning	Post-weaning
Production traits [1]									
Live body weight (kg)	7.48	12.54	15.49	8.07	13.05	17.11	8.23	14.38	17.92
ADWG, 32nd–87th day (g)	145.64			164.21			163.77		
Feeding									
Suckling milk	Ad libitum	Restricted [2]	-	Ad libitum	Restricted [2]	-	Ad libitum	Restricted [2]	-
Feed mixture	-	0% extruded flaxseed or pumpkin seed cake		-	9% extruded flaxseed		-	16% pumpkin seed cake	
		Ad libitum	~200 g		Ad libitum	~200 g		Ad libitum	~200 g
Hay	-	Ad libitum		-	Ad libitum		-	Ad libitum	

ADWG—average daily weight gain. [1] Klir Šalavardić et al. [17]; non-significant differences were observed ($p > 0.05$). [2] Restricted means goat kids were allowed to suck mothers milk between morning and evening milking, throughout the day.

Following kidding, the goat kids suckled colostrum; however, all goat kids were housed with goats until they were about 32 days old and suckled milk ad libitum. After 32 days of age, the goat kids were removed from their mothers before the evening milking and placed back in a pen with the goats after the morning milking. After the first sampling at 32 days of age, the goat kids were offered feed mixtures and a hay mixture of red clover and grass (*Lolium multiflorium* and *Phleum pratense*) ad libitum in an approximate ratio of 50:50 (feed mixture/hay). At the same time, suckling was restricted and only allowed throughout the day between morning and evening milking. The goat kids were completely weaned after 60 days and given feed mixtures and hay ad libitum. The average daily intake of feed mixture by goat kids was ~200 g/day/head during the post-weaning period.

The feed composition used in the study was consistent with that used by Klir et al. [16] and Klir Šalavardić et al. [28], since this trial is a part of wider research carried out with goats and their goat kids. The feed mixtures differed concerning the sources of protein and fat: the control feed mixture with soybean meal and extruded soybean; the feed mixture with 16% pumpkin seed cake (PC-16), which completely replaced soybean; and the feed mixture with 9% extruded flaxseeds (FS-9), which partially replaced soybean. In order to replace soybean as much as possible with PC and FS, without disturbing the metabolism of goat kids, firstly, a study of the chemical composition of PC and FS was carried out (Section 2.2). Then, PC and FS, together with other ingredients, were added in amounts that would balance the feed mixtures to obtain a norm for growing goat kids in terms of protein, fat, and energy content according to the National Research Council [29] (Table 2). It turned out that 16% PC and 9% FS were ideal for the feed mixtures, whose chemical composition was still within the prescribed norms. The chemical composition of the milk consumed by the kids was 3.43, 3.81, and 4.05% milk fat; 3.01, 2.98, and 3.22% protein; and 4.47, 4.40, and 4.29% lactose in goat's milk in CON, PC-16, and FS-9, respectively [30].

Table 2. Dietary ingredients, chemical composition, and major fatty acid proportions of concentrate mixtures and hay used in the diets for goat kids.

Ingredient, %	Concentrate Mixture			Hay
	CON	FS-9	PC-16	
Corn grain	42.9	40.8	45.9	
Barley grain	8.0	8.0	9.0	
Oat grain	10.0	10.0	13.5	
Wheat flour	12.0	9.0	12.0	
Extruded soybean	15.0	-	-	
Extruded linseed	-	9.0	-	
Pumpkin seed cake	-	-	16.0	
Alfalfa dehydrated	-	4.0	-	
Soybean meal (46% crude protein)	8.5	15.7	-	
Calcium carbonate	1.6	1.5	1.6	
Monocalcium phosphate	0.5	0.5	0.5	
Salt	0.4	0.4	0.4	
Pellet binder	0.1	0.1	0.1	
Mineral vitamin premix [1]	1.0	1.0	1.0	
Chemical composition, %				
DM (% fresh matter)	87.6	87.4	87.3	92.5
Crude protein	16.2	16.2	16.3	11.0
Crude fiber	4.14	4.88	3.73	28.7
Crude ash	4.92	5.06	5.23	5.73
Crude lipid	5.64	5.83	5.63	1.33
ME (MJ/kg DM)	13.2	13.0	13.2	8.0
Mineral composition (mg/kg DM)				
Ca	8949	8108	8548	1572
P	7100	6147	6818	1410
Mg	2114	1944	2096	480
Fe	321	278	294	92.8

Table 2. *Cont.*

Ingredient, %	Concentrate Mixture			Hay
	CON	FS-9	PC-16	
	Fatty acids (g/100 g FAME)			
C16:0	10.7	9.43	12.9	29.80
C18:0	4.08	6.11	4.48	2.84
C18:1 n-9	31.9	32.0	34.4	7.71
C18:2 n-6	48.2	38.2	44.4	22.40
C18:3 n-3	2.97	11.50	1.80	24.50

CON—control group; FS-9—feed mixture containing extruded flaxseed, PC-16—feed mixture containing pumpkin seed cake, DM—dry matter, ME—metabolizable energy, FAME—fatty acid methyl ester. [1] Mineral–vitamin premix: iron sulphate monohydrate 4000 mg, copper sulphate pentahydrate 800 mg, manganese oxide 3500 mg, zinc sulphate monohydrate 5000 mg, potassium iodide 80 mg, cobalt sulphate heptahydrate 20 mg, sodium selenite 15 mg, magnesium oxide 5000 mg, vitamin A 1,000,000 IU, vitamin D3 150,000 IU, α-tocopherol 1500 mg, vitamin K3 50 mg, vitamin B1 100 mg, vitamin B2 200 mg, vitamin B6 200 mg, vitamin B12 1 mg, niacin 1000 mg, Ca-pantothenate 500 mg, and choline chloride 10,000 mg.

2.2. Feed Analyses

Standard methods were used to determine the composition of feed [31]. Using the steam distillation unit for Kjeldahl nitrogen (Behr Labor-Technik GmbH, Düsseldorf, Germany) and the Kjeldahl method, crude protein concentrations were determined on the basis of the nitrogen content. The Universal Extractions System B-811 (Büchi, Flawil, Switzerland) was used to analyze crude fat concentrations. According to Menke et al. [32], the metabolic energy (ME, MJ/kg DM) of the feed samples was determined from the gas generation during a 24 h in vitro incubation period using the Hohenheim gas test. An inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500a, Agilent Technologies Inc., Santa Clara, CA, USA) was used to measure the concentrations of mineral elements (Ca, P, Mg, and Fe) in solutions containing digested plant materials. Gas chromatography was used to determine the fatty acid content of food according to the approach of the State Office for Agricultural Chemistry Baden-Württemberg (LaChemie P23-5-008, V. 01).

2.3. Blood Sampling and Analyses

Blood was collected from all animals by jugular venipuncture between 0700 and 0800 h into 10 mL vacuum tubes (Vacutube®, LT Burnik, Vodice, Slovenia) by the same trained professional at goat kids' ages of 32, 60, and 87 days. The blood was sampled in the morning and completed in 1 min to avoid excessive stress. Within an hour of blood collection on each day, a tube from each animal was brought to the Central Agrobiotechnical Analytical Unit (Faculty of Agrobiotechnical Sciences, Osijek, Croatia) for hematology analyses and differential blood counts.

For hematology analysis, the blood of goat kids was sampled into the sterile vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. Before analysis, whole blood was mixed with the Coulter mixer (Coulter Electronics Ltd., Luton Bedfordshire, UK). In whole blood, within 2 h after sampling, the following hematological parameters were determined: the number of leukocytes, the number of erythrocytes, hemoglobin, and hematocrit (WBC, RBC, HGB, and HCT, respectively). The following RBC indices were examined: mean corpuscular volume, average hemoglobin content in erythrocytes, and mean hemoglobin concentration in erythrocytes (MCV, MCH, and MCHC, respectively). The hematological parameters were analyzed on an automatic three differential hematology analyzer (Sysmex PocH-100Iv, Sysmex Europe GmbH, Hamburg, Germany). Blood samples from EDTA tubes were collected to make blood smears on glass slides. The smears were stained according to the Pappenheim method. White blood cells, such as neutrophils (NEUTs) and lymphocytes (LYMs), were identified using a compound microscope (BX53, Olympus Corporation, Tokyo, Japan).

Serum was prepared from the remaining blood tubes using standard procedures. The serum for each animal was aliquoted into 2 mL vials within 2 h of blood collection,

transported in dry ice, and stored at −80 °C until required. Blood serum biochemical parameters were determined, including concentrations of minerals (calcium, phosphorus-inorganic, magnesium, and iron) and concentrations of urea, glucose, total proteins, and albumin, as well as cholesterol, high density lipoprotein, low density lipoprotein, triglyceride, β-hydroxybutyrate, and non-esterified fatty acids (CHOL, HDL, LDL, TGCs, BHB, and NEFAs, respectively). In addition, the following enzymes activities were determined: alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyl-transferase (ALT, AST, and GGT, respectively). Globulin was calculated as the difference between total protein and albumin. Analyses were obtained by Beckman Coulter analyzer (AU400, Brea, CA, USA). Superoxide dismutase activity was measured by the degree to which the xanthine oxidase and superoxide radicals inhibited the following reaction (Randox Laboratories, Crumlin, UK):

$$O_2^{\bullet} + O_2^{\bullet} + 2H \xrightarrow{SOD} O_2 + H_2O_2$$

The glutathione peroxidase enzyme catalyzes the oxidation of glutathione by cumene hydroperoxide (Randox Laboratories, Crumlin, UK):

$$2GSH + ROOH \xrightarrow{GPx} ROH + GSSG + H_2O$$

2.4. Statistical Analyses

Mean values for average daily weight gain and blood parameters were obtained by Proc MEANS for each parameter within each dietary treatment, during different growth periods. The statistical model of these analyses included the fixed effect of diet as a "between-subject factor" and goat kid's growth effect as a "within-subject factor". In the first step, a one-way Proc ANOVA was used to analyze the effect of dietary treatments for each growth period with the following model: $Y_{ij} = \mu + d_i + e_{ij}$, where μ = overall mean, d_i = the fixed effect of diet (three treatments: i = CON, FS-9, and PC-16), and e_{ij} = residual error. In the second step, a repeated measures Proc ANOVA was used to analyze the effect of the growth period on blood parameters for each dietary treatment with the following model: $Y_{ij} = \mu + g_i + e_{ij}$, where μ = overall mean, g_i = the fixed effect of the growth period (three periods: i = suckling, weaning, and post-weaning), and e_{ij} = residual error. Comparisons of mean values among dietary treatments and growth periods were performed using Tukey's tests, while significant differences were declared at $p < 0.05$ and trends were declared at $0.05 < p \leq 0.1$. Standard errors of the mean were reported. Statistical analysis was performed using the statistical software SAS 9.4 [33].

3. Results

3.1. Average Daily Weight Gain

Average daily weight gain is presented in Figure 1 from the suckling to weaning period (32nd–60th day) and from the weaning to post-weaning period (60th–87th day) of goat kids fed FS-9 and PC-16 compared to CON. It is evident that there were no differences observed in ADWG between different dietary treatments in both growth periods. However, the growth period affected ADWG in the PC-16 and CON groups ($p < 0.05$). In PC-16 and CON, ADWG decreased from the weaning to post-weaning period compared to the suckling to weaning growth period, while in FS-9, no differences were estimated.

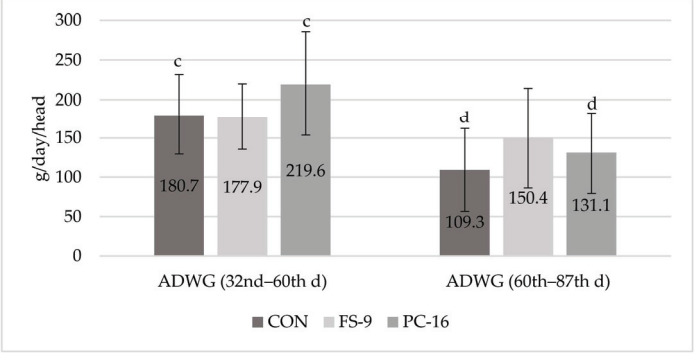

Figure 1. Average daily weight gain (ADWG) of growing goat kids fed diets containing soybean (CON, n = 9), extruded flaxseed (FS-9, n = 11), and pumpkin seed cake (PC-16, n = 11) from the suckling to the weaning period (32nd–60th day) and from the weaning to the post-weaning period (60th–87th day). ^{c,d} Means with different superscripts differ significantly at $p < 0.05$ (growth period effect).

3.2. Hematological Parameters

As presented in Table 3, no specific changes were observed in the majority of hematological parameters in goat kids' blood as affected by dietary treatment. However, the WBC count was higher ($p < 0.05$) in goat kids fed FS-9 and PC-16 compared to CON diets during the weaning. The age of the goat kids affected some hematological parameters, like WBC, RBC, and HGB. The WBC count increased ($p < 0.05$) in all groups as kids were growing, specifically at post-weaning compared to suckling, while in CON, it increased compared to suckling and weaning periods. The RBC count increased only in the CON group post-weaning compared to the suckling period, while in PC-16 a tendency towards an increase was determined ($p = 0.06$). HGB increased ($p < 0.05$) in experimental groups during animal growth at the post-weaning period compared to suckling, while in the CON group a tendency towards an increase ($p = 0.08$) was observed.

Table 3. Hematological parameters in the whole blood of goat kids fed with diets containing soybean (CON, n = 9), extruded flaxseed (FS-9, n = 11), and pumpkin seed cake (PC-16, n = 11).

Parameters	Growth Period	Diet			SEM	p Value [1]
		CON	FS-9	PC-16		
WBCs ($\times 10^9$ L)	Suckling	6.44 ^{d}	8.49 ^{d}	8.76 ^{d}	0.530	0.185
	Weaning	6.61 ^{b,d}	9.84 ^{a,c,d}	9.54 ^{a,c,d}	0.455	0.005
	Post-weaning	10.76 ^{c}	12.09 ^{c}	12.58 ^{c}	0.563	0.468
	p value [2]	0.011	0.003	0.027		
RBCs ($\times 10^{12}$ L)	Suckling	10.18 ^{d}	11.35	11.85	0.503	0.442
	Weaning	11.63 ^{c,d}	12.00	13.03	0.505	0.556
	Post-weaning	13.54 ^{c}	13.43	14.53	0.393	0.481
	p value [2]	0.017	0.232	0.064		
HGB (g/L)	Suckling	85.86	83.20 ^{d}	86.38 ^{d}	3.568	0.927
	Weaning	85.14	90.91 ^{c,d}	96.38 ^{c,d}	3.115	0.410
	Post-weaning	103.0	103.9 ^{c}	110.3 ^{c}	2.715	0.538
	p value [2]	0.076	0.038	0.013		

Table 3. *Cont.*

Parameters	Growth Period	Diet			SEM	*p* Value [1]
		CON	FS-9	PC-16		
HCT (L/L)	Suckling	0.485	0.540	0.408	0.032	0.231
	Weaning	0.558	0.560	0.501	0.043	0.824
	Post-weaning	0.467	0.599	0.417	0.043	0.186
	p value [2]	0.720	0.851	0.323		
MCV (fL)	Suckling	49.90	52.39	38.01	4.829	0.444
	Weaning	56.77	60.33	40.74	5.422	0.302
	Post-weaning	48.20	61.55	36.31	5.200	0.117
	p value [2]	0.804	0.730	0.919		
MCH (pg)	Suckling	8.02	7.38	7.33	0.178	0.050
	Weaning	7.41	7.73	7.45	0.129	0.545
	Post-weaning	7.63	7.83	7.60	0.109	0.638
	p value [2]	0.111	0.203	0.562		
MCHC (g/L)	Suckling	201.6	174.6	216.3	13.652	0.439
	Weaning	159.9	165.1	214.8	14.990	0.290
	Post-weaning	194.0	158.5	241.9	15.001	0.079
	p value [2]	0.570	0.897	0.609		

CON—control group; FS-9—feed mixture containing extruded flaxseed, PC-16—feed mixture containing pumpkin seed cake; RBCs—erythrocytes, WBCs—leukocytes, HGB—hemoglobin, HCT—hematocrit, MCH—average hemoglobin content in erythrocytes, MCV—mean corpuscular volume, MCHC—mean hemoglobin concentration in erythrocytes. [1] Diet effect. [2] Growth period effect. [a,b] Row means with different superscripts differ significantly at $p < 0.05$ (diet effect). [c,d] Column means with different superscripts differ significantly at $p < 0.05$ (growth period effect).

In Figure 2, it is evident that diet influenced the LYM percentage between differently fed groups during the weaning period, when FS-9-fed kids had higher ($p < 0.05$) LYMs compared to PC-16, while the growth effect was noticeable in the PC-16 group by increasing LYMs post-weaning compared to the suckling period. The NEUT content was lower in FS-9 compared to PC-16 during weaning, while in PC-16, NEUTs decreased from the suckling to the post-weaning period.

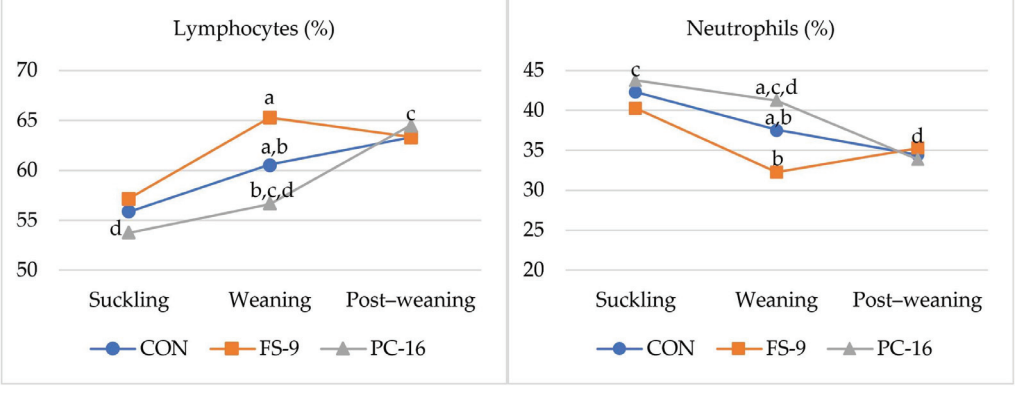

Figure 2. Lymphocytes and neutrophils in growing goat kids fed diets containing soybean (CON, $n = 9$), extruded flaxseed (FS-9, $n = 11$), and pumpkin seed cake (PC-16, $n = 11$). [a,b] Means with different superscripts differ significantly at $p < 0.05$ (diet effect). [c,d] Means with different superscripts differ significantly at $p < 0.05$ (growth period effect).

3.3. Biochemical Parameters

In Table 4, biochemical parameters present mostly no significant changes as affected by FS-9 or PC-16 feeding. The concentration of BHB was the lowest ($p < 0.05$) in the serum of goat kids fed FS-9 compared to CON, while PC-16 feeding did not effect any changes in the post-weaning period. Growth affected a few parameters, like LDL being higher ($p < 0.05$) in serum post-weaning compared to the weaning period in the FS-9 group. The NEFA concentrations were lower ($p < 0.05$) at post-weaning and weaning compared to the suckling period in both experimental groups. Concentrations of BHB were higher ($p < 0.05$) in the suckling period compared to the weaning and post-weaning periods in both FS-9 and PC-16.

Table 4. Biochemical parameters of energy status in the serum of goat kids fed with diets containing soybean (CON, $n = 9$), extruded flaxseed ($n = 11$), and pumpkin seed cake ($n = 11$).

Parameters, mmol/L	Growth Period	Diet			SEM	*p* Value [1]
		CON	FS-9	PC-16		
Glucose	Suckling	3.22	3.34	4.17	0.213	0.163
	Weaning	3.80	3.94	4.29	0.195	0.621
	Post-weaning	3.94	3.94	4.03	0.108	0.926
	p value [2]	0.314	0.316	0.749		
Cholesterol	Suckling	2.54	2.60	2.41	0.159	0.896
	Weaning	2.27	2.21	2.40	0.130	0.830
	Post-weaning	2.66	3.05	2.86	0.191	0.740
	p value [2]	0.689	0.084	0.474		
TGCs	Suckling	0.423	0.422	0.339	0.029	0.430
	Weaning	0.291	0.323	0.384	0.033	0.569
	Post-weaning	0.397	0.395	0.454	0.037	0.780
	p value [2]	0.282	0.387	0.455		
HDL	Suckling	1.49	1.58	1.36	0.066	0.384
	Weaning	1.32	1.30	1.38	0.061	0.856
	Post-weaning	1.48	1.54	1.51	0.066	0.944
	p value [2]	0.633	0.088	0.654		
LDL	Suckling	0.856	0.823 [c,d]	0.900	0.094	0.949
	Weaning	0.813	0.765 [d]	0.846	0.071	0.893
	Post-weaning	0.998	1.33 [c]	1.15	0.124	0.583
	p value [2]	0.788	0.035	0.433		
NEFAs	Suckling	1.37	1.50 [c]	1.69 [c]	0.188	0.819
	Weaning	0.590	0.242 [d]	0.185 [d]	0.077	0.111
	Post-weaning	0.443	0.324 [d]	0.113 [d]	0.103	0.485
	p value [2]	0.104	<0.001	<0.001		
BHB	Suckling	0.463	0.396 [c]	0.498 [c]	0.033	0.436
	Weaning	0.283	0.191 [d]	0.210 [d]	0.029	0.451
	Post-weaning	0.373 [a]	0.229 [b,d]	0.268 [a,b,d]	0.023	0.036
	p value [2]	0.197	<0.001	0.004		

CON—control group; FS-9—feed mixture containing extruded flaxseed, PC-16—feed mixture containing pumpkin seed cake; HDL—high-density lipoprotein, LDL—low-density lipoprotein, TGCs—triglycerides, NEFAs—non-esterified fatty acids, BHB—β-hydroxybutyrate, SEM—standard error of mean. [1] Diet effect. [2] Growth period effect. [a,b] Row means with different superscripts differ significantly at $p < 0.05$ (diet effect). [c,d] Column means with different superscripts differ significantly at $p < 0.05$ (growth period effect).

In all groups, the concentration of proteins increased ($p < 0.05$) during aging, specifically post-weaning, compared to the weaning or suckling periods. Similarity was determined in the concentration of globulin in experimental groups, where higher ($p < 0.05$) values were determined post-weaning compared to weaning or suckling, while in CON, globulin increased only at post-weaning compared to the weaning period (Table 5). Most minerals did not differ in goat kids' serum as affected by different dietary treatments. Only

Mg concentrations tended to increase ($p = 0.05$) with the inclusion of PC-16 in goat kids' diets during the weaning period. It is evident that Mg concentration increased ($p < 0.05$) in the serum of goat kids fed PC-16 post-weaning compared to the weaning or suckling periods. Other minerals, like Ca, Fe, and P-inorganic, did not reveal any significant changes in the serum of goat kids during growth.

Table 5. Biochemical parameters of protein and mineral status in the serum of goat kids fed with diets containing soybean (CON, $n = 9$), extruded flaxseed ($n = 11$), and pumpkin seed cake ($n = 11$).

Parameters (g/L)	Growth Period	Diet			SEM	p Value [1]
		CON	FS-9	PC-16		
Urea (mmol/L)	Suckling	4.15	4.90	4.06	0.236	0.254
	Weaning	3.96	3.86	3.74	0.261	0.953
	Post-weaning	4.72	4.57	3.77	0.297	0.413
	p value [2]	0.614	0.271	0.793		
Proteins	Suckling	53.21 ^{d}	53.48 ^{d}	57.11 ^{d}	0.855	0.129
	Weaning	53.53 ^{d}	54.45 ^{d}	53.64 ^{d}	0.831	0.883
	Post-weaning	61.48 ^{c}	64.42 ^{c}	64.31 ^{c}	0.786	0.303
	p value [2]	0.010	<0.001	<0.001		
Albumin	Suckling	28.84	30.81	29.23	0.472	0.178
	Weaning	29.07	28.75	29.09	0.378	0.766
	Post-weaning	30.45	30.44	31.00	0.362	0.787
	p value [2]	0.305	0.081	0.151		
Globulin	Suckling	24.37 ^{c,d}	22.67 ^{d}	27.88 ^{d}	0.940	0.057
	Weaning	24.07 ^{d}	25.70 ^{d}	24.55 ^{d}	0.679	0.605
	Post-weaning	31.03 ^{c}	33.98 ^{c}	33.31 ^{c}	0.712	0.266
	p value [2]	0.032	<0.001	<0.001		
Ca (mmol/L)	Suckling	2.36	2.45	2.54	0.029	0.053
	Weaning	2.41	2.45	2.38	0.020	0.352
	Post-weaning	2.48	2.41	2.25	0.083	0.589
	p value [2]	0.191	0.680	0.415		
Mg (mmol/L)	Suckling	0.931	0.967	0.880 ^{d}	0.018	0.112
	Weaning	0.869	0.945	0.960 ^{d}	0.015	0.050
	Post-weaning	0.959	0.990	1.06 ^{c}	0.020	0.144
	p value [2]	0.094	0.552	<0.001		
Fe (µmol/L)	Suckling	28.64	26.32	27.09	2.863	0.952
	Weaning	30.67	26.68	33.58	2.567	0.333
	Post-weaning	25.48	23.26	22.69	1.259	0.710
	p value [2]	0.554	0.740	0.123		
P-inorganic (mmol/L)	Suckling	3.06	3.15	3.10	0.078	0.906
	Weaning	3.12	3.08	3.22	0.064	0.669
	Post-weaning	3.49	2.96	3.17	0.087	0.057
	p value [2]	0.187	0.491	0.809		

CON—control group; FS-9—feed mixture containing extruded flaxseed, PC-16—feed mixture containing pumpkin seed cake; SEM—standard error of mean. [1] Diet effect. [2] Growth period effect. ^{c,d} Column means with different superscripts differ significantly at $p < 0.05$ (growth period effect).

The activity of GGT was the lowest in the serum of goat kids fed PC-16 diets compared to CON, while FS-9 did not reveal any changes in the post-weaning period (Table 6). The AST activity tended to decrease ($p = 0.07$) with PC-16 in the post-weaning period, while the activity of GPx was the highest ($p < 0.05$) in the serum of FS-9 goat kids compared to PC-16 post-weaning. The GGT in the serum of CON goat kids was the highest ($p < 0.05$) post-weaning compared to the suckling period, and the GPx was higher in the serum of goat kids during weaning compared to the suckling or post-weaning periods, but only in the FS-9 group.

Table 6. Activities of serum enzymes of goat kids fed with diets containing soybean (CON, $n = 9$), extruded flaxseed ($n = 11$), and pumpkin seed cake ($n = 11$).

Enzyme (U/L)	Growth Period	Diet			SEM	p Value [1]
		CON	FS-9	PC-16		
Aspartate aminotransferase (AST)	Suckling	71.17	71.38	68.73	2.525	0.905
	Weaning	97.15	80.72	74.57	5.984	0.377
	Post-weaning	101.06	82.81	77.19	3.974	0.072
	p value [2]	0.223	0.383	0.369		
Alanine aminotransferase (ALT)	Suckling	11.76	18.78	20.45	2.885	0.498
	Weaning	16.27	17.73	14.07	2.514	0.841
	Post-weaning	25.38	20.54	18.14	3.610	0.771
	p value [2]	0.251	0.927	0.694		
γ-glutamyl transferase (GGT)	Suckling	34.23 [d]	37.52	36.20	1.823	0.791
	Weaning	43.97 [c,d]	43.63	41.56	2.079	0.894
	Post-weaning	48.40 [a,c]	39.52 [a,b]	38.65 [b]	1.606	0.040
	p value [2]	0.009	0.400	0.507		
Superoxide dismutase (SOD, U/mL)	Suckling	0.340	0.402	0.464	0.066	0.803
	Weaning	0.383	0.501	0.609	0.071	0.524
	Post-weaning	0.769	0.617	0.716	0.089	0.789
	p value [2]	0.209	0.323	0.610		
Glutathione peroxidase (GPx)	Suckling	960.1	1061.4 [d]	914.7	62.739	0.610
	Weaning	1011.1	1104.2 [c]	893.6	63.785	0.396
	Post-weaning	700.2 [a,b]	809.7 [a,d]	600.8 [b]	35.716	0.039
	p value [2]	0.154	0.008	0.119		

CON—control group; FS-9—feed mixture containing extruded flaxseed, PC-16—feed mixture containing pumpkin seed cake; SEM—standard error of mean. [1] Diet effect. [2] Growth period effect. [a,b] Row means with different superscripts differ significantly at $p < 0.05$ (diet effect). [c,d] Column means with different superscripts differ significantly at $p < 0.05$ (growth period effect).

4. Discussion

4.1. Average Daily Weight Gain

Feeding goat kids FS-9 and PC-16 resulted in similar ADWG compared to CON from suckling to weaning and from the weaning to post-weaning period. This agrees with Mahouachi et al. [34], who concluded that extruded flaxseed (15%) can be used in lamb diets without adverse effects on average daily gains of lambs fed iso-caloric and iso-nitrogenous feed mixtures. Novoselec et al. [35] concluded that 7% pumpkin seed cake can be used as a protein source in lamb diets without affecting production characteristics such as the average daily gain of lambs. However, it can be seen from Figure 1 that ADWG decreased in PC-16 and CON goat kids after weaning, while there was no significant decrease in FS-9 goat kids. The results showed that the goat kids exhibited a very high growth intensity during the period from suckling to weaning. During this period, ADWG was achieved by both maternal milk consumption and supplemental feed [36,37], such as FS-9, PC-16, or CON feed mixtures.

According to a current study, 16% PC completely replaced the 15% extruded soybeans and 8.5% soybean meal in the feed mixtures for goat kids. This suggests that utilizing PC as an alternative protein source could reduce the high cost of soybeans in feed mixtures. Since PC is one of the affordable and locally available feeds in Europe, it may be financially advantageous for a small-scale goat farm to avoid the import of soybeans [17]. Since PC is primarily produced under organic conditions [22], it can also be used as a premium feed in organic animal farming, supporting sustainable production on small-scale goat farms. In addition, it is essential to maintain the quality of the product, the production output, and the overall health of the livestock [38]. However, 9% FS was combined with 15.7% soybean meal in feed mixtures for goat kids as an alternative source of fat and proteins. Although it is commonly recognized that FS is not very cost-effective for animal nutrition, it may

help enhance the amount of functional compounds, including n-3 fatty acids, which adds additional value to animal products. Consequently, the increased cost of the produced functional foods is the only way to offset the cost of this feed mixture. Functional foods are more expensive than similar products that are not classified as "medical" healthy foods, according to Balogh et al. [39].

4.2. Hematological Parameters

As shown in Table 3, no specific feeding-related changes were observed in most hematological parameters in the blood of goat kids, except for WBC count, which was higher in the blood of goat kids fed FS-9 and PC-16 during the weaning period. In the FS-9 group, this could be explained by the high level of n-3 PUFAs in the FS-9 group, which promoted lymphocyte responses. In the study by Abu El-Hamd et al. [40], the addition of flaxseed oil to the milk of Friesian calves (0.2 mL/kg live weight) during suckling increased the WBC count and improved the immune response of calves without adverse effects on hematological or biochemical parameters. According to Al-Zuhairy and Taher [41], feeding 5 or 10% flaxseed to chickens increased blood WBC counts at 40 days of age. These authors explained that the birds were in good condition and this was not so much a sign of disease but a sign possibly related to the immunomodulatory properties of the active components of flaxseed, like n-3 PUFAs. N-3 PUFAs have an impact on various immune mechanisms, such as lymphocyte proliferation, the production of cytokines by lymphocytes, and natural killer cell activity [42]. Gandra et al. [43] reported that phagocytosis-positive leukocytes were greater in prepartum and postpartum cows fed whole flaxseeds compared to a control group, concluding that n-3 PUFAs seem to have a greater effect on the activity of leukocytes when compared with n-6 PUFAs. These results are consistent with the results of the present research, since α-linolenic acid was the highest in the feed mixture of the FS-9 group (Table 1). Furthermore, membrane phospholipids act as substrates for the release of (non-esterified) PUFAs; these released PUFAs can function as transcription factor ligands, signaling molecules, or precursors for the biosynthesis of lipid mediators, which are involved in the regulation of numerous cell and tissue responses [44]. Lee and Kang [45] showed that n-3 PUFA treatment increased immune cell numbers such as WBCs and LYMs in 100-day-old male miniature pigs fed diets supplemented with n-3 PUFAs. Momeni-Pooya et al. [46] reported that supplementing the diet with n-3 PUFAs from flaxseed oil can be a strategy to improve the immune performance of calves fed barley-based starter diets. As reported by El-Saadany et al. [47], the addition of pumpkin seed oil to the poultry diet (0.5%) can enhance the physiological, antioxidative, and immunological status of birds, owing to its high polyphenol content. The slightly elevated WBC values observed in both experimental groups indicate that the immune system of the goat kids functioned well during the weaning. The values obtained in our study were within the laboratory reference values Jackson and Cockcroft [48] (4–13 $\times$ 10^9/L) obtained for goats. Consistent with the increase in WBCs in the FS-9 group of goat kids, LYM levels increased in the same group compared to PC-16, while PC-16 increased NEUTs compared to FS-9 during weaning. Lee et al. [49] observed a lower NEUT/LYM ratio in the blood of laying hens fed a basal diet containing 3.6% (*w*/*w*) FS product (17% α-linolenic acid), indicating its potential effect on alleviating inflammation and stress conditions in laying hens.

It is known that goat kids are very sensitive to changes in nutrition and rearing, especially at a young age. Therefore, the switch from milk to the feed mixtures' proteins and forage in the diet needs to be gradual [6], and took 28 days in the current study. The WBCs increased in all groups from suckling until the post-weaning period. During the weaning, goat kids are usually under stress. It has been suggested that certain immune cell populations seek refuge in the bone marrow and adipose tissue during metabolic stress [50]. In the experimental groups of the present study, the effect of metabolic stress during weaning was not pronounced, which is also reflected in adequate ADWG. The variation in the total WBC changes from suckling to the post-weaning period reflects the adaptability process of the hematopoietic system to extrauterine life, which brings the WBC

counts of young animals closer to those of adults [51] and the immune system becomes more functional [52]. It is evident that RBCs and HGB increased in all groups during the growth of goat kids, but the RBC count was only significant in CON, and a tendency towards an increase was observed in PC-16, while HGB increased in experimental groups during animal growth with a tendency towards an increase in CON. Souza et al. [52] found that lambs aged 30 to 60 days had higher levels of RBC (12.1–13.6 $\times$ 10^6 cells μL^{-1}) and higher levels of HGB from 30 to 90 days (8.8–11.4 gdL^{-1}) along with a decline at the age of 120 days. They explained that when the amount of solid foods consumed increased, so did the overall RBC count and HGB content. These findings are consistent with the results of the current study on goat kids' growth.

4.3. Biochemical Parameters

When comparing the PC-16 or FS-9 group with the CON group, the concentrations of most blood serum parameters related to energy and protein status, as well as enzyme activities, remained unchanged. This indicates that PC-16 or FS-9 had no discernible negative effects on the health or nutritional status of the goat kids, making it a safe dietary ingredient. However, growth did affect some parameters in goat kids' serum, e.g., serum LDL levels were increased after weaning compared to the weaning period but only in the FS-9 group. This implies that prolonged (>55 days) FS-9 feeding to goat kids may contribute to hyperlipidemic blood traits by increasing serum LDL, as reported in the goat study conducted by Alves Dutra et al. [27]. In this study, increasing flaxseed supplementation from 0 to 15% had a linear effect on LDL levels measured at both 40 and 60 days of the trial period. According to the authors, the effect of flaxseed on lipid concentrations in the plasma in this investigation can be explained by the fact that eating flaxseed increased the number of circulating lipoproteins. In our study, a trend of increasing CHOL concentration in the FS-9 group was reported from the suckling to the post-weaning period, which was slightly above the reference values (1–3 mmol/L) [48]. Most of the CHOL increase occurs in the LDL cholesterol fraction [53]. In a study with 30-month-old lambs fed 10% FS in concentrate mixture, Hossein Abadi et al. [54] discovered higher CHOL when compared to raw flaxseed and the control. According to Huerta et al. [55], some studies observed that supplementation with n-3 PUFAs could raise LDL concentration, while others have found no effect, concluding that the overall n-3 PUFAs' effect on LDL remains unclear.

Regarding the biochemical parameters of energy status, the feeding effect was significant only in the post-weaning period, when feeding FS-9 resulted in decreased BHB compared to CON. Diet, stress, and age have all been shown to influence blood BHB and ketogenesis processes in goats [3]. Probably the lowest mobilization of body fat in the post-weaning period was in FS-9 goat kids, since the ADWG in FS-9 did not decrease as compared to CON or PC-16 from the weaning to the post-weaning period. It is very well known that increased circulating levels of NEFAs and BHB are a result of the increased mobilization of body fat reserves in response to a negative energy balance [56]. Thus, a decreased BHB and NEFAs in both experimental groups during the weaning and post-weaning period, compared to the suckling period, may alleviate the negative energy status of goat kids, during the weaning, more than commercial concentrate mixtures based on soybean. Mohapatra et al. [57] showed that the sudden separation of lambs from their mothers cause stress. However, in the present study, the transition from suckling to weaning took 28 days so that the goat kids had sufficient time to acclimatize to the feed mixture and cope with weaning stress at the age of two months. However, Khan et al. [58] observed that animals weaned gradually over 21 days had lower NEFA levels than animals weaned earlier, which is consistent with the present study. In addition, Qugley et al. [59] reported that NEFA concentrations in the plasma of calves decreased with increasing age (from 5 to 8 weeks). In cases where young animals have a negative energy balance, the BHB measurement in blood could indicate BHB produced in the liver [60]. In the present study, this probably occurred during the suckling period, when BHB levels were higher in all groups, although only significantly higher in the experimental groups than in the other

periods. During the suckling period, goat kids are exclusively dependent on their mother's milk and the fact that they were not suckling during the blood sampling probably led to stress, which could be reflected in a higher BHB value. In addition, there are no reference values for BHB in the serum of growing goat kids, which needs to be investigated further.

The total proteins in goat kids' serum were lower during the suckling period compared to weaning and post-weaning, when goat kids consumed concentrate feed mixture rich in proteins. As reported by Yusuf et al. [61], an adequate supply of protein is a fundamental factor for the proper growth of goats. An adequate proportion of crude proteins and energy provided by the feed mixture (16.2% of crude protein and 13.2 MJ/kg of metabolizable energy) explains the higher serum total proteins in goat kids in the post-weaning period. Osman et al. [62] determined a similar concentration of total proteins in weaned goat kids, which was 61 $\pm$ 4.1 g/L. Lower values for total proteins and globulins at 30 days, according to Souza et al. [63], signify the beginning of the animal's active production of immunoglobulins and the inference of the immunoglobulins' passive degradation process as received via colostrum. After a few months, the increase in globulin concentration in serum mainly comes from the increase in gamma-globulin concentrations related to adaptation to environmental conditions, as at this age, animals already have a mature immune system, as determined in lambs by Santos et al. [64].

GGT activity decreased in the serum of goat kids fed a PC-16 diet compared to CON, while AST activity tended to decrease with PC-16, and ALT showed some numerical decrease in the same group during the post-weaning period. It is known that elevated levels of serum enzymes such as GGT, AST, and ALT are indications of liver injury. According to Makni et al. [65], pumpkin seeds are rich in dietary fiber, antioxidants, and unsaturated fatty acids, which are known to have hepatoprotective and anti-atherogenic properties. In an experiment with rats, Nkosi et al. [66,67] observed a hepatoprotective effect of pumpkin seed protein isolate in the diet by reducing the activity of AST and ALT in plasma. A similar decrease in AST activity in the plasma of dairy cows fed diets in which soybean meal was replaced by PC and dried distillers' grains was found by Li et al. [19]. El-Saadany et al. [47] found that the addition of pumpkin seed oil to the poultry diet (0.5%) decreased AST and ALT activity in the plasma of laying hens, concluding that pumpkin seed oil can be added to laying hens diets to improve metabolism and liver functions. Additionally, PC is rich in polyphenol content [47], which may improve hepatic morphology and antioxidant capacity in the liver, as observed by He et al. [68] in the study with piglets fed dietary holly polyphenol extracts. The decrease in serum GGT and AST activity observed in the goat kids fed PC-16 in the present study suggests that replacing extruded soybeans and soybean meal with PC after feeding for 55 days may have a protective effect on liver cells in the post-weaning period.

Antioxidant enzymes like SOD, GPx, and catalase play a role in preserving an optimal level of antioxidants inside cells [69]. Superoxide dismutase, the first enzyme responding to the presence of oxygen radicals, was not affected by the diet. However, the response of GPx activity in serum was diet-dependent at the post-weaning period. Increased GPx activity in FS-9 compared to PC-16 may be due to the n-3 fatty acids in extruded flaxseed, as shown in Table 2, which indicates that the FS-9 diets are enriched in α-linolenic acid. Higher GPx activity in blood serum could also indicate that the body is ready to cope with reactive oxygen species [70], as determined in FS-9 goat kids during the post-weaning period in our study. Gangal [71] reviewed and explained that n-3 PUFAs regulate gene expressions and thereby influence signaling pathways by interacting with nuclear receptors, e.g., by binding to the peroxisomal proliferator-activated receptor gamma. As reported by Sembratowicz et al. [72], flaxseed oil has a beneficial effect by stimulating antioxidant defense mechanisms and reducing the severity of oxidative stress, which probably occurred in the FS-9 group of the present study compared to PC-16. Al-Maghadi et al. [73] suggested that flaxseed oil exerts an alleviating effect by increasing levels of antioxidant enzymes in the cells of the intestinal mucosa, resulting in improved defense against oxygen-free radicals. In addition, Śpitalniak-Bajerska et al. [70] found higher GPx activity in the serum

of pre-weaning dairy calves fed milk replacer with ethyl esters of flaxseed oil (10 g/day) and lyophilized apples (25 g/day), which improved metabolic and oxidative functions. In our study, a positive effect of FS-9 diets was shown after 55 days of goat kid experimental feeding, which is worth further study. Barda et al. [74] conducted a study on rats with paw edema, who received topical treatment with flaxseed and pumpkin oils. The authors explained that the anti-inflammatory effect of the oils tested was found to be closely linked to their antioxidant properties and their bioactive compounds (PUFAs, vitamin E, and phytosterols). Indeed, the lipophilic aspect of these substances allowed them to diffuse into the cell membrane, which is rich in PUFAs and proteins [74]. In our study, the proportion of palmitic acid was higher in the PC-16 diet than in the FS-9 diet, as palmitic acid is predominant in pumpkin seed cake, together with oleic and linoleic acids. Palmitic acid promotes pro-inflammatory processes [75], while a higher inflammatory status was significantly correlated with lower levels of antioxidant enzymes [76] as observed in PC-16 compared to FS-9 serum in goat kids. Li et al. [77] demonstrated that treatment with palmitic acid promoted the formation of malondialdehyde and simultaneously reduced the activity of GPx determined in bovine endometrial cells. However, the GPx activity in the serum of PC-16 goat kids was similar to that of the CON group and was within normal physiological levels, as reported by Śpitalniak-Bajerska et al. [70].

Despite the promising outcomes regarding the use of alternative protein sources in the nutrition of growing goat kids, the complete replacement of soybean by PC-16 or the partial replacement by FS-9 in feed mixtures addresses some limiting factors in the present experimental setup. Lower sample size was probably a limitation that affected differences among treatments or growth periods, which were not significant but showed some tendencies. The main reason for the current sample size is that the experiment was done on the small-scale goat farm, which requires the production for family consumption and for commercial uses. This is why we tried to minimize the experiment's risks on the farm, especially because goat kids were examined in a very sensitive period during growth. Another reason is that, since this trial is a part of wider research carried out with carefully selected lactating dairy goats, we used only their goat kids in the current experiment. The present study could give us some useful guidelines for further studies regarding PC-16 and FS-9 in diets for goat kids, which will comprise a larger sample size and a longer duration.

5. Conclusions

Regarding the blood parameters, which portray the health and nutritional status, PC-16 and FS-9, in isoproteic, isolipidic, and isoenergetic diets, can be used in goat kids' nutrition as sources of proteins and fats on a small-scale goat farm without compromising goat kids' growth. The current experimental setup demonstrated that pumpkin seed cake is a high-quality ruminant protein feed that contributes to the reduction of soybeans in diets. At the same time, PC-16 decreased GGT activity in serum, thus having a potential hepatoprotective role in the post-weaning period of goat kids. As compared to pumpkin seed cake diets, dietary FS-9 has a more beneficial impact on GPx activity, which is worth further study in which the overall antioxidant status of goat kids shall be studied. To justify these findings further, the meat quality, as well as profitability and environmental issues, like nitrogen excretions, should still be evaluated to study overall sustainability when using pumpkin seed cake and extruded flaxseed in goat kids' diets on a small-scale goat farm.

Author Contributions: Conceptualization, Ž.K.Š. and Z.A.; formal analysis, Ž.K.Š., J.N. and M.Đ.; investigation, Ž.K.Š., J.N. and M.Đ.; writing—original draft preparation, Ž.K.Š.; writing—review and editing, Ž.K.Š.; visualization, Ž.K.Š., J.N., M.Đ. and Z.A.; supervision, Z.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the Faculty of Agrobiotechnical Sciences Osijek (protocol code 2158-94-02-24-18, 19 June 2024).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The study was carried out within the research team Breeding-technological aspects of animal production (AnimTeh, No. 1126) at the Faculty of Agrobiotechnical Sciences Osijek. The authors wish to thank the farmers from the small-scale (family) goat farm "Đurković", who cared for animals.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Habibu, B.; Kawu, M.U.; Makun, H.J.; Aluwong, T.; Yaqub, L.S. Seasonal variation in body mass index, cardinal physiological variables and serum thyroid hormones profiles in relation to susceptibility to thermal stress in goat kids. *Small Rumin. Res.* **2016**, *145*, 20–27. [CrossRef]
2. Antunović, Z.; Mioč, B.; Klir Šalavardić, Ž.; Širić, I.; Držaić, V.; Đidara, M.; Novoselec, J. The effect of lactation stage on the hematological and serum-related biochemical parameters of the Travnik Pramenka ewes. *Poljopr. Agric.* **2021**, *27*, 56–62. [CrossRef]
3. Abdelsattar, M.M.; Vargas-Bello-Pérez, E.; Zhuang, Y.; Fu, Y.; Zhang, N. Effects of age and dietary factors on the blood beta-hydroxybutyric acid, metabolites, immunoglobulins, and hormones of goats. *Front. Vet. Sci.* **2022**, *8*, 793427. [CrossRef]
4. Redlberger, S.; Fischer, S.; Kohler, H.; Diller, R.; Reinhold, P. Age-dependent physiological dynamics in acid-base balance, electrolytes, and blood metabolites in growing goats. *Vet. J.* **2017**, *229*, 45–52. [CrossRef] [PubMed]
5. Fanta, Y.; Kechero, Y.; Yemane, N. Hematological parameters of sheep and goats fed diets containing various amounts of water hyacinth (*Eichhornia crassipes*). *Front. Vet. Sci.* **2024**, *11*, 1286563. [CrossRef]
6. Antunović, Z.; Novoselec, J.; Klir, Ž. Body growth of goat kids in organic farming. *Maced. J. Anim. Sci.* **2015**, *5*, 59–62. [CrossRef]
7. Ripoll, G.; Alcalde, M.J.; Horcada, A.; Panea, B. Suckling kid breed and slaughter weight discrimination using muscle colour and visible reflectance. *Meat Sci.* **2011**, *87*, 151–156. [CrossRef]
8. Alcalde, M.J.; Ripoll, G.; Campo, M.M.; Horcada, A.; Panea, B. Relationship between consumers' perceptions about goat kid meat and meat sensory appraisal. *Animals* **2023**, *13*, 2383. [CrossRef]
9. Longobardi, F.; Sacco, D.; Casiello, G.; Ventrella, A.; Contessa, A.; Sacco, A. Garganica kid goat meat: Physico-chemical characterization and nutritional impacts. *J. Food Compos. Anal.* **2012**, *28*, 107–113. [CrossRef]
10. Ivanović, S.; Pavlović, I.; Pisinov, B. The quality of goat meat and it's impact on human health. *Biotechnol. Anim. Husb.* **2016**, *32*, 111–122. [CrossRef]
11. Gawat, M.; Boland, M.; Singh, J.; Kaur, L. Goat meat: Production and quality attributes. *Foods* **2023**, *12*, 3130. [CrossRef]
12. Pexas, G.; Doherty, B.; Kyriazakis, I. The future of protein sources in livestock feeds: Implications for sustainability and food safety. *Front. Sustain. Food Syst.* **2023**, *7*, 1188467. [CrossRef]
13. Suriyapha, C.; Suntara, C.; Wanapat, M.; Cherdthong, A. Effects of substituting agro-industrial by-products for soybean meal on beef cattle feed utilization and rumen fermentation. *Sci. Rep.* **2022**, *12*, 21630. [CrossRef] [PubMed]
14. Leguizamón, A. Modifying Argentina: GM soy and socio-environmental change. *Geoforum* **2014**, *53*, 149–160. [CrossRef]
15. Nasir, N.A.N.M.; Kamaruddin, S.A.; Zakarya, I.A.; Islam, A.K.M.A. Sustainable alternative animal feeds: Recent advances and future perspective of using azolla as animal feed in livestock, poultry and fish nutrition. *Sustain. Chem. Pharm.* **2022**, *25*, 100581. [CrossRef]
16. Klir, Z.; Castro-Montoya, J.M.; Novoselec, J.; Molkentin, J.; Domacinovic, M.; Mioc, B.; Dickhoefer, U.; Antunovic, Z. Influence of pumpkin seed cake and extruded linseed on milk production and milk fatty acid profile in Alpine goats. *Animal* **2017**, *11*, 1772–1778. [CrossRef]
17. Boldea, I.M.; Dragomir, C.; Gras, M.A.; Ropotă, M. Inclusion of rapeseed and pumpkin seed cakes in diets for Murciano-Granadina goats alters the fatty acid profile of milk. *S. Afrn. J. Anim. Sci.* **2021**, *51*, 262–270. [CrossRef]
18. Antunović, Z.; Klir, Ž.; Šperanda, M.; Sičaja, V.; Čolović, D.; Mioč, B.; Novoselec, J. Partial replacement of soybean meal with pumpkin seed cake in lamb diets: Effects on carcass traits, haemato-chemical parameters and fatty acids in meat. *S. Afr. J. Anim. Sci.* **2018**, *48*, 695–704. [CrossRef]
19. Li, Y.; Zhang, G.N.; Fang, X.P.; Zhao, C.; Wu, H.Y.; Lan, Y.X.; Che, L.; Sun, Y.K.; Lv, J.Y.; Zhang, Y.G.; et al. Effects of replacing soybean meal with pumpkin seed cake and dried distillers grains with solubles on milk performance and antioxidant functions in dairy cows. *Animal* **2021**, *15*, 100004. [CrossRef]
20. Patel, S. Pumpkin (*Cucurbita* sp.) seeds as nutraceutic: A review on status quo and scopes. *Mediterr. J. Nutr. Metab.* **2013**, *6*, 183–189. [CrossRef]
21. Valdez-Arjona, L.P.; Ramírez-Mella, M. Pumpkin waste as livestock feed: Impact on nutrition and animal health and on quality of meat, milk, and egg. *Animals* **2019**, *9*, 769. [CrossRef] [PubMed]
22. Pospišil, M. *Ratarstvo, II-Dio-Industrijsko Bilje [Plant Production, IInd Part-Industrial Plants]*; Zrinski d.d.: Čakovec, Croatia, 2013; p. 84.
23. Colonna, M.A.; Karatosidi, D.; Cosentino, C.; Freschi, P.; Carbonara, C.; Giannico, F.; Losacco, C.; Tufarelli, V.; Tarricone, S.; Selvaggi, M.; et al. Dietary supplementation with oregano and linseed in autochthonous "Facciuta Lucana" goats: Effects on meat quality traits in suckling kids. *Animals* **2023**, *13*, 3050. [CrossRef] [PubMed]

24. Hao, X.Y.; Yu, S.C.; Mu, C.T.; Wu, X.D.; Zhang, C.X.; Zhao, J.X.; Zhang, J.X. Replacing soybean meal with flax seed meal: Effects on nutrient digestibility, rumen microbial protein synthesis and growth performance in sheep. *Animal* **2020**, *14*, 1841–1848. [CrossRef] [PubMed]
25. Ababakri, R.; Dayani, O.; Khezri, A.; Naserian, A.A. Effects of extruded flaxseed and dietary rumen undegradable protein on reproductive traits and the blood metabolites in Baluchi ewes. *J. Anim. Feed. Sci.* **2021**, *30*, 214–222. [CrossRef]
26. Nudda, A.; Battacone, G.; Atzori, A.S.; Dimauro, C.; Rassu, S.P.G.; Nicolussi, P.; Bonelli, P.; Pulina, G. Effect of extruded linseed supplementation on blood metabolic profile and milk performance of Saanen goats. *Animal* **2013**, *7*, 1464–1471. [CrossRef]
27. Alves Dutra, P.; Batista Pinto, L.F.; Cardoso-Neto, B.M.; Silva Mendes, C.; Moraes Pinheiro, A.; Pires Barbosa, L.; de Jesus Pereira, T.C.; Pinto de Carvalho, G.G. Flaxseed added to the diet of Alpine goats affects the nutrients intake and blood parameters. *Trop. Anim. Health Prod.* **2022**, *54*, 104. [CrossRef]
28. Klir Šalavardić, Ž.; Novoselec, J.; Ronta, M.; Antunović, Z. Influence of pumpkin seed cake and extruded linseed on production traits of goat kids. *Krmiva* **2022**, *1*, 13–22. [CrossRef]
29. National Research Council. *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids*; The National Academies Press: Washington, DC, USA, 2007.
30. Klir Šalavardić, Ž.; Novoselec, J.; Castro-Montoya, J.M.; Šperanda, M.; Đidara, M.; Molkentin, J.; Mioč, B.; Dickhoefer, U.; Antunović, Z. The effect of dietary pumpkin seed cake and extruded linseed on blood haemato-chemicals and milk quality in Alpine goats during early lactation. *Mljekarstvo* **2021**, *71*, 13–24. [CrossRef]
31. Association of Official Agricultural Chemists, AOAC. *Official Methods of Analysis of AOAC International*; Association of Analytical Communities: Arlington, VA, USA, 2006.
32. Menke, K.H.; Raab, L.; Salewski, A.; Steingass, H.; Fritz, D.; Schneider, W. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. *J. Agr. Sci.* **1979**, *93*, 217–222. [CrossRef]
33. *SAS*; Version 9.4; SAS Institute Inc.: Cary, NC, USA.
34. Mahouachi, M.; Mathlouthi, N.; Saïdi, C.; Atti, N. The effect of increasing extruded linseed level on nutrient digestibility, growth, carcass characteristics, and non-carcass components of lambs from two genotypes. *Trop. Anim. Health Prod.* **2023**, *56*, 1. [CrossRef]
35. Novoselec, J.; Klir, Ž.; Steiner, Z.; Ronta, M.; Sičaja, V.; Antunović, Z. Production—Hematological parameters of lambs fed with diets containing pumpkin seed cake. *Krmiva* **2018**, *59*, 85–94. [CrossRef]
36. Călin, I.; Răducută, I.; Dărăban, S.; Vlad, I.; Priseceanu, H.I.; Pascal, C.; Pădeanu, I. Research on quantitative skills in meat production direction at youth goats from Carpathian breed in relation with the rearing system. *Agric. Agric. Sci. Procedia* **2015**, *6*, 191–196. [CrossRef]
37. Panayotov, D.; Sevov, S.; Georgiev, D. Live weight and intensity of growth of lambs from Lacaune breed raised in Bulgaria. *Bulg. J. Agric. Sci.* **2018**, *24*, 88–94.
38. Vasta, V.; Nudda, A.; Cannas, A.; Lanza, M.; Priolo, A. Alternative feed resources and their effects on the quality of meat and milk from small ruminants. *Anim. Feed. Sci. Technol.* **2008**, *147*, 223–246. [CrossRef]
39. Balogh, T.; Kőszegi, I.; Hoyk, E. The market of functional foods. *Gradus* **2020**, *7*, 161–166. [CrossRef]
40. Abu El-Hamd, M.A.; El-Diahy, Y.M.; El-Maghraby, M.M.; Elshora, M.A. Effect of flaxseed oil on digestibility, blood parameters, immuno-response and productive performance of suckling friesian calves. *J. Anim. Poult. Prod Mansoura Univ.* **2015**, *6*, 755–765. [CrossRef]
41. Al-Zuhairy, M.A.; Taher, M.G. Effects of feeding different levels of flaxseed on Performance traits and blood parameters in broiler. *Diyala Agr. Sci. J. DASJ* **2014**, *6*, 1–10.
42. Calder, P.C.; Yaqoob, P.; Thies, F.; Wallace, F.A.; Mile, E.A. Fatty acids and lymphocyte functions. *Brit. J. Nutr.* **2002**, *87*, S31–S48. [CrossRef]
43. Gandra, J.R.; Barletta, R.V.; Mingoti, R.D.; Verdurico, L.C.; Freitas, J.E.; Oliveira, L.J.; Takiya, C.S.; Kfoury, J.R.; Wiltbank, M.C.; Renno, F.P. Effects of whole flaxseed, raw soybeans, and calcium salts of fatty acids on measures of cellular immune function of transition dairy cows. *J. Dairy Sci.* **2016**, *99*, 4590–4606. [CrossRef]
44. Calder, P.C. Mechanisms of action of (n-3) fatty acids. *J. Nutr.* **2012**, *142*, 592S–599S. [CrossRef]
45. Lee, S.I.; Kang, K.S. Omega-3 fatty acids modulate cyclophosphamide induced markers of immunosuppression and oxidative stress in pigs. *Sci. Rep.* **2019**, *9*, 2684. [CrossRef] [PubMed]
46. Momeni-Pooya, F.; Kazemi-Bonchenari, M.; Mirzaei, M.; Hossein Yazdi, M. Effects of linseed oil supplementation in Holstein dairy calves received starters based on either corn or barley grain on growth performance and immune response. *J. Anim. Physiol. Anim. Nutr.* **2023**, *107*, 329–339. [CrossRef] [PubMed]
47. El-Saadany, A.S.; El-Barbary, A.M.; Shreif, E.Y.; Elkomy, A.; Khalifah, A.M.; El-Sabrout, K. Pumpkin and garden cress seed oils as feed additives to improve the physiological and productive traits of laying hens. *Ital. J. Anim. Sci.* **2022**, *21*, 1047–1057. [CrossRef]
48. Jackson, P.G.G.; Cockcroft, P.D. *Clinical Examination of Farm Animals*; Blackwell Science Ltd.: Hoboken, NJ, USA, 2002; pp. 302–305.
49. Lee, S.M.; Kyum Kim, H.; Lee, H.B.; Kwon, O.D.; Lee, E.B.; Cho, C.S.; Choi, Y.J.; Kang, S.K. Effects of flaxseed supplementation on omega-6 to omega-3 fatty acid ratio, lipid mediator profile, proinflammatory cytokines and stress indices in laying hens. *J. Appl. Anim. Res.* **2021**, *49*, 460–471. [CrossRef]

50. Wijffels, G.; Sullivan, M.L.; Stockwell, S.; Briscoe, S.; Pearson, R.; Li, Y.; Macs, A.M.; Sejian, V.; McCulloch, R.; Olm, J.C.W.; et al. Comparing the responses of grain-fed feedlot cattle under moderate heat load and during subsequent recovery with those of feed-restricted thermoneutral counterparts: Blood cells and inflammatory markers. *Int. J. Biometeorol.* **2024**, *68*, 211–227. [CrossRef]
51. Jain, N.C. *Essentials of Veterinary Hematology*; Lea & Febiger: Philadelphia, PA, USA, 1993; p. 417.
52. Souza, D.F.; Paula, E.F.E.; Fernandes, S.R.; Regonato Franco, D.; Oliveira Koch, M.; Locatelli-Dittrich, R.; Barros Filho, I.R.; Gomes Monteiro, A.L. Dynamics of hematological parameters in female lambs during the first four months of life. *Semin. Ciências Agrárias Londrina* **2018**, *39*, 2465–2476. [CrossRef]
53. Grundy, S.M. Cholesterol: Factors Determining Blood Levels. In *Encyclopedia of Human Nutrition*, 3rd ed.; Caballero, B., Ed.; Academic Press: Cambridge, MA, USA, 2013; pp. 335–340.
54. Hossein Abadi, M.; Ghoorchi, T.; Amirteymouri, E.; Poorghasemi, M. The effect of different processing methods of linseed on growth performance, nutrient digestibility, blood parameters and ruminate behaviour of lambs. *Vet. Med. Sci.* **2023**, *9*, 1771–1780. [CrossRef]
55. Huerta, A.E. Role of Omega-3 Fatty Acids in Metabolic Syndrome. In *Omega-3 Fatty Acids. Keys to Nutritional Health*; Hegdje, M.W., Zanwar, A.A., Adekar, S.P., Eds.; Springer International Publishing: Cham, Switzerland, 2016; p. 198.
56. Ockenden, E.M.; Russo, V.M.; Leury, B.J.; Giri, K.; Wales, W.J. Preweaning nutrition and its effects on the growth, immune competence and metabolic characteristics of the dairy calf. *Animals* **2023**, *13*, 829. [CrossRef]
57. Mohapatra, A.; De, K.; Kumar Saxena, V.; Kumar Mallick, P.; Devi, I.; Singh, R. Behavioral and physiological adjustments by lambs in response to weaning stress. *J. Vet. Behav.* **2021**, *41*, 47–51. [CrossRef]
58. Khan, M.A.; Lee, H.J.; Lee, W.S.; Kim, H.S.; Ki, K.S.; Hur, T.Y.; Suh, G.H.; Kang, S.J.; Choi, Y.J. Structural growth, rumen development, and metabolic and immune responses of Holstein male calves fed milk through step-down and conventional methods. *J. Dairy Sci.* **2007**, *90*, 3376–3387. [CrossRef]
59. Qugley, J.D.; Caldwell, L.A.; Sinks, D.; Heitmann, R.N. Changes in blood glucose, nonesterified fatty acids, and ketones in response to weaning and feed intake in young calves. *J. Dairy Sci.* **1991**, *74*, 74250–74257. [CrossRef] [PubMed]
60. Deelen, S.M.; Leslie, K.E.; Steele, M.A.; Eckert, E.; Brown, H.E.; DeVries, T.J. Validation of a calf-side β-hydroxybutyrate test and its utility for estimation of starter intake in dairy calves around weaning. *J. Dairy Sci.* **2016**, *99*, 7624–7633. [CrossRef] [PubMed]
61. Yusuf, A.O.; Ajayi, T.O.; Ajayi, O.S.; Yusuf, O.A. Nutritional manipulation in goats: Supplementation of high protein concentrate, effect on performance and resilience of internal parasites. *Niger. J. Anim. Prod.* **2019**, *46*, 193–201. [CrossRef]
62. Osman, O.A.; Elkhair, N.M.; Abdoun, K.A. Effects of dietary supplementation with different concentration of molasses on growth performance, blood metabolites and rumen fermentation indices of Nubian goats. *BMC Vet. Res.* **2020**, *16*, 411. [CrossRef]
63. Souza, D.F.; Reijers, T.S.S.S.; Gilaverte, S.; Cruz, T.A.; Hentz, F.; Castilhos, B.Q.; Dittrich, R.L.; Monteiro, A.L.G. Dynamics of biochemical parameters in lambs during the first four months of life. *Rev. Bras. Zootec.* **2020**, *49*, e20190167. [CrossRef]
64. Santos, R.P.; Lima Macedo, G.J.; Silva, P.S.; Fernandes de Sousa, L.; Barbosa Andrade, M.E. Inclusion of propylene glycol in the diet of sheep and its effect on their lambs' protein and mineral metabolites. *Acta Sci. Anim. Sci.* **2017**, *39*, 297–302. [CrossRef]
65. Makni, M.; Fetoui, H.; Gargouri, N.K.; Garoui, M.; Jaber, H.; Makni, J.; Boudawara, T.; Zeghal, N. Hypolipidemic and hepatoprotective effects of flax and pumpkin seed mixture rich in ω-3 and ω-6 fatty acids in hypercholesterolemic rats. *Food Chem. Toxicol.* **2008**, *46*, 3714–3720. [CrossRef] [PubMed]
66. Nkosi, C.Z.; Opoku, A.R.; Terblanche, S.E. Effect of pumpkin seed (*Cucurbita pepo*) protein isolate on the activity levels of certain plasma enzymes in CCl4-induced liver injury in low-protein fed rats. *Phytother. Res.* **2005**, *19*, 341–345. [CrossRef]
67. Nkosi, C.Z.; Opoku, A.R.; Terblanche, S.E. In Vitro antioxidative activity of pumpkin seed (*Cucurbita pepo*) protein isolate and its In Vivo effect on alanine transaminase and aspartate transaminase in acetaminophen-induced liver injury in low protein fed rats. *Phytother. Res.* **2006**, *20*, 780–783. [CrossRef]
68. He, P.; Hua, H.; Tian, W.; Zhu, H.; Liu, Y.; Xu, X. Holly (*Ilex latifolia* Thunb.) polyphenols extracts alleviate hepatic damage by regulating ferroptosis following diquat challenge in a piglet model. *Front. Nutr.* **2020**, *7*, 604328. [CrossRef]
69. Peng, K.; Shirley, D.C.; Xu, Z.; Huang, Q.; McAllister, T.A.; Chaves, A.V.; Acharya, S.; Liu, C.; Wang, S.; Wang, Y. Effect of purple prairie clover (*Dalea purpurea* Vent.) hay and its condensed tannins on growth performance, wool growth, nutrient digestibility, blood metabolites and ruminal fermentation in lambs fed total mixed rations. *Anim. Feed. Sci. Technol.* **2016**, *222*, 100–110. [CrossRef]
70. Śpitalniak-Bajerska, K.; Szumny, A.; Pogoda-Sewerniak, K.; Kupczyński, R. Effects of n-3 fatty acids on growth, antioxidant status, and immunity of preweaned dairy calves. *J. Dairy Sci.* **2020**, *103*, 2864–2876. [CrossRef] [PubMed]
71. Gangal, S. Modulation of immune response by omega-3 in health and disease. In *Omega-3 Fatty Acids. Keys to Nutritional Health*; Hegdje, M.W., Zanwar, A.A., Adekar, S.P., Eds.; Springer International Publishing: Cham, Switzerland, 2016; p. 308.
72. Sembratowicz, I.; Zięba, G.; Cholewinska, E.; Czech, A. Effect of dietary flaxseed oil supplementation on the redox status, haematological and biochemical parameters of horses' blood. *Animals* **2020**, *10*, 2244. [CrossRef] [PubMed]
73. Al-Madhagy, S.; Ashmawy, N.S.; Mamdouh, A.; Eldahshan, O.A.; Farag, M.A. A comprehensive review of the health benefits of flaxseed oil in relation to its chemical composition and comparison with other omega-3-rich oils. *Eur. J. Med. Res.* **2023**, *28*, 240. [CrossRef] [PubMed]
74. Barda, S.; Turki, M.; Khedir, S.B.; Mzid, M.; Rebai, T.; Ayadi, F.; Sahnoun, Z. The Effect of prickly pear, pumpkin, and linseed oils on biological mediators of acute inflammation and oxidative stress markers. *BioMed Res. Int.* **2020**, *5643465*, 11. [CrossRef]

75. Domínguez-López, I.; Arancibia-Riveros, C.; Casas, R.; Tresserra-Rimbau, A.; Razquin, C.; Martínez-González, M.Á.; Hu, F.B.; Ros, E.; Fitó, M.; Estruch, R.; et al. Changes in plasma total saturated fatty acids and palmitic acid are related to pro-inflammatory molecule IL-6 concentrations after nutritional intervention for one year. *Biomed. Pharmacother.* **2022**, *150*, 113028. [CrossRef]
76. Chen, S.J.; Yen, C.H.; Huang, Y.C.; Lee, B.J.; Hsia, S.; Lin, P.T. Relationships between inflammation, adiponectin, and oxidative stress in metabolic syndrome. *PLoS ONE* **2012**, *7*, e45693. [CrossRef]
77. Li, P.; Li, L.; Zhang, C.; Cheng, X.; Zhang, Y.; Guo, Y.; Long, M.; Yang, S.; He, J. Palmitic acid and β-hydroxybutyrate induce inflammatory responses in bovine endometrial cells by activating oxidative stress-mediated NF-κB signaling. *Molecules* **2019**, *24*, 2421. [CrossRef]

agriculture

MDPI

Article

The Effect of Grape Seed Cake as a Dietary Supplement Rich in Polyphenols on the Quantity and Quality of Milk, Metabolic Profile of Blood, and Antioxidative Status of Lactating Dairy Goats

Zvonko Antunović [1], Josip Novoselec [1], Željka Klir Šalavardić [1], Zvonimir Steiner [1], Mato Drenjančević [1], Valentina Pavić [2,*], Mislav Đidara [1], Mario Ronta [1], Lidija Jakobek Barron [3] and Boro Mioč [4]

[1] Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, V. Preloga 1, 31000 Osijek, Croatia; zantunovic@fazos.hr (Z.A.); jnovoselec@fazos.hr (J.N.); zeljka.klir@fazos.hr (Ž.K.Š.); zsteiner@fazos.hr (Z.S.); mato.drenjancevic@fazos.hr (M.D.); mdidara@fazos.hr (M.Đ.); mronta@fazos.hr (M.R.)

[2] Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8, 31000 Osijek, Croatia

[3] Faculty of Food and Technology Osijek, Josip Juraj Strossmayer University of Osijek, V. Preloga 1, 31000 Osijek, Croatia; lidija.jakobek@ptfos.hr

[4] Department of Animal Science and Technology, Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, 10000 Zagreb, Croatia; bmioc@agr.hr

* Correspondence: vpavic@biologija.unios.hr; Tel.: +38-5912241413

Abstract: The objective of this study was to assess the impact that diets supplemented with grape seed cake rich in polyphenols had on lactating goats. The study investigated the quantity and quality of goat milk, the metabolic profile of blood, and the antioxidative status. The study involved 24 French Alpine dairy goats throughout their lactation period. The goats were, on average, 5 years old (±three months) and in the fourth lactation. The experiment lasted for 58 days. The control group (CON) had a diet without grape seed cake (GSC). The experimental groups were given a diet containing 5% and 10% GSC on a dry matter basis (GSC5 and GSC10, respectively). A slightly higher milk production, as well as protein and fat milk content, were found in GSC5 and GSC10, but the differences were not significant. Goat milk in the GSC10 group exhibited significantly higher activity of superoxide dismutase and glutathione reductase, as well as decreased concentrations of GUK and SCC. The feeding treatments did not affect significant differences in hematological and biochemical indicators, except for the BHB content, which can be associated with a higher energy value of feed containing GSC. There was an observed elevation in the activity of SOD within the blood of GSC5, and GSC10 was measured as well. The determined changes justify the supplementation of GSC rich in polyphenols to goat feed, especially in the amount of 10%, as it can reduce stress caused by lactation, which is known as a very stressful production period for animals.

Keywords: grape seed cake; goat; milk; blood; antioxidative status; blood metabolic profile

Citation: Antunović, Z.; Novoselec, J.; Klir Šalavardić, Ž.; Steiner, Z.; Drenjančević, M.; Pavić, V.; Đidara, M.; Ronta, M.; Jakobek Barron, L.; Mioč, B. The Effect of Grape Seed Cake as a Dietary Supplement Rich in Polyphenols on the Quantity and Quality of Milk, Metabolic Profile of Blood, and Antioxidative Status of Lactating Dairy Goats. *Agriculture* **2024**, *14*, 479. https://doi.org/10.3390/agriculture14030479

Academic Editors: Tatiana Dumitra Panaite and Mihaela Hăbeanu

Received: 10 February 2024
Revised: 9 March 2024
Accepted: 14 March 2024
Published: 15 March 2024

1. Introduction

Lactating dairy goats play a crucial role in providing milk, a valuable source of nutrition for human consumption. However, the physiological demands of lactation can often lead to stress and metabolic challenges for these animals. Investigating nutritional strategies to improve the production and well-being of lactating goats has drawn more attention in recent years [1]. Polyphenols found abundantly in various plant sources have been recognized for their antioxidant properties and potential health-promoting effects [2]. Grape seed cake, a by-product of the winemaking industry, is rich in polyphenols and has been increasingly considered as a potential feed supplement for livestock [3,4].

Grapevine (*Vitis vinifera* L.) ranks among the most widely cultivated crops globally, primarily renowned for its nutritional benefits, largely attributed to its abundant polyphenol content [5]. Global wine production reached 260 million hectoliters in 2021 [6], causing millions of tonnes of by-products to be produced each year. Mediterranean countries such as Italy, France, and Spain are prominent contributors to this production. In Croatia, wine production amounted to approximately 726,000 hectoliters in 2022. The processing of grapes results in various by-products, one of which is grape seeds. The wine industry's by-products frequently contain beneficial nutrients and chemicals that can be used as additives for livestock feed, thereby reducing waste and promoting resource efficiency [7,8].

The share of seeds in grape pomace amounts to 15–52% of DM [9]. Grape seeds are complex in structure; their chemical composition usually depends on ecological conditions (growing and harvesting of grapes, etc.). According to Bucić-Kojić et al. [10], grape seeds contain around 40% fibre (with a significant share of cellulose), 16% essential oils, 11% protein, 7% polyphenols (flavonols, flavanols, anthocyanins, phenolic acids, and resveratrol) and other compounds (minerals, sugars, and neo-phenolic antioxidant-β-carotene). The most common minerals in grape seeds are iron (Fe) and copper (Cu), but their use in food is limited because of the high levels of lignin, acid detergent fibre (ADF), and neutral detergent fibre (NDF). In addition to the above-mentioned ingredients, grape seeds are rich in vitamin E, which contributes to their significant antioxidant activity [11]. The food, cosmetic, and pharmaceutical industries regularly utilize grape seeds [12]. In recent years, grape seeds have also been processed for oil extraction. After crushing the grape seeds for oil extraction, the resulting by-product, known as grape seed cake (GSC), can be utilized as a nutritional supplement in human nutrition. More recently, GSC as a by-product has been used in animal feeding as well. Although the potentials of GSC supplementation to animal feed are still not fully recognized, farmers are slowly starting to consider GSC as a dietary supplement because of its nutritional value and rich source of natural antioxidants. Lutterodt et al. [13] determined that total phenol content was 100 times lower in the oils than in grape seed flour, thus confirming it to be a valuable source of total phenols. Incorporating large quantities of grape by-products into the diet of monogastric animals may negatively impact growth features since anti-nutritional substances have been present [3,14]. There are few studies referring to the use of grape seed cake in the feeding of lactating ruminants, yet none of them are conducted on lactating goats. Alba et al. [15] investigated the impact of supplementing sheep diet with grape residue flour (GRF) at levels of 0%, 1%, and 2%. They found no effect on the produced quantities and composition of milk despite the fact that the experimental sheep groups produced a greater amount of milk than the control. However, the indicators of antioxidative status were better in the experimental groups. Correddu et al. [16] pointed out that feeding ruminants with agro-industrial by-products, including grape by-products, has many advantages. These include reducing storage and disposal costs, adding value to dairy products by improving their quality, enhancing animal health by enriching animal's diets with polyphenols, and contributing to the preservation of environmental biodiversity.

Lactation is a highly demanding and stressful period for animals, often disrupting homeostasis maintenance and leading to the occurrence of various diseases. In order to prevent such conditions during highly demanding production phases, various antioxidants are often added to animal feed. The purpose of this study is to establish the feasibility and efficacy of using grape seed cake, rich in polyphenols, as a dietary supplement for lactating goats and to assess its effects on the quantity and quality of milk, metabolic profile and antioxidative status of goats. Utilizing industry by-products, such as grape seed cake, in animal nutrition presents a promising avenue for advancing both animal welfare and sustainable agricultural practices. Furthermore, integrating industry by-products into animal nutrition strategies can contribute to the development of circular economies within the agricultural sector [17].

2. Materials and Methods

2.1. Experimental Design and Bioethics Standard

This study involved 24 French Alpine lactating goats at a small-scale family-operated farm in Osijek-Baranja County (Croatia). The selected goats were approximately 5 years of age (±three months), in the fourth lactation. Every goat was in good health and physical condition, had a starting body weight of 49.4 kg, and morning milk production was 1.53 kg. A herd of fifty goats was used to select the goats, and all goats had given birth within one week. The trial started on day 40 after kidding, with an adaptation period of eight days for experimental nutrition. According to the dietary treatment, three groups were formed, with eight goats in each. Goats were kept in a barn, and milking was completed in a separate parlour. The trial lasted for 58 days, during which goats were weighed at the beginning of the experiment (the preparatory period lasted for 7 days) on days 1, 29, and 58. On the same days, goats' body measures were recorded, and milk and blood samples were collected. According to Santucci and Maestrini [18], the body condition score (BCS) was measured on a 1-to-5-point scale, with the intervals between 1 (thin) and 5 (obesity) being 0.25. Every employee handling live goats had the necessary training and education. The Faculty of Agrobiotechnical Sciences Osijek's Committee for Animal Welfare gave the study their approval (644-01/22-01/03 from 30 June 2022). The declaration of Helsinki and the legislative guidelines established by the Animal Protection Act (the Republic of Croatia Official Gazette Nos. 133 (2006), No. 37 (2013) and No. 125 (2013)) were followed throughout.

2.2. Feed and Analysis of Feedstuffs

The goats were fed with a feed mixture offered twice a day (during machine milking), individually (1.5 kg per day) in separate feeding troughs, according to NRC [19]. In addition, goats were fed with alfalfa hay ad libitum. The ratio of the voluminous and concentrated parts of the diet was 60:40. The diet was isonitrogenous and isoenergetic. Kids were removed from the mothers 24 h prior to each milk sample collection day (1st, 29th, and 58th day of trial). The ratio was formulated according to body weight before the experiment started, 49.4 kg, and morning milk production of 1.53 kg. Diets differed in the amount of grape seed cake (GSC) supplemented to the feed mixture. The pomace of Cabernet franc (*Vitis vinifera* L.) grapes was purified after maceration lasting for 5 days. Seeds were separated in a separating machine manufactured by Crystal-Mezőtúr Kft., model S800. After separation, seeds were washed of impurities and dried for 10 h at a temperature of 50 °C. After drying, seeds were pressed in the Komet oil press, model CA59G, at a screw conveyor temperature of 80 °C, screw conveyor diameter of 10 mm, and frequency of 60°/min, with the aim of separating the oil. The residue (grape seed cake) was ground in the Albrigi Luigi hammer mill. A sieve with holes of 1.5 mm diameter was used to grind the cake. The ground grape seed cake was used as a supplement in the feed mixture for goats.

The control diet did not contain GSC. In the first experimental group (GSC5), grape seed cake was added to the feed mixture in the amount of 5%/DM. In the second experimental group (GSC10), grape seed cake was added to the feed mixture in the amount of 10%/DM. Grape seed cake was added to the feed mixture of group GSC5 in the amount of 75 g/day and to the feed mixture of GSC10 in the amount of 150 g/day. Feed mixture, hay, and GSC were dehydrated and pulverized by using an ultra-centrifugal mill (heavy-metal-free). To ascertain feed composition, AOAC [20] standard procedures were applied. The raw material and chemical composition of the feed are presented in Table 1.

Table 1. Ingredients and chemical composition of feed mixture, grape seed cake, and hay.

Ingredient (g/kg Feed Mixture)	Feed Mixture			GSC	Hay
	CON	GSC5	GSC10		
Corn	487	569	553		
Oat	100	100	100		
Wheat flour	100	-	-		
Soybean meal (46% CP)	179	197	199		
Soybean hulls	100	50	-		
Grape seed cake	-	50	100		
Fat	-	-	14		
Salt	4	4	4		
Mineral vitamin premix [1]	30	30	30		
Chemical content (g/kg DM)					
DM	889	888	889	909	900
Crude protein	165	161	159	119	143
Crude fibre	54	51	53	422	293
Crude ash	54	54	51	32	61
EE	28	29	38	46	10
NDF	386	273	367	614	686
ADF	85	79	88	501	395
ADL	21	8.6	37	422	68
ME (MJ/kg DM)	11.36	11.20	11.21	3.50	7.21
Polyphenols (total), mg/kg	56.06	418.22	812.06	5065.36	-
Anthocyanins (total), mg/kg	144.23	155.80	240.46	105.53	-

CON control group; GSC5: grape seed cake 5%, GSC10: grape seed cake 10%; DM: dry matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; ME: metabolic energy; [1] The mineral vitamin premix1 is composed of 21% calcium, 5% phosphorus, 6% sodium, 5% magnesium, 1,200,000 IU/kg vitamin A, 140,000 IU/kg vitamin D3, 3500 mg/kg vitamin E, 600 mg/kg iron sulphate monohydrate, 490 mg/kg copper sulphate pentahydrate, 500 mg/kg copper (in the form of chelates), 5 mg/kg manganese sulphate pentahydrate, 6500 mg/kg zinc oxide, 1500 mg/kg zinc (in the form of chelates), 60 mg/kg iodine in the form of anhydrous calcium iodate, 40 mg/kg cobalt carbonate monohydrate, and 50 mg/kg selenium selenite.

All feed samples (grape seed cake, alfalfa hay, and feed mixture) were dried before being processed into a powder in a knife mill (GM 200, Retsch GmbH, Haan, Germany) or heavy metal-free ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany). The Association of Official Analytical Chemists'-established procedures were used to determine the feed composition [20]. Table 1 displays the ingredients and chemical compositions of the diets. The Kjeldahl method was used to assess the crude protein content of the feed using a Kjeldahl steam distillation apparatus (Behr, Stuttgart, Germany). The universal extraction system B-811 (Buchi, Flawil, Switzerland) was used to estimate the ether extract. The Weende technique was used to calculate the crude fibre content, and INRAE-CIRAD-AFZ was used to determine the ME [21]. Using the instruments and methods outlined by Jakobek et al. [22], the total polyphenols in the feed were extracted and determined. The same procedure was repeated to obtain three extracts of feed. The total polyphenol concentration was presented as mg of gallic acid equivalents (GAE) per kg of sample weight. Total anthocyanins and polyphenols analyses were carried out by using a Shimadzu UV-1280 spectrophotometer (Shimadzu Europe GmbH) following the method by Jakobek et al. [22].

2.3. Milk Sampling and Analysis

During routine milking on experiment days 1, 29, and 58, three samples of milk were obtained from each goat at 7:00 a.m. None of the goats had mastitis throughout the experiment, as observed by forestripping before each milk sampling. One of these two milk samples obtained from each goat was transferred into bottles (30 mL) and cooled to 4 °C. The milkoScan FT 6000 analyzer (Foss Electric, Hillerød, Denmark) was used to obtain the

chemical composition of milk following HRN ISO 9622:2017. The following equation was used to calculate fat-corrected milk at 3.5% (FCM, kg/day; [23]):

$$\text{FCM} = \text{milk yield} \times (0.634 + 0.1046 \times \text{fat}) \quad (1)$$

A Fossomatic 5000 Analyser (Foss Electric, Hillerød, Denmark) was used to determine somatic cell count (SCC) with fluoro-opto-electronic method (HRN ISO 13366-2/Ispr.1:2007). According to Wiggans and Shook [24], the results were transformed to log values via the following equation:

$$\text{SCC} = 3 + \log2(\text{SCC}/100{,}000) \quad (2)$$

In order to determine biochemical indicators, fresh milk was centrifuged at 5000× *g* for 30 min to separate the fat, and until analysis, the milk plasma was stored at −80 °C. Biochemical markers, such as ALT (alanine aminotransferase), GGT (γ-glutamyl transferase) and AST (aspartate aminotransferase), GR (glutathione reductase), urea, albumin, glucose, and TP (total protein)) were determined using a biochemical analyser Olympus AU 400 (Olympus, Tokyo, Japan). Glutathione peroxidase (GPx) activity in milk was determined by Ransel® kit (Randox, Crumlin, UK) and SOD activity by Ransod® kit (Randox Laboratories, Crumlin, UK).

Milk antioxidant compounds were extracted in three replicates using a slightly modified method, according to Alyaqouba et al. [25]. Briefly, 10 parts of extraction solvent (1 N HCl/95% ethanol (*v*/*v*, 15/85)) were used for 1 part of milk and shaken for 1 h using a rotary shaker (Brunswick™ Innova® 43R Console Incubator Shaker, Eppendorf AG, Hamburg, Germany) at 30 °C and 300 rpm, while protected from light. The supernatants separated after centrifugation at 4400× *g* and 5 °C for 40 min (Hermle Z 326 K, Hermle Labortechnik GmbH, Wehingen, Germany) and were then stored at −20 °C until further analysis. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the milk extracts was assessed in three replicates using a modified method [26]. Briefly, 9 parts of methanolic solution of DPPH radical at 0.2 mM were mixed with 1 part of milk extract and left at room temperature for 30 min in the dark after being shaken vigorously. In brief, 9 parts of a 0.2 mM methanol DPPH radical solution and 1 part of milk extracts were combined and, after being vigorously shaken, were allowed to sit at room temperature for 30 min in the dark. Utilizing a spectrophotometer (Lambda 25, Perkin Elmer, Waltham, MA, USA), absorbance was measured at 517 nm. Equation (3) was used in order to compute the DPPH scavenging activity:

$$\text{DPPH scavenginming activity (\%)} = ((\text{Ab} + \text{As}) - \text{Am})/\text{Ab} \times 100)) \quad (3)$$

where Ab is the negative control absorbance, As is the milk sample control absorbance, and Am is the tested milk extracts and DPPH radical absorbance.

The method used for Thiobarbituric Acid Reactive Substances measurement was adapted from Oancea et al. [27] and Sun et al. [28]. To remove proteins, a mixture of 1 part of 0.1% trichloroacetic acid (TCA) and milk samples (3 parts) was prepared. The supernatants were collected and incubated with TBA/TCA reagent (0.5% thiobarbituric acid—TBA in 20% TCA) in a 2:1 ratio for 90 min at 80 °C, after centrifugation at 3000× *g* for 5 min at 4 °C (Hermle Z 326 K, Hermle Labortechnik GmbH, Wehingen, Germany). The incubation was followed by cooling on ice. Absorbance readings were taken at different wavelengths: 450 nm for saturated aldehydes, 532 nm for malondialdehyde (MDA), and 600 nm for non-specific absorption. Results were then calculated by subtracting non-specific absorbance at 600 nm and expressed as absorbance values at 450 nm ($TBARS_{450}$).

2.4. Blood Sampling and Analysis

Samples of blood (10 mL) were drawn from each goat's jugular vein for hematological analysis, and they were placed into sterile Venoject® (Sterile Terumo Europe, Leuven, Belgium) vacuum tubes that contained EDTA (ethylenediamine tetra-acetic acid). After

collection, blood samples were set on ice and stored in chilled conditions (0–6 °C). To ascertain the hematological parameters in goat whole blood (WBC (number of leukocytes), RBC (erythrocytes), HGB (the content of hemoglobin), HCT (hematocrit), MCV (mean corpuscular volume), MCH (average hemoglobin content in erythrocytes), and MCHC (mean hemoglobin concentration in erythrocytes)), the three-part differential veterinary hematology analyzer (Sysmex PocH-100iV, Sysmex Europe GmbH, Norderstedt, Germany) was utilized.

Following that, blood samples that had been obtained in sterile vacuum tubes devoid of EDTA were centrifuged using a centrifuge ROTOFIX 32A (Hettich GmbH & Co. KG, Tuttlingen, Germany) at 1609.92× *g* for ten minutes. The obtained serum samples were placed into the Olympus AU400. ALB (albumin), CHOL (cholesterol), BHB (β-hydroxybutyrate), TGC (triglycerides), GUK (glucose), PROT (total proteins), ALB (low-density lipoprotein), HDL (high-density lipoprotein), NEFA (non-esterified fatty acids), and minerals (calcium, magnesium, phosphorus, and iron) were among the biochemical parameters determined in the serum. Enzyme activities were measured for GGT (γ-glutamyl transferase), AST (aspartate aminotransferase), ALT (alanine aminotransferase), and GR (glutathione reductase) by using Olympus System reagents (Olympus Diagnostic GmbH, Ballymount, Ireland). The difference between total protein and albumin was expressed as globulin (GLOB) content. The GPx in the serum was determined using a Ransel® kit (Randox, Crumlin, UK), and SOD in serum was determined using a Ransod® kit (Randox, Crumlin, UK) on an Olympus AU 400 analyzer (Olympus, Tokyo, Japan).

2.5. Statistical Analyses

Mean values for milk performance, as well as hematological and antioxidative status, were estimated for each goat and for each time of sampling. These values were then subjected to a repeated-measure analysis using PROC MIXED (SAS 9.4; [29]), with the following model: $Y_{ijk} = \mu + d_i + h_{ij} + w_k + dw_{ik} + e_{ijk}$, where μ means overall mean, d_i means fixed effect of diet (i = C, GSC5, GSC10), h_{ij} means animal within diet as subject (j = C, GSC5, GSC10), w_k means fixed effect of sampling time in lactation (k = 1–3), d_{wik} means interaction between diet and sampling time (diet × sampling), and e_{ijk} means residual error. Diet × sampling time interactions were considered fixed effects. Mean values were compared with the Tukey significant difference test, where $p < 0.05$ indicated significant differences.

3. Results

Data on recorded body weight, body condition score, milk production, and composition of lactating dairy goats fed a diet enriched with varying amounts of grape seed cake are shown in Table 2.

Analysis of data presented in Table 2 confirmed a slightly higher amount of milk, as well as protein and fat content, in milk produced by goats that were fed diets supplemented with GSC; however, those differences were not significant. When comparing the milk of GSC10 goats to the control (CON) and GSC5 groups, the SCC was significantly reduced. Furthermore, when compared to the CON group, GSC5 and GSC10 milk had a significantly lower concentration of GUK. A significant influence on the periods of milk sampling was also determined for the concentration of ALB, and there was also a significant interaction of D × P for concentrations of TP, ALB, and ALT and AST activities in goat milk.

Table 3 provides an overview of the hematological parameters of dairy goats fed diets with different amounts of GSC.

The content of hematological indicators was not affected by the supplementation of GSC to goats' diets.

Analysis of the data presented in Table 4 confirmed a significant decrease only in the concentrations of BHB in experimental GSC10 groups as well as a non-significant increase in the concentrations of ALB, TGC, and CHOL, HDL, and LDL cholesterol, and a decrease

in the concentration of Fe in blood in GSC groups. A significant influence of the lactation stage was determined for concentrations of Ca, TP, TGC, and NEFA in goats' blood.

Table 2. Milk yield and composition and production traits of goats fed diets supplemented with GSC.

	Diets			SEM	*p* Value		
	CON	**GSC5**	**GSC10**		**D**	**S**	**D × S**
Milk yield (kg)	1.34	1.63	1.59	0.075	0.214	0.094	0.843
Body weight (kg)	50.02	49.57	47.74	0.881	0.588	0.982	0.986
BCS (point)	2.61	2.53	2.45	0.053	0.518	0.094	0.534
Milk composition (g/100 g)							
Fat	3.00	3.14	3.27	0.105	0.572	0.420	0.901
Protein	2.77	2.98	3.02	0.052	0.157	0.796	0.946
Lactose	4.29	4.40	4.39	0.023	0.088	0.100	0.174
Non-fat dry matter	8.25	8.31	8.49	0.050	0.146	0.470	0.763
AST (U/L)	18.70	21.56	26.22	1.674	0.083	0.095	0.024
ALT (U/L)	2.87	4.14	7.40	4.109	0.051	0.076	<0.001
GGT (U/L)	286.86	334.85	329.55	14.134	0.223	0.621	0.066
ALB (g/L)	12.62	11.65	11.40	0.645	0.732	0.027	0.021
TP (g/L)	18.64	17.93	18.30	0.741	0.947	0.054	0.015
GUK (mmol/L)	0.28 ^{a}	0.19 ^{b}	0.16 ^{b}	0.014	0.001	0.460	0.377
Urea (mmol/L)	8.95	8.65	8.45	0.274	0.834	0.174	0.010
SCC (log)	5.95 ^{a}	5.85 ^{a}	5.15 ^{b}	0.077	<0.001	0.052	0.411

^{a,b} Means in rows with different letters differ significantly ($p < 0.05$). CON: control group; GSC5: grape seed cake 5%, GSC10: grape seed cake 10%; D: diet, S: time of sampling, D × S: interaction (diet × sampling); SEM: standard error of mean; BCS: body condition score; TP: total proteins; ALB: albumins; GUK: glucose; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-glutamyl transferase; SCC: somatic cells count.

Table 3. Hematological parameters of goats fed diets with different amounts of GSC.

Parameter	Diets			SEM	*p* Value			*Reference Values* *
	CON	**GSC5**	**GSC10**		**D**	**S**	**D × S**	
RBC ($\times 10^{12}$ L)	8.47	9.47	9.20	0.283	0.305	0.831	0.672	8.00–18.00
WBC ($\times 10^{9}$ L)	10.06	9.61	10.48	0.422	0.733	0.563	0.803	4.00–13.00
HGB (g/L)	67.09	78.76	71.95	2.078	0.073	0.892	0.723	80.00–120.00
HCT (L/L)	0.24	0.27	0.26	0.006	0.222	0.869	0.624	0.22–0.38
MCH (pg)	8.27	8.37	7.85	0.135	0.230	0.052	0.796	5.20–8.00
MCV (fL)	30.10	28.71	28.57	0.486	0.262	0.059	0.328	16.00–25.00
MCHC (g/L)	276.39	293.10	275.29	3.443	0.068	0.357	0.835	300.00–360.00

CON: control group; GSC5: grape seed cake 5%; GSC10: grape seed cake 10%; D: diet; S: time of sampling; D × S: interaction (diet × sampling); SEM: standard error of mean; RBC: erythrocytes; WBC: number of leukocytes; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: average hemoglobin content in erythrocytes; MCHC: mean hemoglobin concentration in erythrocytes; * [30].

The activities of blood enzymes did not vary depending on feeding treatment, except for SOD activity, which was significantly increased in GSC5 and GSC10 (Table 5). The milk sampling period had a significant influence on GPx activity in goats' blood.

The $TBARS_{450}$ content and DPPH scavenging activity did not differ significantly when influenced by the feeding treatment, although the $TBARS_{450}$ content was decreased in the milk of goats fed diets supplemented with GSC (Table 6). Milk from GSC10 goats had a significantly higher activity of SOD. Furthermore, when compared to goat milk from C and GSC5, milk from GSC10 had a significantly higher activity of GR. The milk sampling period had a significant influence on DPPH scavenging as well as the activities of SOD and GR.

Table 4. Blood biochemical parameters in goats fed diets with different amounts of GSC.

Parameter (mmol/L)	Diets			SEM	*p* Value			*Reference Values* *
	CON	GSC5	GSC10		D	S	D × S	
Urea	9.28	9.35	9.57	0.215	0.870	0.575	0.714	4.00–8.60
TP (g/L)	70.44	74.97	73.58	1.332	0.820	<0.001	0.232	62.00–79.00
ALB (g/L)	25.20	28.50	26.52	0.563	0.060	0.783	0.838	29.00–43.00
GLOB (g/L)	45.23	46.47	47.06	0.906	0.701	0.362	0.740	35.00–57.00 [1]
Ca	2.21	2.18	2.10	0.028	0.221	0.017	0.503	2.30–2.90
P-inorganic	3.29	3.26	3.39	0.098	0.286	0.056	0.550	1.00–2.40
Mg	1.44	1.50	1.41	0.023	0.286	0.056	0.550	0.80–1.30
Fe (µmol/L)	23.60	22.64	16.96	1.233	0.078	0.850	0.609	11.60–38.10 [1]
GUK	4.26	4.23	4.37	0.060	0.619	0.072	0.699	2.40–4.00
CHOL	2.64	3.02	2.84	0.078	0.159	0.938	0.991	1.00–3.00
HDL	1.44	1.70	1.49	0.039	0.616	0.314	0.754	1.05–1.76
LDL	1.11	1.23	1.33	0.070	0.420	0.916	0.906	0.77–1.25
TGC	0.17	0.20	0.21	0.012	0.440	0.022	0.537	0.20
NEFA	0.16	0.19	0.19	0.012	0.410	<0.001	0.392	>0.2 [2]
BHB	0.54 [a]	0.50 [a]	0.41 [b]	0.016	0.003	0.150	0.750	0–1.20

CON: control group; GSC5: grape seed cake 5%; GSC10: grape seed cake 10%; D: diet; S: time of sampling; D × S: interaction (diet × sampling); SEM: standard error of mean; TP: total proteins, ALB: albumins; GLOB: globulins; GUK: glucose; CHOL: cholesterol; HDL: HDL cholesterol; LDL: LDL cholesterol; TGC: triglycerides; NEFA: non-esterified fatty acids; BHB: β-hydroxybutyrate; [a,b] means in rows with different letters differ significantly ($p < 0.05$).* [31]; [1] [32]; [2] [33].

Table 5. Blood enzyme activities of goats fed diets with different amounts of GSC.

Parameter (U/L)	Diets			SEM	*p* Value		
	CON	GSC5	GSC10		D	S	D × S
AST	125.51	121.35	119.76	3.296	0.772	0.725	0.570
ALT	27.34	26.10	26.82	0.895	0.857	0.425	0.974
GGT	46.52	50.17	48.35	1.165	0.466	0.965	0.972
CK	172.87	154.05	166.90	4.719	0.274	0.621	0.953
GPx	768.25	829.09	849.90	23.662	0.330	0.006	0.961
SOD (U/mL)	0.26 [b]	0.48 [a]	0.51 [a]	0.021	0.001	0.126	0.851
GR	90.48	83.90	82.16	2.512	0.336	0.500	0.411

CON: control group; GSC5: grape seed cake 5%; GSC10: grape seed cake 10%; D: diet; S: time of sampling; D × S: interaction (diet×sampling); SEM: standard error of mean; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-glutamyl transferase; CK: creatine kinase; GPx: glutathione peroxidase; SOD: superoxide dismutase; GR: glutathione reductase; [a,b] means in rows with different letters differ significantly ($p < 0.05$).

Table 6. Antioxidant status of milk of goats given diets with various amounts of GSC.

Parameter	Diets			SEM	*p* Value		
	CON	GSC5	GSC10		D	S	D × S
$TBARS_{450}$	0.039	0.036	0.035	0.0017	0.724	0.604	0.233
DPPH scavenging (%)	92.87	92.19	92.14	0.247	0.240	<0.001	0.425
SOD (U/mL)	3.41 [a]	4.31 [ab]	5.30 [b]	0.250	0.002	0.002	0.765
GPx (U/L)	426.64	441.17	472.75	36.043	0.866	0.302	0.200
GR (U/L)	3.37 [a]	3.25 [a]	6.57 [b]	0.426	<0.001	0.047	0.072

CON: control group; GSC5: grape seed cake 5%, GSC10: grape seed cake 10%; D: diet, S: time of sampling, D × S: interaction (diet × sampling); SEM: standard error of the mean; $TBARS_{450}$—Thiobarbituric acid reactive substances; DPPH—radical scavenging activity at final milk concentration 10 uL/mL; SOD—superoxide dismutase; GPx—glutathione peroxidase; GR—glutathione reductase; [a,b] means in rows with different letters differ significantly ($p < 0.05$).

4. Discussion

There is a growing interest of scientists exploring the potential of using grape waste products in the feeding of small ruminants. Flavonoids and proanthocyanidins, as bioactive compounds obtained from wine industry by-products, are used to enhance the immune response. They modulate the immune system of ruminants via binding to proteins, as well as to boost interference at the active site, thus exerting antioxidant effects [34]. Feeding goats with polyphenol-rich diets leads to the presence of proline-rich proteins (PRPs) in their saliva [35], as well as in a better capacity of saliva to bind tannins [36]. In the present research, supplementation of GSC to goats' diets resulted in a slight, nonsignificant increase in the quantity of milk, fat, and protein milk content but a significant decrease in SCC in the milk of the GSC10 group (Table 2). In a study on sheep fed a diet supplemented with 5% grape seeds during mid-pregnancy and lactation, Pascual-Alonso et al. [37] found similar content of milk fat, protein, and somatic cell count. Smaller changes in the lactose content in sheep milk were determined in all feeding treatments. A study by Gessner et al. [38] with dairy cows administered pomace meal extract and grape seeds describes the effect of grape phenols on enhancing the flow of protein into the small intestine, which results in an increase in milk production. Similarly, high doses of winery by-products (grape seeds and pomace) could reduce the breakdown of dietary protein [39]. When compared to CON, significantly higher activity of SOD and reduced SCC were found in the milk from GSC10 goats in the present study. Likewise, when compared to CON and GSC5, GSC10 had a significantly lower concentration of GUK in the milk. Alba et al. [15] supplemented grape residue flour (GRF) in the amount of 1% and 2% (10 and 20 g/kg) to sheep diets and determined a non-significant increase in milk quantity without changes in its composition (except for reduced BSC and increased fat content). The SCC was significantly lowered in the milk from sheep fed diet with 2% grape residue flour (by 18.01%) and nonsignificant lowered in groups fed with 1% (by 10.20%), which was linked to the anti-inflammatory capability caused by the ingredients present in the dietary supplement. The aforementioned changes align with the results obtained in the present study, where a reduction in SCC in milk by 13.5% was determined in the GSC10 group. The reduction in milk GUK in the GSC groups in the present study may be related to slightly higher milk production and higher lactose content in the milk. The reason for the high turnover of glucose in lactating ruminants is that the demand for the udder is high, and glucose is almost the only precursor of lactose [40]. Mokni et al. [41] investigated grape seed and skin supplemented to lactating sheep feed in the amount of 20% and proved increased milk production, but without significant differences in the composition of milk, except for the increased content of urea, Fe and Ca. Resconi et al. [42] did not confirm the influence of grape seeds (5%) in feeding lactating sheep on the quality of milk and meat of sheep and their lambs. Similarly, Manso et al. [43] and Nudda et al. [44] also did not confirm changes in the quantity of milk produced by sheep fed diet with grape pomace. The absence of a significant increase in the amount of milk in the goats of the experimental groups that consumed feed mixtures with added GSC can be connected with similar ME content in feed mixtures (Table 1). Mokni et al. [41] obtained similar results with sheep but with significantly higher milk production influenced by feeding sheep diets supplemented with grape seed and skin (20%). In the present research, a non-significant increase in GPx activity was observed in the milk and blood of goats fed diets supplemented with GSC. Alba et al. [15] also reported increased activity of GPx in sheep blood, which was more pronounced in sheep fed a diet supplemented with 2% GRF, and the authors associated this with the presence of polyphenols in grape residue.

In this research, the determined blood metabolic profile (concentrations of hematological and biochemical indicators) was within reference values [31–33,45,46], with a slight influence on the milk sampling period, which was expected (Tables 3 and 4). We determined a lower content of HGB, MCHC, ALB, and Ca as well as higher value of MCV, urea, and P. The content of hematological indicators was not influenced by GSC supplemented in goats' diets. When feeding sheep diet supplemented with grape pomace, Nudda et al. [47]

obtained similar results for hematochemical indicators in blood. The present research resulted in a non-significant increase in TGC and CHOL concentrations and a significant decrease in BHB in goats' blood, which points out the fact that feeding mixtures of the experimental groups were enriched with GSC ether extract (Table 1). Specifically, it is known that grape seeds have higher polysaccharide content, which can lower serum BHB concentrations and also inhibit the mobilization of adipose tissue and liver glycogen in the GSC10 group [48]. The amount of NEFA in the serum indicates how fat is metabolized, and the goats from the GSC groups' consistent concentration might be connected to the absence of alterations in CHOL. Similar results for blood TGC in investigation with dried pomace feeding of wethers were confirmed by Juráček et al. [49]. In a study on sheep fed a diet supplemented with 5% grape seeds during mid-pregnancy and lactation, Pascual-Alonso et al. [37] found higher concentrations of NEFA and CK activity in the blood. Alba et al. [15] published similar conclusions referring to concentrations of TGC in the blood of sheep fed diets supplemented with grape residue flour. It is very well known that both flavonoids and proanthocyanidins are free radical scavengers; they promote vasodilation by inhibiting phospholipase, cyclooxygenase, and lipoxygenase. They also reduce the peroxidation process of lipids [50]. This research did not confirm the variation in enzyme activities in goats' blood that depended on the feeding treatment, except for SOD activity, which was significantly increased in GSC5 and GSC10 (Table 5). SOD is an enzyme that is essential to the body's defence against oxidative stress because it removes superoxide radicals from inside cells [51]. When feeding sheep diets supplemented with grape residue flour (10 and 20 g/kg), Alba et al. [15] also found that there was an increase in GPx, a decrease in lipid peroxidation, an increase in total antioxidant status (TAS), and enhanced SOD activity in the serum. In this study, the absence of notable alterations in the activity of other antioxidant enzymes in the blood of goats could be linked to the restricted absorption of anthocyanins in small ruminants relative to monogastric animals [52]. Previous studies have shown a high correlation between the oxidation of dairy products and the yellow colour of TBARS at 450 nm, which have been associated with the oxidation of monounsaturated fatty acids [27,53,54]. The objectives of the TBARS analysis were to assess potential lipid oxidation in milk. Milk lipids are susceptible to chemical and physical changes, including autooxidation, oxidation, and the development of trans fatty acids, aldehydes, ketones, and lactones. These changes can have adverse effects on the properties of dairy products, such as undesirable odour, flavour, and colour. By conducting TBARS analysis, it becomes possible to quantify the extent of lipid oxidation, thereby evaluating the quality and stability of dairy products. The TBARS are indeed primary by-products of lipid peroxidation. Previous studies demonstrated that antioxidants, such as polyphenols found in grape extracts or grape pomaces, seeds, skins, and stems, have the capability to regulate and reduce the levels of TBARS [55]. Polyphenols gain their antioxidant properties via scavenging free radicals and preventing lipid peroxidation reactions, thus mitigating the formation of TBARS. Consequently, the incorporation of grape-derived antioxidants into food products may help preserve their quality by minimizing lipid oxidation and subsequent TBARS formation. While polyphenols have low plasma and tissue levels and limited bioavailability in healthy animals, they still exhibit a favourable effect on antioxidative capacity, particularly in stressed animals [56]. The content of $TBARS_{450}$ and DPPH scavenging activity determined in this research did not differ significantly depending on the feeding treatment, although the content of $TBARS_{450}$ was decreased in milk of goats fed with GSC supplementation (from 0.039 to 0.035; Table 6).

When compared to CON, significantly higher activity of SOD was determined in GSC10 goat milk. In contrast to CON and GSC5, GSC10 had significantly higher activity of GR in milk. A significantly greater GR activity in the milk of goats fed with 10% GSC in food indicates the stimulation of oxidation-reduction potential. Similar results were found in the research with dried apple pomace by Bartel et al. [57]. GR is the primary enzyme involved in the metabolism of glutathione and plays a crucial role in the glutathione redox cycle, which sustains appropriate amounts of reduced cellular GSH [58]. For example,

adding grape extracts from different cultivars to goat feed confirmed increased GR activity in the liver tissue of goats [59].

According to Santos et al. (2014) [60], dairy cows fed silage with the greatest addition of grape residue had milk with a positive antioxidant influence. Significant rises in SOD activity in the serum and GPx in milk were confirmed by Alba et al. [15]. In an experiment with pigs fed a diet with an additional 5% GSC throughout the 24-day finishing period, Taranu et al. [14] found no influence on blood biochemistry or pig characteristics, but they did find a modulatory effect on the antioxidative status (substantially reduced TBARS). Paraskevakis [61] identified the beneficial effects of polyphenols on the oxidative status of goats. This is attributed to the direct antioxidative effect of polyphenols, stemming from their absorption in the gastrointestinal tract and subsequent tissue deposition [62].

5. Conclusions

Considering the preservation of goat milk production and quality, as well as the metabolic profile of goats' blood, a significant increase in antioxidant enzymes in milk (SOD and GR) and in blood (SOD) confirms that using grape seed cake rich in polyphenols as a supplement in goats' feed is justified because it reduces oxidative stress caused by lactation, which is very stressful for animals. Supplementation of 10% GSC rich in polyphenols to the diet fed to lactating goats can be recommended because GSC can prevent oxidative stress and reinforce the antioxidative response of animals. By bolstering the antioxidative response and mitigating oxidative stress during this demanding production period, GSC supplementation may contribute to the overall well-being of lactating goats. Further research should explore the effects of various groups of polyphenols in the diet of lactating goats.

Author Contributions: Investigation, writing—original draft preparation conceptualization, visualization, Z.A.; writing—review and editing, V.P.; investigation, formal analysis, methodology, J.N.; investigation, statistical analyses, visualization, Ž.K.Š.; formal analysis and methodology, visualization, M.Đ., V.P., M.D., Z.S. and L.J.B.; field investigation, M.R.; investigation, visualization, B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was approved by the Committee for Animal Welfare of the Faculty of Agrobiotechnical Sciences Osijek (644-01/22-01/03 from 30 June 2022). It was carried out in accordance with the Declaration of Helsinki and followed the legal provisions determined by the Animal Protection Act (Republic of Croatia Official Gazette No. 133 (2006), No. 37 (2013) and No. 125 (2013)).

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: The research was carried out with the Innovative Breeding and Technological Processes in Animal Production (no. 1126) research team at the Faculty of Agrobiotechnical Sciences Osijek.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Castro, N.; Suarez-Trujillo, A.; Gonzalez-Cabrera, M.; Hernandez-Castellano, L.E.; Argüello, A. Goat Lactation Research as a Gateway for the Development of the Dairy Goat Industry. *Anim. Front.* **2023**, *13*, 108–111. [CrossRef] [PubMed]
2. Pandey, K.B.; Rizvi, S.I. Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxid. Med. Cell Longev.* **2009**, *2*, 270–278. [CrossRef]
3. Costa, M.M.; Alfaia, C.M.; Lopes, P.A.; Pestana, J.M.; Prates, J.A.M. Grape By-Products as Feedstuff for Pig and Poultry Production. *Animals* **2022**, *12*, 2239. [CrossRef]
4. Kalli, E.; Lappa, I.; Bouchagier, P.; Tarantilis, P.A.; Skotti, E. Novel Application and Industrial Exploitation of Winery By-Products. *Bioresour. Bioprocess.* **2018**, *5*, 46. [CrossRef]

5. Turcu, R.P.; Panaite, T.D.; Untea, A.E.; Vlaicu, P.A.; Badea, I.A.; Mironeasa, S. Effects of Grape Seed Oil Supplementation to Broilers Diets on Growth Performance, Meat Fatty Acids, Health Lipid Indices and Lipid Oxidation Parameters. *Agriculture* **2021**, *11*, 404. [CrossRef]
6. OIV. *State of the World Vine and Wine Sector 2021*; OIV: Paris, France, 2022; pp. 3–19.
7. Ferrer-Gallego, R.; Silva, P. The Wine Industry By-Products: Applications for Food Industry and Health Benefits. *Antioxidants* **2022**, *11*, 2025. [CrossRef]
8. Maicas, S.; Mateo, J.J. Sustainability of Wine Production. *Sustainability* **2020**, *12*, 559. [CrossRef]
9. Teixeira, A.; Baenas, N.; Dominguez-Perles, R.; Barros, A.; Rosa, E.; Moreno, D.A.; Garcia-Viguera, C. Natural Bioactive Compounds from Winery By-Products as Health Promoters: A Review. *Int. J. Mol. Sci.* **2014**, *15*, 15638–15678. [CrossRef]
10. Bucić-Kojić, A.; Planinić, M.; Tomas, S.; Tišma, M. Grape Pomace—Waste and High-Value Raw Material. In *Some Possibilities of Using By-Products of the Food Industry*; Šubarić, D., Ed.; Faculty of Food Technology Osijek: Osijek, Croatia, 2017; pp. 111–131, ISBN 978-953-7005-52.
11. Messina, C.M.; Manuguerra, S.; Catalano, G.; Arena, R.; Cocchi, M.; Morghese, M.; Montenegro, L.; Santulli, A. Green Biotechnology for Valorisation of Residual Biomasses in Nutraceutic Sector: Characterization and Extraction of Bioactive Compounds from Grape Pomace and Evaluation of the Protective Effects in Vitro. *Nat. Prod. Res.* **2021**, *35*, 331–336. [CrossRef] [PubMed]
12. Dragović-Uzelac, V.; Planinić, M.; Bursać Kovačević, D.; Putnik, P. Possibilities of Using Waste from Fruit and Vegetable Processing. In *Some Possibilities of Using By-Products of the Food Industry*; Šubarić, D., Ed.; Faculty of Food Technology Osijek: Osijek, Croatia, 2017; pp. 39–55, ISBN 978-953-7005-52.
13. Lutterodt, H.; Slavin, M.; Whent, M.; Turner, E.; Yu, L.L. Fatty Acid Composition, Oxidative Stability, Antioxidant and Antiproliferative Properties of Selected Cold-Pressed Grape Seed Oils and Flours. *Food Chem.* **2011**, *128*, 391–399. [CrossRef]
14. Taranu, I.; Habeanu, M.; Gras, M.A.; Pistol, G.C.; Lefter, N.; Palade, M.; Ropota, M.; Sanda Chedea, V.; Marin, D.E. Assessment of the Effect of Grape Seed Cake Inclusion in the Diet of Healthy Fattening-Finishing Pigs. *J. Anim. Physiol. Anim. Nutr.* **2017**, *102*, e30–e42. [CrossRef] [PubMed]
15. Alba, D.F.; Campigotto, G.; Cazarotto, C.J.; Dos Santos, D.S.; Gebert, R.R.; Reis, J.H.; Souza, C.F.; Baldissera, M.D.; Gindri, A.L.; Kempka, A.P.; et al. Use of Grape Residue Flour in Lactating Dairy Sheep in Heat Stress: Effects on Health, Milk Production and Quality. *J. Therm. Biol.* **2019**, *82*, 197–205. [CrossRef]
16. Correddu, F.; Lunesu, M.F.; Buffa, G.; Atzori, A.S.; Nudda, A.; Battacone, G.; Pulina, G. Can Agro-Industrial By-Products Rich in Polyphenols Be Advantageously Used in the Feeding and Nutrition of Dairy Small Ruminants? *Animals* **2020**, *10*, 131. [CrossRef]
17. Rațu, R.N.; Veleșcu, I.D.; Stoica, F.; Usturoi, A.; Arsenoaia, V.N.; Crivei, I.C.; Postolache, A.N.; Lipșa, F.D.; Filipov, F.; Florea, A.M.; et al. Application of Agri-Food By-Products in the Food Industry. *Agriculture* **2023**, *13*, 1559. [CrossRef]
18. Santucci, P.M.; Maestrini, O. Body Conditions of Dairy Goats in Extensive Systems of Production: Method of Estimation. *Ann. De Zootech.* **1985**, *34*, 473–474. [CrossRef]
19. National Research Council (NRC). *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids*; National Academies Press: Washington, DC, USA, 2007; p. 293. ISBN 978-0-309-10213-1.
20. Association of Official Analytical Chemists, (AOAC). Official Methods of Analysis (2023). In *Official Methods of Analysis (OMA) Is a Publication of AOAC INTERNATIONAL Comprising over 3000 Validated Methods for the Scientific Analysis of Food and Agriculture Products*, 22nd ed.; AOAC: Arlington, VA, USA, 2006; pp. 26–43.
21. INRAE-CIRAD-AFZ. Tables of Composition and Nutritional Values of Feed Materials. Available online: https://www.feedtables.com/ (accessed on 9 February 2024).
22. Jakobek, L.; Matić, P.; Ištuk, J.; Barron, A.R. Study of Interactions Between Individual Phenolics of Aronia with Barley Beta-Glucan. *Pol. J. Food Nutr. Sci.* **2021**, *71*, 187–196. [CrossRef]
23. Pulina, G.; Cannas, A.; Serra, A.; Vallebella, R. Determination and Estimation of the Energy Value in Sardinian Goat Milk. In Proceedings of the Congress of Società Italiana Scienze Veterinarie (SISVet), Altavilla Milicia, Italy, 25–28 September 1991; pp. 1779–1781.
24. Wiggans, G.R.; Shook, G.E. A Lactation Measure of Somatic Cell Count. *J. Dairy Sci.* **1987**, *70*, 2666–2672. [CrossRef] [PubMed]
25. Alyaqoubi, S.; Abdullah, A.; Addai, Z.R. Antioxidant Activity of Goat's Milk from Three Different Locations in Malaysia. *AIP Conf. Proc.* **2014**, *1614*, 198–201. [CrossRef]
26. Qwele, K.; Hugo, A.; Oyedemi, S.O.; Moyo, B.; Masika, P.J.; Muchenje, V. Chemical Composition, Fatty Acid Content and Antioxidant Potential of Meat from Goats Supplemented with Moringa (Moringa Oleifera) Leaves, Sunflower Cake and Grass Hay. *Meat Sci.* **2013**, *93*, 455–462. [CrossRef]
27. Oancea, A.-G.; Untea, A.E.; Dragomir, C.; Radu, G.L. Determination of Optimum TBARS Conditions for Evaluation of Cow and Sheep Milk Oxidative Stability. *Appl. Sci.* **2022**, *12*, 6508. [CrossRef]
28. Sun, Q.; Faustman, C.; Senecal, A.; Wilkinson, A.L.; Furr, H. Aldehyde Reactivity with 2-Thiobarbituric Acid and TBARS in Freeze-Dried Beef during Accelerated Storage. *Meat Sci.* **2001**, *57*, 55–60. [CrossRef]
29. SAS 9.4 Copyright (c) 2002–2012. by SAS Institute Inc., Cary, NC, USA. Available online: https://go.documentation.sas.com/api/docsets/fndigunx/9.4/content/fndigunx.pdf?locale=en (accessed on 9 February 2024).
30. Kramer, J.W. Normal Hematology of Cattle, Sheep and Goats. In *Schalm's Veterinary Hematology*; Feldman, B.F., Zinkl, J.G., Jain, N.C., Eds.; Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2000; pp. 1075–1084.

31. Jackson, P.G.G.; Cockcroft, P.D. Laboratory Reference Values: Biochemistry. In *Clinical Examination of Farm Animals*; Blackwell Science Ltd.: Oxford, UK, 2002; pp. 303–305, ISBN 978-0-470-75242-5.
32. Kaneko, J.; Harvey, J.; Bruss, M. *Clinical Biochemistry of Domestic Animals*; Elsevier Academic Press: Amsterdam, The Netherlands, 2008; p. 963.
33. Antunović, Z.; Šperanda, M.; Novoselec, J.; Đidara, M.; Mioč, B.; Klir, Ž.; Samac, D. Blood Metabolic Profile and Acid-Base Balance of Dairy Goats and Their Kids during Lactation. *Vet. Arh.* **2017**, *87*, 43–55.
34. Provenza, F.D.; Villalba, J.J. The Role of Natural Plant Products in Modulating the Immune System: An Adaptable Approach for Combating Disease in Grazing Animals. *Small Rumin. Res.* **2010**, *89*, 131–139. [CrossRef]
35. Alonso-Díaz, M.A.; Torres-Acosta, J.F.J.; Sandoval-Castro, C.A.; Capetillo-Leal, C.M. Amino Acid Profile of the Protein from Whole Saliva of Goats and Sheep and Its Interaction with Tannic Acid and Tannins Extracted from the Fodder of Tropical Plants. *Small Rumin. Res.* **2012**, *103*, 69–74. [CrossRef]
36. Lamy, E.; Rawel, H.; Schweigert, F.J.; Capela e Silva, F.; Ferreira, A.; Costa, A.R.; Antunes, C.; Almeida, A.M.; Coelho, A.V.; Sales-Baptista, E. The Effect of Tannins on Mediterranean Ruminant Ingestive Behavior: The Role of the Oral Cavity. *Molecules* **2011**, *16*, 2766–2784. [CrossRef] [PubMed]
37. Pascual-Alonso, G.A.M.; Resconi, V.C.; Aguayo-Ulloa, L.; Miranda-de la Lama, G.C.; Olleta, J.L.; Villarroel, M.; Acenjo, B.; María, G.A. Wine By-Products Feeding on Ewe Physiological Traits, Milk Quality and the Meat Quality of Their Suckling Lambs. *Large Anim. Rev.* **2018**, *24*, 149–154.
38. Gessner, D.K.; Koch, C.; Romberg, F.-J.; Winkler, A.; Dusel, G.; Herzog, E.; Most, E.; Eder, K. The Effect of Grape Seed and Grape Marc Meal Extract on Milk Performance and the Expression of Genes of Endoplasmic Reticulum Stress and Inflammation in the Liver of Dairy Cows in Early Lactation. *J. Dairy Sci.* **2015**, *98*, 8856–8868. [CrossRef] [PubMed]
39. Khiaosa-ard, R.; Mahmood, M.; Mickdam, E.; Pacífico, C.; Meixner, J.; Traintinger, L.-S. Winery By-Products as a Feed Source with Functional Properties: Dose–Response Effect of Grape Pomace, Grape Seed Meal, and Grape Seed Extract on Rumen Microbial Community and Their Fermentation Activity in RUSITEC. *J. Anim. Sci. Biotechnol.* **2023**, *14*, 92. [CrossRef]
40. Bickerstaffe, R.; Annison, E.F.; Linzell, J.L. The Metabolism of Glucose, Acetate, Lipids and Amino Acids in Lactating Dairy Cows. *J. Agric. Sci.* **1974**, *82*, 71–85. [CrossRef]
41. Mokni, M.; Amri, M.; Limam, F.; Aouani, E. Effect of Grape Seed and Skin Supplement on Milk Yield and Composition of Dairy Ewes. *Trop. Anim. Health Prod.* **2017**, *49*, 131–137. [CrossRef] [PubMed]
42. Resconi, V.C.; Pascual-Alonso, M.; Aguayo-Ulloa, L.; Miranda-de la Lama, G.C.; Alierta, S.; Campo, M.M.; Olleta, J.L.; Villarroel, M.; María, G.A. Effect of Dietary Grape Pomace and Seed on Ewe Milk and Meat Quality of Their Suckling Lambs. *J. Food Qual.* **2018**, *2018*, e2371754. [CrossRef]
43. Manso, T.; Gallardo, B.; Salvá, A.; Guerra-Rivas, C.; Mantecón, A.R.; Lavín, P.; de la Fuente, M.A. Influence of Dietary Grape Pomace Combined with Linseed Oil on Fatty Acid Profile and Milk Composition. *J. Dairy Sci.* **2016**, *99*, 1111–1120. [CrossRef]
44. Nudda, A.; Correddu, F.; Marzano, A.; Battacone, G.; Nicolussi, P.; Bonelli, P.; Pulina, G. Effects of Diets Containing Grape Seed, Linseed, or Both on Milk Production Traits, Liver and Kidney Activities, and Immunity of Lactating Dairy Ewes. *J. Dairy Sci.* **2015**, *98*, 1157–1166. [CrossRef] [PubMed]
45. Antunović, Z.; Novoselec, J.; Klir, Ž.; Đidara, M. Hematological parameters in the Alpine goats during lactation. *Poljoprivreda* **2013**, *19*, 40–43.
46. Antunović, Z.; Novaković, K.; Klir, Ž.; Šerić, V.Š.; Mioč, B.; Šperanda, M.; Ronta, M.; Novoselec, J. Blood Metabolic Profile and Acid-Base Status of Istrian Goats—A Critically Endangered Croatian Goat—in Relation to Age. *Vet. Arh.* **2020**, *90*, 27–38. [CrossRef]
47. Nudda, A.; Buffa, G.; Atzori, A.S.; Cappai, M.G.; Caboni, P.; Fais, G.; Pulina, G. Small Amounts of Agro-Industrial Byproducts in Dairy Ewes Diets Affects Milk Production Traits and Hematological Parameters. *Anim. Feed Sci. Technol.* **2019**, *251*, 76–85. [CrossRef]
48. Du, X.; Cheng, X.; Dong, Q.; Zhou, J.; Degen, A.A.; Jiao, D.; Ji, K.; Liang, Y.; Wu, X.; Yang, G. Dietary Supplementation of Fruit from Nitraria Tangutorum Improved Immunity and Abundance of Beneficial Ruminal Bacteria in Hu Sheep. *Animals* **2022**, *12*, 3211. [CrossRef] [PubMed]
49. Juráček, M.; Vašeková, P.; Massányi, P.; Kováčik, A.; Bíro, D.; Šimko, M.; Gálik, B.; Rolinec, M.; Hanušovský, O.; Kolláthová, R.; et al. The Effect of Dried Grape Pomace Feeding on Nutrients Digestibility and Serum Biochemical Profile of Wethers. *Agriculture* **2021**, *11*, 1194. [CrossRef]
50. Fascina, V.B.; Sartori, J.R.; Gonzales, E.; Carvalho, F.B.d.; Souza, I.M.G.P.d.; Polycarpo, G.d.V.; Stradiotti, A.C.; Pelícia, V.C. Phytogenic Additives and Organic Acids in Broiler Chicken Diets. *R. Bras. Zootec.* **2012**, *41*, 2189–2197. [CrossRef]
51. Fridovich, I. Superoxide Dismutases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1986**, *58*, 61–97. [CrossRef] [PubMed]
52. Dijkstra, J.; Forbes, J.M.; France, J. Introduction. In *Quantitative Aspects of Ruminant Digestion and Metabolism*; CABI Publishing: Wallingford, UK, 2005; pp. 1–10. ISBN 978-0-85199-814-5.
53. Semeniuc, C.A.; Mandrioli, M.; Rodriguez-Estrada, M.T.; Muste, S.; Lercker, G. Thiobarbituric Acid Reactive Substances in Flavored Phytosterol-Enriched Drinking Yogurts during Storage: Formation and Matrix Interferences. *Eur. Food Res. Technol.* **2016**, *242*, 431–439. [CrossRef]

54. Hedegaard, R.V.; Kristensen, D.; Nielsen, J.H.; Frøst, M.B.; Østdal, H.; Hermansen, J.E.; Kröger-Ohlsen, M.; Skibsted, L.H. Comparison of Descriptive Sensory Analysis and Chemical Analysis for Oxidative Changes in Milk. *J. Dairy Sci.* **2006**, *89*, 495–504. [CrossRef] [PubMed]
55. Goutzourelas, N.; Stagos, D.; Demertzis, N.; Mavridou, P.; Karterolioti, H.; Georgadakis, S.; Kerasioti, E.; Aligiannis, N.; Skaltsounis, L.; Statiri, A.; et al. Effects of Polyphenolic Grape Extract on the Oxidative Status of Muscle and Endothelial Cells. *Hum. Exp. Toxicol.* **2014**, *33*, 1099–1112. [CrossRef]
56. Gessner, D.K.; Ringseis, R.; Eder, K. Potential of Plant Polyphenols to Combat Oxidative Stress and Inflammatory Processes in Farm Animals. *J. Anim. Physiol. Anim. Nutr.* **2017**, *101*, 605–628. [CrossRef] [PubMed]
57. Bartel, I.; Koszarska, M.; Wysocki, K.; Kozłowska, M.; Szumacher-Strabel, M.; Cieślak, A.; Wyrwał, B.; Szejner, A.; Strzałkowska, N.; Horbańczuk, J.O.; et al. Effect of Dried Apple Pomace (DAP) as a Feed Additive on Antioxidant System in the Rumen Fluid. *Int. J. Mol. Sci.* **2022**, *23*, 10475. [CrossRef]
58. Kerasioti, E.; Terzopoulou, Z.; Komini, O.; Kafantaris, I.; Makri, S.; Stagos, D.; Gerasopoulos, K.; Anisimov, N.Y.; Tsatsakis, A.M.; Kouretas, D. Tissue Specific Effects of Feeds Supplemented with Grape Pomace or Olive Oil Mill Wastewater on Detoxification Enzymes in Sheep. *Toxicol. Rep.* **2017**, *4*, 364–372. [CrossRef]
59. Singha, I.; Das, S.K. Scavenging and Antioxidant Properties of Different Grape Cultivars against Ionizing Radiation-Induced Liver Damage Ex Vivo. *Indian J. Exp. Biol.* **2016**, *54*, 280–285.
60. Santos, N.W.; Santos, G.T.D.; Silva-Kazama, D.C.; Grande, P.A.; Pintro, P.M.; de Marchi, F.E.; Jobim, C.C.; Petit, H.V. Production, Composition and Antioxidants in Milk of Dairy Cows Fed Diets Containing Soybean Oil and Grape Residue Silage. *Livest. Sci.* **2014**, *159*, 37–45. [CrossRef]
61. Paraskevakis, N. Effects of Dietary Dried Greek Oregano (*Origanum Vulgare* ssp. *Hirtum*) Supplementation on Blood and Milk Enzymatic Antioxidant Indices, on Milk Total Antioxidant Capacity and on Productivity in Goats. *Anim. Feed Sci. Technol.* **2015**, *209*, 90–97. [CrossRef]
62. Jordán, M.J.; Moñino, M.I.; Martínez, C.; Lafuente, A.; Sotomayor, J.A. Introduction of Distillate Rosemary Leaves into the Diet of the Murciano-Granadina Goat: Transfer of Polyphenolic Compounds to Goats' Milk and the Plasma of Suckling Goat Kids. *J. Agric. Food Chem.* **2010**, *58*, 8265–8270. [CrossRef] [PubMed]

agriculture

MDPI

Article

Performance, Carcass Traits and Meat Quality of Lambs Fed with Increasing Levels of High-Oleic Sunflower Cake

Daviane M. Costa [1], Tharcilla I. R. C. Alvarenga [2,*], Isabela J. dos Santos [1], Paulo C. G. Dias Junior [1], Flavio A. P. Alvarenga [2], Nadja G. Alves [1] and Iraides F. Furusho-Garcia [1]

[1] Department of Animal Science, Federal University of Lavras, (DZO/UFLA), P.O. Box 3037, Lavras CEP 37200-000, Minas Gerais, Brazil; davianecosta@yahoo.com.br (D.M.C.); isabelajorge1@yahoo.com.br (I.J.d.S.); paulocsar_dias@hotmail.com (P.C.G.D.J.); nadja@dzo.ufla.br (N.G.A.); iraides.ufla@gmail.com (I.F.F.-G.)

[2] Armidale Livestock Industries Centre, NSW Department of Primary Industries and Regional Development, Armidale, NSW 2351, Australia; flavio.alvarenga@dpi.nsw.gov.au

* Correspondence: tharcilla.alvarenga@dpi.nsw.gov.au

Abstract: The aim of this study was to evaluate the effect of sunflower cake from high-oleic seeds on performance, carcass characteristics, meat quality, and intramuscular fatty acid composition of finishing lambs. Thirty-six crossbred ewe lambs were assigned to four treatments (nine lambs/treatment) in a completely randomized design: 0 (control), 150, 300 and 450 g/kg DM of high-oleic sunflower cake. The lambs were weighed weekly and slaughtered with 42.3 ± 0.18 kg body weight and 270 ± 10.8 days of old. The inclusion of sunflower cake did not affect weight gain, dry matter intake and metabolizable energy intake ($p > 0.05$). There was an increase in neutral detergent fiber and EE intake ($p < 0.01$) with the inclusion of sunflower cake in the diet of the lambs. The inclusion of sunflower cake reduced hot and cold carcass yields ($p < 0.01$). Intramuscular fat content, L^*, oleic acid, rumenic acid and EPA fatty acids linearly increased ($p < 0.01$) with the inclusion of high-oleic sunflower cake. The inclusion of high-oleic sunflower cake reduced saturated fatty acids ($p < 0.01$), except stearic acid, which linearly increased ($p < 0.01$). Up to 450 g/kg DM of high-oleic sunflower cake in the diet of lambs did not affect animal performance while providing a higher deposition of fat with better fatty acid composition for human consumption.

Keywords: ruminant nutrition; by-product; fatty acids; PUFA; saturated fatty acids; ovine

Citation: Costa, D.M.; Alvarenga, T.I.R.C.; dos Santos, I.J.; Dias Junior, P.C.G.; Alvarenga, F.A.P.; Alves, N.G.; Furusho-Garcia, I.F. Performance, Carcass Traits and Meat Quality of Lambs Fed with Increasing Levels of High-Oleic Sunflower Cake. *Agriculture* **2025**, *15*, 191. https://doi.org/10.3390/agriculture15020191

Academic Editors: Tatiana Dumitra Panaite and Mihaela Hâbeanu

Received: 10 December 2024
Revised: 10 January 2025
Accepted: 14 January 2025
Published: 16 January 2025

Citation: Costa, D.M.; Alvarenga, T.I.R.C.; dos Santos, I.J.; Dias Junior, P.C.G.; Alvarenga, F.A.P.; Alves, N.G.; Furusho-Garcia, I.F. Performance, Carcass Traits and Meat Quality of Lambs Fed with Increasing Levels of High-Oleic Sunflower Cake. *Agriculture* **2025**, *15*, 191. https://doi.org/10.3390/agriculture15020191

1. Introduction

The search for more productive and sustainable food production systems, in addition to the elevated costs of animal feed, especially in the off-season, has been the fundamental driver for adhering to the use of alternative foods in animal feeding systems. By-products of the biodiesel industry have been prominent among the potential nutrient sources for animal feed, especially due to their lower price compared to traditional feeding ingredients and nutritious composition [1,2].

Sunflower (*Helianthus annuus* L.) is an oilseed with potential for biodiesel production due to its high oil concentration [3]. Currently, high-oleic sunflower oil is the most used in the food sector and as a raw material for non-food applications such as biofuels, due to its industrial properties, mainly high oxidative stability [4,5]. High-oleic sunflower has approximately 80% oleic acid, but some linoleic acid is still present in the achenes [4]. Unlike sunflower meal, sunflower cake is a by-product with a high fat content. It is produced when sunflower seeds are crushed for physical strength without the use of heat or solvent [6,7].

Meat fatty acid composition is one of the determining factors affecting fat quality [8], especially with regard to human health, emphasizing the recommendation of reduced intake of saturated fatty acids and increased intake of long-chain n3 polyunsaturated fatty acids (PUFA) in response to their effect in lipid-related diseases [9]. The inclusion of alternative animal feeds rich in unsaturated fatty acids is a strategy to reduce saturated fatty acids deposited in tissues and increase polyunsaturated fatty acids in red meat [9], primarily conjugated linoleic acid (CLA) isomers [10–12]. However, ruminal fatty acid biohydrogenation challenges the increase in PUFA content in the meat of ruminants through dietary intake, in addition to the activity of enzymes involved in fatty acid synthesis in the muscle (e.g., Δ9 desaturase and elongase) [13,14].

The application progress of sunflower cake has gained increased attention as a viable ingredient in ruminant nutrition due to its favorable nutrient profile and availability. High-oleic sunflower cake can be a high-energy feed ingredient in the diet of feedlot lambs and improve the profile of fatty acids in the meat, providing a direct deposition of monounsaturated fatty acids and, through biohydrogenation, convert to stearic fatty acid, which has a protective effect against cardiovascular disease [15]. However, due to the fat content associated with the amount of fiber in sunflower cake, the levels of inclusion of this by-product in the diet can reduce dry matter intake and animal performance [2,16] while others have shown that the inclusion of sunflower cake can support intake, nitrogen balance and ruminal fermentation parameters [17]. Given that, the objective of the present study was to assess the effects of increased levels of high-oleic sunflower cake (0, 150, 300 and 450 g/kg DM) in the diet of feedlot crossbred lambs on their performance, carcass traits, meat quality (pH, color, shear force and composition), and intramuscular fatty acid composition.

2. Materials and Methods

2.1. Facilities and Experimental Diets

This study was conducted in 2013 at the Sheep Sector in the Department of Animal Sciences of the Federal University of Lavras. Animal experimental procedures were approved and performed according to the Animal Care Guidelines (Protocol number 105/12).

Thirty-six female lambs ($\frac{1}{2}$ Santa Inês × $\frac{1}{2}$ Dorper, randomly selected from a larger herd), with an initial body weight of 21.52 ± 0.27 kg and initial age of 138 ± 2.62 days, were individually allocated to covered pens (1 m^2). This experiment was carried out in a completely randomized design. Animals were allocated among the four treatments, totaling 9 replicates per treatment, which consisted of the inclusion of high-oleic sunflower cake (Table 1) in the proportions of 0, 150, 300, and 450 g/kg DM (Table 2). The diets were calculated to be isoproteic (181 g/kg DM), with a 20:80 roughage:concentrate ratio. The diets were formulated to meet the nutritional requirements of female lambs with 20 kg live weight and average daily weight gain of 250 g, according to the National Research Council [18]. Sunflower cake was obtained through cold pressing of high-oleic sunflower seeds from a Brazilian company (Parecis Alimentos S/A, Campo Novo do Parecis, Brazil).

Table 1. Chemical and main fatty acid composition of the sunflower cake.

Chemical Composition	g/kg DM
Dry matter, g/kg as fed	968.8
Organic matter	943.3
Crude protein	276.0
Neutral detergent fiber	440.0

Table 1. *Cont.*

Acid detergent fiber	357.0
Ether extract	160.0
Ash	57.0
Non fibrous carbohydrates	72.0
Metabolizable energy [a], (Mcal/kg)	2.77
Fatty acid composition	g/100 g total fatty acids
C12:0 (Lauric)	0.00
C16:0 (Palmitic)	4.40
C18:0 (Stearic)	2.21
C18:1c9 (Oleic)	81.02
C18:2 n6 (Linoleic)	5.30
C18:3 n3 (α-Linolenic)	0.23
Saturated fatty acids	7.90
Unsaturated fatty acids	91.30
Monounsaturated fatty acids	85.64
Polyunsaturated fatty acids	5.66

[a] Estimated according to [18], where metabolizable energy = 0.82 × digestible energy.

Table 2. Diet composition, chemical analysis and main fatty acids composition of the experimental diets.

	High-Oleic Sunflower Cake (g/kg DM)			
	0	**150**	**300**	**450**
Ingredients (g/kg DM)				
Tifton 85 hay (*Cynodon* spp.)	200.0	200.0	200.0	200.0
Ground corn grain	560.0	440.0	380.0	310.3
Soybean meal	210.0	180.0	90.0	7.70
Sunflower cake	0.00	150.0	300.0	450.0
Premix mineral-vitamin supplement [a]	20.0	20.0	20.0	20.0
Limestone	10.0	10.0	10.0	10.0
Chemical composition (g/kg DM)				
Dry matter, g/kg as fed	947.7	950.0	952.2	955.5
Organic matter	933.3	932.2	934.4	933.3
Crude protein	181.5	182.0	180.2	180.5
Neutral detergent fiber	357.4	388.1	418.6	449.0
Acid detergent fiber	130.3	163.6	196.8	229.8
Ether extract	30.0	49.8	69.5	89.1
Ash	67.2	68.6	66.6	68.6
Non fibrous carbohydrates	364.0	311.5	265.4	212.8
Metabolizable energy [b], (Mcal/kg)	2.70	2.72	2.67	2.62
Fatty acid composition (g/100g total fatty acids)				
C12:0 (Lauric)	0.04	0.02	0.02	0.01
C16:0 (Palmitic)	18.51	11.63	8.50	6.80
C18:0 (Stearic)	3.07	2.76	2.61	2.61
C18:1c9 (Oleic)	25.60	52.77	63.40	68.80
C18:2 n6 (Linoleic)	41.22	25.41	17.00	12.00
C18:3 n3 (α-Linolenic)	1.08	0.83	0.63	0.42
Saturated fatty acids	26.23	17.50	12.65	11.46
Unsaturated fatty acids	74.95	83.13	86.67	88.00
Monounsaturated fatty acids	32.40	56.61	68.90	75.40
Polyunsaturated fatty acids	42.56	26.52	17.84	12.60

[a] Composition per kg of mixture: 122 g Calcium, 87 g Phosphorus, 18 g Sulfur, 147 g Sodium, 3800 mg Zinc, 590 mg Copper, 2000 mg Manganese. [b] Estimated according to [18], where metabolizable energy = 0.82 × digestible energy.

2.2. Animal Performance, Slaughter and Carcass Traits

The lambs were subjected to 15 days of an adaptation protocol to adapt to facilities and experimental diets. The total mixed ration was offered *ad libitum* twice daily (07:00 and 15:00 h). The amount of diet provided everyday was adjusted according to the recorded intake of the previous day, calculated to achieve 5% refusals. Feeding leftovers were weighed daily

to determine the average daily intake. One sample of the feed refusals from each animal was collected daily and combined into a weekly sampling and taken to a dry-forced ventilation oven for drying to determine daily dry matter intake. The samples were ground using a Wiley-type mill with a sieve size of 1 mm to determine the concentrations of total dry matter (DM) (method 967.03), ash (method 942.05), crude protein (CP) (method 981.10), and ether extract (EE) (method 920.29) [19]. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the procedure described by Van Soest et al. [20], with an NDF correction for ash (NDFa). Non-fibrous carbohydrate (NFC) concentration was calculated using the following equation: NFC = 100 − (CP + NDF + EE + ash).

Lambs were weighed weekly until they reached a body weight of 42.3 ± 0.18 kg. The animals were submitted to feed and water fasting for 16 h and weighed to assess the pre-slaughter weight (SW). Then, the animals were transported to a commercial slaughterhouse, where they were slaughtered according to the slaughterhouse regulations. Non-carcass body parts were removed and weighed to determine the non-carcass traits (NCTs). Stomach and intestinal contents were weighed to determine gastrointestinal tract content (GITC) weight, and empty body weight (EBW) was estimated according to the equation: EBW = SW − GITC. The carcasses were weighed to obtain hot carcass weight (HCW) and hot carcass yield (HCY = HCW/SW × 100); subsequently, the carcasses were cooled for 24 h at 4 °C. After cooling, the carcasses were weighed again, obtaining the cold carcass weight (CCW) and cold carcass yield (CCY = CCW/SW × 100). The cooling loss was calculated according to the equation: CL = (HCW − CCW)/HCW) × 100. Then, the carcasses were split longitudinally, and an incision was made in the left half carcass, between the 12th and 13th ribs for exposure of the longissimus muscle. Subcutaneous fat thickness (SFT) was measured with a digital caliper. At the same point, the eye muscle area (EMA) of the muscle was drawn on a transparency with the aid of a permanent pen and measured using the software Universal Desktop Ruler (AVPSoft®). Percentage yields of carcass cuts (loin, ribs, leg, shoulder, rack and neck) were calculated relative to the cold carcass weight of the lambs.

2.3. Meat Quality Analysis

The longissimus thoracis et lumborum muscle was sampled and divided into three steak samples, vacuum-packed with polyethylene packages and kept in refrigerator at 2 °C for 3 days before further analyses. One steak was used for the measurement of meat color. After 30 min of opening the vacuum bags at room temperature, color measurements were taken using a CM-700 Minolta spectrophotometric colorimeter (Konica Minolta, Osaka, Japan). Three readings were recorded on each steak to calculate the average values of the lightness (L^*), redness (a^*) and yellowness (b^*). The saturation index (Chroma) and hue angle were calculated according to MacDougall [21], using the following equations: Chroma = ((a^*)2 + (b^*)2)0.5 and hue = arc tan (b^*/a^*). Meat pH was measured in triplicate using a digital pH meter (TESTO-205 pH meter, Campinas, Brazil). Additionally, the same steak sample was also analyzed for moisture, CP, EE, and ash concentration, according to the AOAC [19]. The second steak was used to measure cooking loss. In summary, the weight of the samples was recorded, and the samples were placed on a rack above a glass baking dish. A digital thermometer was inserted into the geometric center of the samples to monitor their internal temperature. The steaks were cooked in an electric oven, and once the internal temperature of the sample reached 40 °C, the steak was flipped and cooked further until the internal temperature reached 71 °C, as previously described [22]. After cooking, the samples were kept at room temperature for 15 min, weighed again to determine cooking loss, and cooled at 4 °C for 24 h. After refrigeration, for determination of shear force (SF) three subsamples measuring 1 cm^2 each were cut parallel to the muscle

fiber. The subsamples were sectioned using a texturometer (ModleTA-TX2, Stable Micro Systems Ltd., Godalming, Surrey, UK) attached to a Warner–Bratzler slide and calibrated using a weight of 2 kg with an adjusted speed of 200 mm/min. The results were presented in kgf/cm^2 and five replicate measurements were taken per steak.

The third steak was used for the quantification of intramuscular fatty acid composition according to Hara and Radin [23]. The fatty acid profile was assessed using the methods reported by Rodrígues-Ruiz et al. [24]. After extraction and methylation, each sample was injected into a gas chromatograph (model Focus CG, Finnigan) equipped with a flame ionization detector and a 100 m long CP-Sil 88 capillary column (Varian) with an internal diameter of 0.25 μm and a film thickness of 0.20 μm. Hydrogen was used as the carrier gas at a 1.8 mL/min flow rate. The oven temperature program started at 70 °C with a 4 min wait time. Subsequently, the temperature increased to 175 °C at 13 °C/min, with a 27 min wait time, followed by an increase to 215 °C at 4 °C/min, with a 9 min wait time. The temperature was increased by 7 °C/min until it reached 230 °C, which was held for 5 min, totaling 65 min. The vaporizer temperature was set at 250 °C, and the detector temperature was set at 300 °C. The fatty acids were identified by comparing the retention times of methyl esters with a predefined pattern, and then quantified by area normalization of the methyl esters, and were expressed as percentages of total methylated fatty acids.

The Δ9-desaturase and elongase enzymatic activity indices were calculated as reported by Malau-Aduli et al. [25], using the following equations: Δ9-Desaturase 16 = 100 [(C16:1cis-9)/(C16:1cis-9 + C16:0)]; Δ9-Desaturase 18 = 100 [(C18:1cis-9)/(C18:1cis-9 + C18:0)]; and Elongase = 100 [(C18:0 + C18:1cis-9)/(C16:0 + C16:1cis-9 + C18:0 + C18:1cis-9)]. The atherogenicity and thrombogenicity indices were calculated according to Ulbricht and Southgate [26], as indicators of the risk for cardiovascular disease: Atherogenicity = {[C12:0 + 4 × (C14:0) + C16:0]/ (MUFA + n3 + n6)} and Thrombogenicity = {(C14:0 + C16:0 + C18:0)/[(0.5 × MUFA) + (05 × n6) + (3 × n3) + (n3/n6)]}.

2.4. Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS Version 9.1, SAS Institute, Cary, NC, USA) to determine the significant effects of the inclusion of high-oleic sunflower cake in the diet. The initial body weight of the animals was included as a covariate, according to the following model: Yij = μ + Ti + Pj + eij; where Yij is the observed value, μ is the constant associated with each observation, Ti is the effect of using sunflower cake (i = 0 to 450 g/kg DM), Pj is the effect of the covariate initial live weight and eij is the experimental error. When significant effects were detected ($p < 0.05$), linear regression models were fitted using the REG procedure of SAS, testing linear and quadratic models ($\alpha - 0.05$).

3. Results and Discussion

3.1. Growth Performance and Carcass Characteristics

The inclusion of high-oleic sunflower cake up to 450 g/kg DM in the diet of the lambs did not affect dry matter intake, crude protein intake, daily weight gain, or gain/feed ratio ($p > 0.05$) (Table 3). The intake of EE and NDF linearly increased ($p < 0.01$) with the inclusion of high-oleic sunflower cake due to the high concentrations of these nutrients in the by-product. Conversely, a reduction in the NFC intake was observed. These outcomes were anticipated, as including sunflower cake in the diet reduced the proportion of soybean meal, thereby maintaining isoproteic condition. Sunflower cake contains higher levels of EE and NDF but lower levels of NFC compared to soybean meal. The primary rationale for incorporating fat into ruminant diets is to enhance energy intake without utilizing rapidly fermentable feeds. However, reductions in dry matter intake and NDF digestibility are commonly reported due to the inhibitory effect of fatty acids on ruminal microorganisms [27].

These fatty acids include medium-chain (10 to 14 carbons) and long-chain polyunsaturated fatty acids [18,28]. However, there was an elevated EE intake, especially in the diet with 450 g/kg DM of high-oleic sunflower cake inclusion (89 g/kg of DM). The by-product used in the present study is composed mostly of oleic acid (81.0 g/100 kg total fatty acids), a monounsaturated fatty acid which is less toxic to ruminal microorganisms [29]. Previous studies have shown that antiprotozoal and antibacterial effects from feed oils are associated with the degree of unsaturation of their fatty acids [30,31]. A reduction in the detrimental effects of unsaturated fatty acids on digestibility through the use of oleic acid was also observed by Weld and Armentano [32] using plenish high-oleic soybeans, which moderately increased milk fat content compared with conventional soybeans. Therefore, the performance of lambs fed with increased levels of high-oleic sunflower cake was not greatly affected apart from an increase in EE and NDF intake due to their superior concentration in the diet.

Table 3. Performance of lambs fed diets with increased levels of high-oleic sunflower cake.

	High-Oleic Sunflower Cake (g/kg DM)				SEM	*p*-Value			Equation	R^2
	0	150	300	450		T	L	Q		
Intake										
Dry matter, kg/day	1.07	1.15	1.11	1.09	0.03	0.42	0.86	0.14	Y = 1.11	-
Crude protein, g/day	189.00	197.23	188.12	180.44	3.90	0.06	0.08	0.06	Y = 188.70	-
Neutral detergent fiber, g/day	361.41	426.63	453.83	496.13	12.00	<0.01	<0.01	0.07	Y = 370.64 + 2.85x	0.95
Ether extract, g/day	35.63	64.07	86.36	105.91	2.51	<0.01	<0.01	0.07	Y = 38.12 + 15.5x	0.98
NFC, g/day	406.51	389.28	332.28	251.78	8.22	<0.01	<0.01	0.05	Y = 422.97 − 34.4x	0.94
Weight gain, kg	22.35	22.15	21.61	21.68	0.37	0.40	0.73	0.86	Y= 21.95	-
Daily weight gain, g	176.87	200.09	206.21	184.41	8.90	0.09	0.45	0.08	Y = 191.90	-
Gain: feed, kg/kg	6.29	5.75	5.38	5.92	0.23	0.14	0.31	0.07	Y= 5.84	-
Gastrointestinal tract content, kg	3.63	4.29	4.78	5.20	0.21	<0.01	<0.01	0.46	Y = 3.71 + 0.03x	0.90
Empty body weight, kg	39.06	38.01	37.26	36.90	0.34	<0.01	<0.01	0.28	Y = 38.88 − 0.048x	0.73
Slaughter weight, kg	43.00	42.41	41.70	42.15	0.36	0.10	0.12	0.20	Y = 42.32	-

NFC: non-fibrous carbohydrates; SEM: standard error of the mean; R^2: coefficient of determination; T: treatment effect; L: linear effect; Q: quadratic effect.

A significant linear decrease in empty body weight ($p < 0.01$) and a linear increase in gastrointestinal tract content ($p < 0.01$) were observed with the inclusion of high-oleic sunflower cake (Table 3). Diets containing higher levels of NDF can prolong rumen retention time and increase total digesta volume [33], ultimately leading to a proportional decrease in empty body weight once gastrointestinal contents are removed [34].

Hot and cold carcass weights and their yield percentage linearly decreased ($p < 0.01$) as a result of the observed increase in gastrointestinal tract content, even though there was no change in slaughter weight nor in the sum of non-carcass components with the inclusion of high-oleic sunflower cake in the lambs' diet ($p > 0.05$) (Table 4). Lima et al. [2] also reported a reduction in hot and cold carcass yields with the inclusion of up to 300 g/kg of sunflower cake in the diet of male Santa Ines lambs. Additionally, Lima et al. [2] observed a decrease in weight gain and slaughter weight of the lambs with the inclusion of sunflower cake in the diet, which is not supported by our findings. In diets with up to 9.5% EE in which cottonseed meal was replaced by sunflower cake, Junior et al. [35] did not observe effects on weight gain and empty body weight of confined lambs despite reductions in hot and cold carcass yields. This effect can be attributed to the varying levels of sunflower cake included in the diet, which can increase gastrointestinal fill. As a result, a larger proportion of the animal's live weight consists of digesta, leaving a smaller share for carcass tissue once the gastrointestinal tract is emptied at slaughter. Consequently, both hot and cold carcass weights are reduced.

Table 4. Carcass characteristics of lambs fed diets with increased levels of high-oleic sunflower cake.

	High-Oleic Sunflower Cake (g/kg DM)				SEM	*p*-Value			Equation	R^2
	0	150	300	450		T	L	Q		
Non-carcass traits, kg	21.07	20.83	21.41	20.60	0.49	0.74	0.69	0.32	Y = 20.98	-
Hot carcass weight, kg	23.52	22.44	21.80	21.45	0.34	<0.01	<0.01	0.21	Y = 23.64 − 0.05x	0.65
Hot carcass yield, %	54.70	52.93	52.39	50.97	0.77	0.02	<0.01	0.16	Y = 23.17 − 0.04x	0.73
Cold carcass weight, kg	23.03	22.06	21.40	21.00	0.31	<0.01	<0.01	0.17	Y = 54.43 − 0.075x	0.64
Cold carcass yield, %	53.57	52.02	51.33	49.91	0.74	<0.01	<0.01	0.23	Y = 53.39 − 0.078x	0.72
Cooling loss, %	2.06	1.82	1.82	2.08	0.15	0.54	0.84	0.07	Y = 1.94	-
Eye muscle area, cm^2	17.96	17.01	17.00	16.94	0.76	0.70	0.34	0.58	Y = 17.23	-
Subcutaneous fat thickness, mm	2.51	2.74	2.93	3.11	0.11	0.01	<0.01	0.90	Y = 2.54 + 0.013x	0.99
Kidney fat, kg	1.14	1.13	1.15	1.34	0.05	0.04	0.02	0.08	Y = 1.09 + 0.004x	0.66
Gastrointestinal tract fat, kg	2.52	2.77	2.85	3.39	0.10	<0.01	<0.01	0.17	Y= 2.48 + 0.017x	0.90
Cut yield, %										
Loin	6.42	6.44	6.33	6.61	0.15	0.68	0.47	0.30	Y = 6.45	-
Ribs	13.31	13.48	14.47	13.70	0.34	0.15	0.42	0.09	Y = 13.74	-
Leg	31.17	31.36	30.52	31.03	0.48	0.70	0.45	0.55	Y = 31.02	-
Shoulder	17.30	17.85	16.63	17.47	0.36	0.15	0.44	0.94	Y = 17.31	-
Rack	22.50	21.68	22.45	22.53	0.46	0.52	0.52	0.26	Y = 22.29	-
Neck	5.70	6.94	5.31	6.12	0.43	0.08	0.88	0.83	Y = 6.02	-

SEM: standard error of the mean; R^2: coefficient of determination; T: treatment effect; L: linear effect; Q: quadratic effect.

The inclusion of up to 450g/kg DM of high-oleic sunflower cake in the diet of the lambs did not affect eye muscle area and cooling loss ($p > 0.05$) (Table 4). Eye muscle area is an indicator of muscular tissue development in animals and is highly correlated with valuable commercial cuts. In the present study, the yields of commercial cuts were not affected by the treatment effect ($p > 0.05$) (Table 4). Similar findings have been reported in the literature with the use of sunflower cake in the diet of male Santa Ines lambs and male Boer goats [35,36]. The inclusion of high-oleic sunflower cake in the diet linearly increased subcutaneous fat thickness ($p < 0.01$), reaching 3.11 mm in lambs fed 450 g/kg DM of high-oleic sunflower cake (Table 4). Regardless of treatment, the fat thickness was presumably insufficient to affect the percentage of cooling loss. Hristov et al. [37] did not observe effects on eye muscle area or fat thickness when adding 5% of high-oleic acid safflower oil to the diet of beef cattle. Lima et al. [2] also did not observe an effect on subcutaneous fat thickness of lambs fed up to 6% EE from sunflower cake. Kidney fat also increased with the inclusion of high-oleic sunflower cake in the diet of the lambs ($p < 0.01$) (Table 4). Therefore, the increase in subcutaneous fat thickness and kidney fat can be attributed to a higher energy intake from increased levels of lipids in the high-oleic sunflower cake diets which support fat storage in the body [38]. However, these dietary changes did not significantly alter eye muscle area or cooling loss, suggesting that the inclusion of high-oleic sunflower cake in the diet of the lambs appears to affect fat deposition more than muscle growth due to the high-fat content and energy-dense nature of the sunflower cake.

3.2. Meat Quality

Meat pH was not significantly affected by the experimental diets ($p > 0.05$), with a mean value of 5.56 (Table 5). The observed pH values indicate that the glycogen concentration available in the animals was satisfactory at slaughter and was unaffected by the increased EE intake, which can otherwise prevent the pH from lowering to optimal levels [39]. Similar results were observed by Junior et al. [35] when sunflower cake was included in lamb diets containing up to 9.5% EE, and by Oliveira et al. [40] in the goat diets.

Table 5. Meat quality of lambs fed diets with increased levels of high-oleic sunflower cake.

	High-Oleic Sunflower Cake (g/kg DM)				SEM	*p*-Value			Equation	R^2
	0	150	300	450		T	L	Q		
pH	5.57	5.57	5.60	5.56	0.02	0.79	0.86	0.86	Y = 5.58	-
Cooking loss, %	21.56	21.85	20.23	20.81	0.87	0.55	0.32	0.32	Y = 21.11	-
Shear force, kgf/cm^2	3.93	4.09	3.49	3.99	0.23	0.29	0.61	0.61	Y = 3.88	-
*L**	37.66	37.54	37.96	38.87	0.19	<0.01	<0.01	0.03	Y = 37.47 + 0.025x	0.61
*a**	21.76	21.12	20.87	20.91	0.48	0.55	0.17	0.17	Y = 21.17	-
*b**	13.22	13.13	13.20	13.59	0.26	0.62	0.37	0.37	Y = 13.29	-
Chrome	25.47	24.88	24.71	24.96	0.48	0.70	0.37	0.37	Y = 25.00	-
Hue	31.32	31.90	32.31	33.08	0.59	0.21	0.04	0.87	Y = 32.15	-
Moisture, %	73.15	72.90	72.60	72.08	0.14	<0.01	<0.01	0.40	Y = 73.18 − 0.022x	0.95
Crude protein, %	21.18	20.95	19.80	21.59	0.57	0.09	0.74	0.07	Y = 20.88	-
Intramuscular fat, %	4.52	4.52	4.95	5.37	0.13	<0.01	<0.01	0.13	Y = 4.39 + 0.020x	0.89
Ash, %	1.18	1.19	1.17	1.15	0.02	0.73	0.31	0.77	Y = 1.17	-

SEM: standard error of the mean; R^2: coefficient of determination; T: treatment effect; L: linear effect; Q: quadratic effect.

The inclusion of high-oleic sunflower cake did not significantly affect cooking loss or shear force values ($p > 0.05$) (Table 5). Shear force is commonly used as an index of meat toughness and is associated with higher concentrations of intramuscular fat [41]. Although no difference was observed in shear force, there was a linear increase in intramuscular fat concentration and *L** with the inclusion of up to 450 g/kg DM of high-oleic sunflower cake in the diet of lambs ($p < 0.01$) (Table 5). The increase in *L** is related to the increase in fat content, as this component contributes to higher luminosity in the meat [42]. This effect on luminosity through the addition of fat in the meat was also reported by Brito et al. [43] and Holman et al. [44]. Higher nutritional levels during the finishing period are associated with increased subcutaneous and intramuscular fat. Elevated intramuscular fat concentration has been shown to reduce the toughness of beef, which Nakamura et al. [45] suggested is due to changes in collagen architecture. Additionally, this elevation could affect the consumer's perception of toughness by improving meat juiciness and flavor. There was no treatment effect on *a**, *b**, Chroma and hue values ($p > 0.05$) (Table 5). The inclusion of high-oleic sunflower cake lamb diets linearly decreased meat moisture content ($p < 0.01$). This result is associated with the increase in fat as lipid content in meat is negatively associated with moisture content [46]. There was no treatment effect on ash and crude protein concentrations of lamb meat ($p > 0.05$) (Table 5). Oliveira et al. [40] observed the same pattern in goats fed sunflower cake. A significant increase in intramuscular fat concentration was observed by Qwele et al. [6] with the inclusion of 170g of sunflower cake in goats' diet.

3.3. Fatty Acid Profile

Inclusion of up to 450 g/kg DM of high-oleic sunflower cake in the diet of lambs did not significantly affect lauric (C12:0) and myristic (C14:0) fatty acids ($p > 0.05$) (Table 6). Chikwanha et al. [12] showed that even-chain fatty acids are frequently found in ovine meat and their adverse effects on human health have been widely debated. Fatty acids with 12 to 16 C are undesirable for human health because they raise the serum concentration of low-density lipoprotein, contributing to increased coagulation, inflammation, and insulin resistance [47]. In the present study, palmitic acid (C16:0) concentration decreased ($p < 0.01$) with the inclusion of high-oleic sunflower cake; on the other hand, an increase in stearic acid (C18:0) concentration ($p < 0.01$) was observed. These results are beneficial to human health as palmitic acid is associated with the development of cardiovascular diseases (CVDs) [48], and stearic acid is associated with protection against CVDs [15].

Table 6. Fatty acid composition (g/100g fatty acid methyl esters) of the longissimus muscle of lambs fed diets with increased levels of high-oleic sunflower cake.

	High-Oleic Sunflower Cake (g/kg DM)				SEM	*p*-Value Q			Equation	R^2
	0	150	300	450		T	L	Q		
C12:0, lauric	0.07	0.08	0.08	0.07	0.003	0.13	0.98	0.23	Y = 0.08	-
C14:0, myristic	1.97	2.04	2.07	2.06	0.065	0.70	0.59	0.68	Y = 2.04	-
C16:0, palmitic	23.94	22.65	22.09	21.70	0.290	<0.01	<0.01	0.09	Y = 29.15−0.70x	0.99
C17:0, margaric	1.28	0.96	0.88	0.70	0.035	<0.01	<0.01	0.06	Y = 1.44 − 0.18x	0.93
C18:0, stearic	12.80	12.55	13.91	14.80	0.412	<0.01	<0.01	0.18	Y = 8.48 + 0.73x	0.87
∑ Saturated	41.06	39.11	39.28	39.09	0.245	<0.01	<0.01	0.03	Y = 42.96 − 0.57x	0.80
C16:1c9, palmitoleic	2.46	2.43	2.14	2.06	0.092	<0.01	<0.01	0.77	Y = 3.73 − 0.15x	0.99
C17:1, heptadecenoic	0.92	0.71	0.57	0.47	0.022	<0.01	<0.01	0.02	Y = 1.43 − 0.15x	0.98
C18:1c9, oleic	46.53	48.88	49.60	49.40	0.490	<0.01	<0.01	0.08	Y = 46.92 + 0.90x	0.78
∑ Monounsaturated	53.97	55.46	55.88	56.31	0.484	0.01	<0.01	0.27	Y = 56.34 + 0.75x	0.99
C18:2 n6, linoleic	2.31	2.23	1.86	1.88	0.118	<0.01	<0.01	0.70	Y = 2.90 − 0.16x	0.74
C18:2 c9 t11 rumenic	0.22	0.26	0.26	0.28	0.012	0.01	<0.01	0.43	Y = 0.18 + 0.01	0.77
C18:3 n6, γ-linolenic	0.03	0.03	0.04	0.04	0.002	<0.01	<0.01	0.61	Y = −0.006 + 0.004	0.99
C18:3 n3, α-linolenic	0.07	0.06	0.07	0.07	0.008	0.65	0.50	0.55	Y = 0.07	-
C20:4 n6, araquidonic	0.560	0.536	0.532	0.583	0.027	0.553	0.77	0.36	Y = 0.553	-
C20:5 n3, EPA	0.004	0.004	0.007	0.007	0.0008	0.01	<0.01	0.80	Y= −0.013 + 0.001x	0.99
C22:5 n3, DPA	0.06	0.07	0.06	0.05	0.004	<0.01	<0.01	0.10	Y = 0.03 − 0.006x	0.64
C22:6 n3, DHA	0.007	0.008	0.006	0.009	0.001	0.43	0.47	0.55	Y = 0.007	-
∑ Polyunsaturated	3.38	3.30	2.79	2.95	0.147	0.02	0.01	0.45	Y = 3.50 − 0.18x	0.69
∑ Unsaturated	57.29	59.00	59.00	59.23	0.365	<0.01	<0.01	0.05	Y = 58.42 + 0.58x	0.81
∑ UFA /∑ SFA	1.37	1.49	1.50	1.51	0.015	<0.01	<0.01	0.02	Y = 1.29 + 0.04x	0.99
∑ n3	0.36	0.37	0.33	0.31	0.008	<0.01	<0.01	0.19	Y = 0.37 − 0.02x	0.81
∑ n6	3.01	2.90	2.37	2.38	0.077	<0.01	<0.01	0.51	Y = 2.95 − 0.24x	0.71
n6: n3	8.54	8.00	6.96	7.32	0.314	<0.01	<0.01	0.09	Y = 8.43 − 0.45x	0.66
Δ9-desaturase 16	9.91	9.80	9.46	8.50	0.112	<0.01	<0.01	0.04	Y = 0.37 − 0.02x	0.78
Δ9-desaturase 18	79.18	79.30	78.07	76.89	0.353	<0.01	<0.01	0.07	Y = 79.70 − 0.81x	0.59
Elongase	0.69	0.71	0.72	0.72	0.003	<0.01	<0.01	0.03	Y = 0.64 + 0.01x	0.95
Atherogenicity	0.54	0.53	0.53	0.51	0.004	<0.01	<0.01	0.87	Y = 0.56 − 0.008x	0.81
Thrombogenicity	1.32	1.30	1.24	1.24	0.019	0.02	<0.01	0.64	Y = 1.50 − 0.03x	0.63

SEM: standard error of the mean; R^2: coefficient of determination; T: treatment effect; L: linear effect; Q: quadratic effect.

Ruminal biohydrogenation can be negatively affected by the high inclusion of polyunsaturated fatty acids in the diet [49]. Additionally, the conversion of C18:1 to C18:0 is impaired by the reduction in rumen pH, resulting from the high intake of rapidly fermentable carbohydrates [50]. In the present study, the fat source and the supply of fibrous carbohydrates, both provided by sunflower cake, contributed to the maintenance of rumen metabolism, favoring complete biohydrogenation and the formation of stearic acid, which was subsequently deposited in the meat. Furthermore, the significant increase in C18:1 dietary intake with the elevated levels of high-oleic sunflower cake may have potentiated the final stage of the ruminal biohydrogenation process by continuously providing a high concentration of the C18:0 precursor.

The inclusion of high-oleic sunflower cake in the diet significantly decreased the sum of the SFA ($p < 0.01$). There was an increase in oleic acid (C18:1 c9) concentration in meat with the inclusion of high-oleic sunflower cake ($p < 0.01$) as a result of the high concentration of this fatty acid in the by-product used. A significant decrease in enzymatic activity of Δ9-desaturase 18 ($p < 0.01$) and Δ9-desaturase 16 ($p < 0.01$) was observed with the inclusion of high-oleic sunflower cake. The reduction in Δ9-desaturase 18 is associated with the modulatory effect of C18: 1c9 on sterol regulatory element-binding protein-1c (SREBP-1c), the transcription factor that encodes genes for the enzyme Δ9-desaturase (SCD1) [51]. Choi et al. [52] reported that oleic acid downregulated SCD1 expression in bovine subcutaneous and intramuscular preadipocytes.

The inclusion of up to 450 g/kg DM of high-oleic sunflower cake in the diet reduced the concentration of palmitoleic acid (C16:1c9) in the meat ($p < 0.01$). This monounsaturated fatty acid is associated with increased insulin resistance [53]. This occurrence may have

caused a linear increase in stearic acid ($p < 0.01$), which is the fatty acid with the highest ruminal flow for absorption in the small intestine [54].

The essential fatty acids series n6 (linoleic) and n3 (linolenic) are the main fatty acids that contribute to human well-being and health [55]. In the present study, the inclusion of high-oleic sunflower cake reduced the concentration of these fatty acids in the diets (Table 2), which resulted in a decrease in n6 concentration in meat ($p < 0.01$) (Table 6). Although there was a reduction in linoleic acid concentration in the diets with the inclusion of high-oleic sunflower cake, this result did not affect linolenic acid concentration in the meat ($p > 0.05$). A study with cattle fed sunflower cake up to 27% in DM supports the results for linolenic acid in the meat; however, there were opposite findings for the concentration of linoleic acid and EPA [56]. The inclusion of high-oleic sunflower cake in the diet reduced the sum of PUFA in the meat ($p < 0.02$) as a result of the intrinsic characteristics of the by-product used, which presented a small amount of PUFA (5.66 g/100 g).

The inclusion of high-oleic sunflower cake in the diet resulted in a linear increase in rumenic acid (C18:2 c9 t11) and conjugated linoleic acid (CLA), achieving an approximate 27% increase compared to the control diet. The CLA is a fatty acid associated with the prevention of diseases such as cancer, atherosclerosis, alterations in protein and energy metabolism, and reduced immune response [12,57]. The CLA can be formed through the ruminal biohydrogenation process or by the action of Δ9-desaturase on vaccenic acid in the tissues [58]. In the present study, there was a reduction in Δ9-desaturase activity with the inclusion of high-oleic sunflower cake in the diet, which suggests that the greatest contribution to the increase in CLA in the meat was due to the biohydrogenation process. Possibly, a higher lipid concentration in the diets with the inclusion of high-oleic sunflower cake compared to the control diet increased the supply of fatty acid intermediates of ruminal biohydrogenation in the small intestine. During FA biohydrogenation, including the CLA intermediate, FA continually leaves the rumen, is absorbed across the small intestine, and can be deposited in muscle tissue [59].

The inclusion of high-oleic sunflower cake in the diet of lambs did not affect DHA ($p > 0.05$); however, it increased EPA ($p < 0.01$) and reduced DPA ($p < 0.01$). The conversion of C18: 2 and C18: 3 to PUFA is dependent of the n6/n3 ratio in the diet [60]. Elongase activity increased with the inclusion of high-oleic sunflower cake in the diet ($p < 0.01$); however, it did not increase the concentration of DPA and DHA. This result is associated with greater proportions of C18: 0 and a lower proportion of C16: 0 in lamb meat fed with high-oleic sunflower cake compared to the control diet. The sum of n3 and n6 fatty acids decreased ($p < 0.01$) with the inclusion of sunflower cake, reducing the ratio n6/n3 ($p < 0.01$). Omega-3 fatty acids are precursors to a series of bioactive fatty acids important in reducing the risk of cardiovascular, cancer, and Alzheimer's disease [61]. Desirable values for the n6/n3 ratio in foods for human consumption are between 2 and 6 [62,63]. Although the inclusion of high-oleic sunflower cake reduced the concentration of n3 and n6 fatty acids in the meat, a linear decrease in the atherogenicity and thrombogenicity indices was observed ($p < 0.01$). Lower values of atherogenicity and thrombogenicity indices are associated with a greater amount of anti-atherogenic fatty acids [64]. The inclusion of high-oleic sunflower cake in the diet of the lambs improved the fatty acid profile, as evidenced by a reduction in saturated fatty acids in the meat.

4. Conclusions

Up to 450 g/kg DM dietary supplementation of feedlot lambs with sunflower cake rich in oleic acid did not affect animal performance, even in those lambs that consumed diets containing approximately 9% ether extract. Although the increased levels of high-oleic sunflower cake in lambs diet decreased carcass yield and increased fat deposits, the

inclusion of up to 450 g/kg DM of high-oleic sunflower cake in the diet of the lambs significantly reduced the concentration of hypercholesteremic fatty acids (C14:0 and C16:0), increased the concentration of fatty acids with neutral or protective effects on cardiovascular diseases (C18:0 and C18:1), and that of EPA and CLA in the meat. This study demonstrates the potential use of up to 450 g/kg DM of high-oleic sunflower cake in feedlot diets for lambs to improve nutritional properties of red meat without compromising animal performance.

Author Contributions: Conceptualization, D.M.C. and I.F.F.-G.; Methodology, D.M.C. and I.F.F.-G.; Validation, D.M.C. and I.F.F.-G.; Formal Analysis, D.M.C. and I.F.F.-G.; Investigation, D.M.C., I.F.F.-G., I.J.d.S. and P.C.G.D.J.; Resources, I.F.F.-G.; Data Curation, D.M.C.; Writing—Original Draft Preparation, D.M.C.; Writing—Review and Editing, D.M.C., I.F.F.-G., T.I.R.C.A. and F.A.P.A.; Visualization, D.M.C., I.F.F.-G. and N.G.A.; Supervision, I.F.F.-G.; Project Administration, I.F.F.-G.; Funding Acquisition, I.F.F.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CNPq-Brazil (National Council for Scientific and Technological Development, grant number 483631/2010-8) for project financial support.

Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee from Federal University of Lavras (protocol number 105/12).

Data Availability Statement: The data presented in this study will be available upon reasonable request to the second author, I.F.F.-G.

Acknowledgments: The authors would like to thank the GAO/UFLA-Brazil (Grupo de Apoio à Ovinocaprinocultura, Federal University of Lavras) research team for supporting data collection and laboratory analysis of this study.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Duca, D.; Toscano, G.; Riva, G.; Mengarelli, C.; Rossini, G.; Pizzi, A.; Pedretti, E.F. Quality of residues of the biodiesel chain in the energy field. *Ind. Crops Prod.* **2015**, *75*, 91–97. [CrossRef]
2. Lima, A.G.V.O.; Silva, T.M.; Bezerra, L.R.; Pereira, E.S.; Barbosa, A.M.; Ribeiro, R.D.X.; Rocha, T.C.; Trajano, J.S.; Oliveira, R.L. Intake, digestibility, nitrogen balance, performance and carcass traits of Santa Ines lamb fed with sunflower cake from biodiesel production. *Small Rumin. Res.* **2018**, *168*, 19–24. [CrossRef]
3. FAO. The State of Food and Agriculture. In *FAO Biofuels: Prospects, Risks and Opportunities*; FAO: Rome, Italy, 2008.
4. Ferfuia, C.; Vanozzi, G.P. Maternal effect on seed fatty acid composition in a reciprocal cross of high oleic sunflower (*Helianthus annuus* L.). *Euphytica* **2015**, *205*, 325–336. [CrossRef]
5. Melo, M.A.R.; Maria, A.M.F.; Silva, E.V.; Filho, J.R.C.; Souza, A.G. Study of the oxidative stability of oils vegetables for production of biodiesel. *Rev. Verde Agroecologia Desenvolv. Sustent.* **2014**, *9*, 84–88.
6. Qwele, K.; Hugo, A.; Oyedemi, S.O.; Moyo, B.; Masika, P.J.; Muchenje, V. Chemical composition, fatty acid content and antioxidant potential of meat from goats supplemented with Moringa (*Moringa oleifera*) leaves, sunflower cake and grass hay. *Meat Sci.* **2013**, *93*, 455–462. [CrossRef] [PubMed]
7. Selvam, T.A.; Manikantan, M.R.; Chand, T.; Sharma, R.; Seerangrayar, S. Compression loading behaviour of sunflower seeds and kernels. *Int. Agrophys.* **2014**, *28*, 543–548. [CrossRef]
8. Wood, J.D.; Richardson, R.I.; Nute, G.R.; Fisher, A.V.; Campo, M.M.; Kasapidou, E.; Sheard, P.R.; Enser, M. Effects of fatty acids on meat quality: A review. *Meat Sci.* **2004**, *66*, 21–32. [CrossRef]
9. Alvarenga, T.I.R.C.; Chen, Y.; Furusho-Garcia, I.F.; Perez, J.R.O.; Hopkins, D.L. Manipulation of Omega-3 PUFAs in lamb: Phenotypic and genotypic views. *Compr. Rev. Food Sci. Food Saf.* **2015**, *14*, 189–204. [CrossRef] [PubMed]
10. Daley, C.A.; Abbott, A.; Doyle, P.S.; Nader, G.A.; Larson, S. A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. *Nutr. J.* **2010**, *9*, 2891–2899. [CrossRef]
11. Shingfield, K.J.; Bonnet, M.; Scollan, N.D. Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal* **2013**, *7*, 132–162. [CrossRef]
12. Chikwanha, O.C.; Vahmani, P.; Muchenje, V.; Dugan, M.E.; Mapiye, C. Nutritional enhancement of sheep meat fatty acid profile for human health and wellbeing. *Food Res. Int.* **2018**, *104*, 25–38. [CrossRef]

13. Bressan, M.C.; Rossato, L.V.; Rodrigues, E.C.; Alves, S.P.; Bessa, R.J.B.; Ramos, E.M.; Gama, L.T. Genotype x environment interactions for fatty acid profiles in Bos indicus and Bos taurus finished on either pasture or grain. *J. Anim. Sci.* **2011**, *89*, 221–232. [CrossRef]
14. Wood, J.D.; Enser, M.; Fisher, A.V.; Nute, G.R.; Sheard, P.R.; Richardson, R.I.; Hughes, S.I.; Whittington, F.M. Fat deposition, fatty acid composition and meat quality: A review. *Meat Sci.* **2008**, *78*, 343–358. [CrossRef]
15. Hunter, J.E.; Zhang, J.; Kris-Etherton, P.M. Cardiovascular disease risk of dietary stearic acid compared with trans, other saturated, and unsaturated fatty acids: A systematic review. *Am. J. Clin. Nutr.* **2010**, *91*, 46–63. [CrossRef] [PubMed]
16. Jenkins, T.C. Lipid Metabolism in the Rumen. *J. Dairy Sci.* **1993**, *76*, 3851–3863. [CrossRef]
17. Moura, E.S.; Silva, L.D.F.; Peixoto, E.L.T.; Bumbieris Junior, V.H.; Ribeiro, E.L.A.; Mizubuti, I.Y.; Fortaleza, A.P.S. Sunflower cake in diets for lambs: Intake, digestibility, nitrogen balance and rumen parameters. *Semina Ciênc. Agrár.* **2015**, *36*, 2247–2258. [CrossRef]
18. NRC. *Nutrient Requirements of Dairy Cattle*, 7th ed.; National Academy Press: Washington, DC, USA, 2001.
19. AOAC. *Association of Official Analytical Chemists, 18th ed*; Official Methods of Analysis; AOAC: Gaithersburg, MD, USA, 1990.
20. Van Soest, P.V.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber, neutral detergent fiber, and no starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef]
21. MacDougall, D.B. Colour of meat. In *Quality Attributes and Their Measurement in Meat, Poultry and Fish Products*; Pearson, A.M., Dutson, T.R., Eds.; Advances in Meat Research Series; Blackie Academic and Professional: London, UK, 1994; pp. 79–93.
22. Wheeler, T.L.; Shackelford, S.D.; Koohmaraie, M. Sampling, Cooking, and Coring Effects on Warner-Bratzler Shear Force Values in Beef. *J. Anim. Sci.* **1996**, *74*, 1553–1562. [CrossRef] [PubMed]
23. Hara, A.; Radin, N.S. Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **1978**, *90*, 420–426. [CrossRef]
24. Rodríguez-Ruiz, J.; Belarbi, E.H.; Sánchez, J.L.G.; Alonso, D.L. Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotechnol. Tech.* **1998**, *12*, 689–691. [CrossRef]
25. Malau-Aduli, A.E.O.; Siebert, B.D.; Bottema, C.D.K.; Pitchford, W.S. A comparison of the fatty acid composition of triacylglycerols in adipose tissue from Limousin and Jersey cattle. *Aust. J. Agric. Res.* **1997**, *48*, 715–722. [CrossRef]
26. Ulbricht, T.L.V.; Southgate, D.A.T. Coronary heart disease: Seven dietary factors. *Lancet* **1991**, *338*, 985–992. [CrossRef] [PubMed]
27. Palmquist, D.L.; Jenkins, T.C. A 100-Year Review: Fat feeding of dairy cows. *J. Dairy Sci.* **2017**, *100*, 10061–10077. [CrossRef]
28. Palmquist, D.L.; Mattos, W.R.S. Metabolismo de lipídeos. In *Nutrição de Ruminantes*; Funep: Jaboticabal, Brazil, 2006.
29. Maia, M.R.G.; Chaudhary, L.C.; Bestwick, C.S.; Richardson, A.J.; McKain, N.; Larson, T.R.; Graham, I.A.; Wallace, R.J. Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. *BMC Microbiol.* **2010**, *10*, 52. [CrossRef]
30. Hristov, A.N.; Grandeen, K.L.; Ropp, J.K.; McGuire, M.A. Effect of sodium laurate on ruminal fermentation and utilization of ruminal ammonia nitrogen for milk protein synthesis in dairy cows. *J. Dairy Sci.* **2004**, *87*, 1820–1831. [CrossRef] [PubMed]
31. Oldick, B.S.; Firkins, J.L. Effects of degree of fat saturation on fiber digestion and microbial protein synthesis when diets are fed twelve times daily. *J. Anim. Sci.* **2000**, *78*, 2412–2420. [CrossRef] [PubMed]
32. Weld, K.; Armentano, L.E. Milk fat secretion in lactating dairy cattle is influenced by soybean particle size and fatty acid profile. *J. Dairy Sci.* **2016**, *94*, 344.
33. Kendall, C.; Leonardi, C.; Hoffman, P.C.; Combs, D.K. Intake and milk production of cows fed diets that differed in dietary neutral detergent fiber and neutral detergent fiber digestibility. *J. Dairy Sci.* **2009**, *92*, 313–323. [CrossRef]
34. Morril, J.L.; Van Horn, C.J.W. (Eds.) *Large Dairy Herd Management*; American Dairy Science Association: Savoy, IL, USA, 1992; pp. 401–410.
35. Junior, F.F.; Ribeiro, E.L.A.; Mizubuti, I.Y.; Silva, L.D.F.; Barbosa, M.A.A.F.; Prado, O.P.P.; Pereira, E.S.; Pimentel, P.G.; Constantino, C. Características de carcaça e qualidade da carne de cordeiros Santa Inês alimentados com torta de girassol em substituição ao farelo de algodão. *Semin. Cienc. Agrar.* **2013**, *34*, 3999–4014. [CrossRef]
36. Palmieri, A.D.; Oliveira, R.L.; Ribeiro, C.V.D.M.; Ribeiro, M.D.; Ribeiro, R.D.X.; Leão, A.G.; Agy, M.S.F.A.; Ribeiro, O.L. Effects of substituting soybean meal for sunflower cake in the diet on the growth and carcass traits of crossbred boer goat kids. *Asian-Aust. J. Anim. Sci.* **2012**, *25*, 59–65. [CrossRef] [PubMed]
37. Hristov, A.N.; Kennington, L.R.; McGuire, M.A.; Hunt, C.W. Effect of diets containing linoleic acid- or oleic acid-rich oils on ruminal fermentation and nutrient digestibility, and performance and fatty acid composition of adipose and muscle tissues of finishing cattle. *J. Anim. Sci.* **2005**, *83*, 1312–1321. [CrossRef]
38. Torres, R.N.S.; Ghedini, C.P.; Chardulo, L.A.L.; Baldassini, W.A.; Curi, R.A.; Pereira, G.L.; Schoonmaker, J.P.; Almeida, M.T.C.; Costa, C.; Machado Neto, O.R. Potential of different strategies to increase intramuscular fat deposition in sheep: A meta-analysis study. *Small Rumin. Res.* **2024**, *234*, 107258. [CrossRef]
39. Apple, J.K.; Dikeman, M.E.; Minton, J.E.; McMurphy, R.M.; Fedde, M.R.; Leith, D.E.; Unruh, J.A. Effects of restraint and isolation stress and epidural blockade on endocrine and blood metabolite status, muscle glycogen metabolism, and incidence of dark-cutting Longissimus muscle of sheep. *J. Anim. Sci.* **1995**, *73*, 2295–2307. [CrossRef] [PubMed]

40. Oliveira, R.L.; Palmieri, A.D.; Carvalho, S.T.; Leão, A.G.; Abreu, C.L.; Ribeiro, C.V.D.M.; Pereira, E.S.; Carvalho, G.G.P.; Bezerra, L.R. Commercial cuts and chemical and sensory attributes of meat from crossbred Boer goats fed sunflower cake-based diets. *Anim. Sci. J.* **2015**, *86*, 557–562. [CrossRef]
41. Minick, J.A.; Dikeman, M.E.; Pollak, E.J.; Wilson, D.E. Heritability and correlation estimates of Warner-Bratzler shear force and carcass traits from Angus-, Charolais-, Hereford-, and Simmental-sired cattle. *Can. J. Anim. Sci.* **2004**, *84*, 599–609. [CrossRef]
42. Realini, C.E.; Duckett, S.K.; Brito, G.W.; Dalla Rizza, M.; De Mattos, D. Effect of pasture vs. concentrate feeding with or without antioxidants on carcass characteristics, fatty acid composition, and quality of Uruguayan beef. *Meat Sci.* **2004**, *66*, 567–577. [CrossRef] [PubMed]
43. Brito, G.F.; Ponnampalam, E.N.; Hopkins, D.L. The effect of extensive feeding systems on growth rate, carcass traits, and meat quality of finishing lambs. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 23–38. [CrossRef]
44. Holman, B.W.B.; Ven, R.V.; Mao, Y.; Coombs, C.E.O.; Hopkins, D.L. Using instrumental (CIE and reflectance) measures to predict consumers' acceptance of beef colour. *Meat Sci.* **2017**, *127*, 57–62. [CrossRef] [PubMed]
45. Nakamura, Y.-N.; Tsuneishi, E.; Kamiya, M.; Yamada, A. Histological contribution of collagen architecture to beef toughness. *J. Food Sci.* **2020**, *75*, E73–E77. [CrossRef]
46. D'Alessandro, A.G.; Palazzo, M.; Petrotos, K.; Goulas, P.; Martemucci, G. Fatty acid composition of light lamb meat from Leccese and Comisana dairy breeds as affected by slaughter age. *Small Rumin. Res.* **2015**, *127*, 36–43. [CrossRef]
47. Calder, P.C. Functional roles of fatty acids and their effects on human health. *J. Parenter. Enteral Nutr.* **2015**, *39*, 18S–32S. [CrossRef] [PubMed]
48. Jiang, J.; Xiong, Y.L. Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. *Meat Sci.* **2016**, *120*, 107–117. [CrossRef] [PubMed]
49. Ladeira, M.M.; Schoonmaker, J.P.; Swanson, K.C.; Duckett, S.K.; Gionbelli, M.P.; Rodrigues, L.M.; Teixeira, P.D. Review: Nutrigenomics of marbling and fatty acid profile in ruminant meat. *Animal* **2018**, *12*, s282–s294. [CrossRef] [PubMed]
50. Fievez, V.; Vlaeminck, B.; Jenkins, T.; Enjalbert, F.; Doreau, M. Assessing rumen biohydrogenation and its manipulation in vivo, in vitro and in situ. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 740–756. [CrossRef]
51. Waters, S.M.; Kelly, J.P.; O'Boyle, P.; Moloney, A.P.; Kenny, D.A. Effect of level and duration of dietary n-3 polyunsaturated fatty acid supplementation on the transcriptional regulation of Δ9-desaturase in muscle of beef cattle. *J. Anim. Sci.* **2009**, *87*, 244–252. [CrossRef]
52. Choi, S.H.; Park, S.K.; Johnson, B.J.; Chung, K.Y.; Choi, C.W.; Kim, K.H.; Kim, W.Y.; Smith, S.B. AMPKα, C/EBPβ, CPT1β, GPR43, PPARγ, and SCD gene expression in single-and co-cultured bovine satellite cells and intramuscular preadipocytes treated with palmitic, stearic, oleic, and linoleic acid. *Asian-Australas. J. Anim. Sci.* **2015**, *28*, 411–419. [CrossRef] [PubMed]
53. Mozaffarian, D.; Micha, R.; Wallace, S. Effects on coronary heart disease of increasing polyunsaturated fat in place of saturated fat: A systematic review and meta-analysis of randomized controlled trials. *PLoS Med.* **2010**, *7*, e1000252. [CrossRef] [PubMed]
54. Bauman, D.E.; Lock, A.L. Milk fatty acid composition: Challenges and opportunities related to human health. In Proceedings of the 26th World Buiatrics Congress, Santiago, Chile, 14–18 November 2010; pp. 278–289.
55. Marangoni, F.; Agostoni, C.; Borghi, C.; Catapano, A.L.; Cena, H.; Ghiselli, A.; Poli, A. Dietary linoleic acid and human health: Focus on cardiovascular and cardiometabolic effects. *Atherosclerosis* **2020**, *292*, 90–98. [CrossRef]
56. Oliveira, V.S.; Oliveira, R.L.; Goes, R.H.T.B.; Silva, T.M.; Silva, L.F.; Freitas, L.S.; Pereira, E.S.; Bezerra, L.R. Physicochemical composition, fatty acid profile and sensory attributes of the meat of young Nellore bulls fed sunflower cake from the biodiesel industry. *Livest. Sci.* **2019**, *227*, 97–104. [CrossRef]
57. Jóźwiak, M.; Filipowska, A.; Fiorino, F.; Struga, M. Anticancer activities of fatty acids and their heterocyclic derivatives. *Eur. J. Pharmacol.* **2020**, *871*, 172937. [CrossRef]
58. Nute, G.R.; Richardson, R.I.; Wood, J.D.; Hughes, S.I.; Wilkinson, R.G.; Cooper, S.L.; Sinclair, L.A. Effect of dietary oil source on the flavour and the colour and lipid stability of lamb meat. *Meat Sci.* **2007**, *76*, 715–720. [CrossRef]
59. Enjalbert, F.; Combes, S.; Zened, A.; Meynadier, A. Rumen microbiota and dietary fat: A mutual shaping. *J. Appl. Microbiol.* **2017**, *123*, 782–797. [CrossRef]
60. Harnack, K.; Andersen, G.; Somoza, V. Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids. *Nutr. Metab.* **2009**, *19*, 6–8. [CrossRef]
61. Nunes, B.; Pinho, C.; Sousa, C.; Melo, A.R.; Bandarra, N.; Silva, M.C. Relevance of omega-3 and omega-6/omega-3 ratio in preventing cognitive impairment. *Acta Médica Portuguesa* **2017**, *30*, 213–223. [CrossRef]
62. French, P.; Stanton, C.; Lawless, F.; O'riordan, E.G.; Monahan, F.J.; Caffrey, P.J.; Moloney, A.P. Fatty acid composition, including conjugated linoleic acid, of intramuscular fat from steers offered grazed grass, grass silage, or concentrate-based diets. *J. Anim. Sci.* **2000**, *78*, 2849–2855. [CrossRef]

63. Wood, J.D.; Enser, M. Factors influencing fatty acids in meat and the role of antioxidants in improving meat quality. *Br. J. Nutr.* **1997**, *78*, S49–S60. [CrossRef]
64. Turan, H.; Sönmez, G.; Kaya, Y. Fatty acid profile and proximate composition of the thornback ray (*Raja clavata*, L. 1758) from the Sinop coast in the Black Sea. *J. Fish. Sci.* **2007**, *1*, 97–103. [CrossRef]

MDPI AG
Grosspeteranlage 5
4052 Basel
Switzerland
Tel.: +41 61 683 77 34

Agriculture Editorial Office
E-mail: agriculture@mdpi.com
www.mdpi.com/journal/agriculture

Academic Open
Access Publishing

mdpi.com

ISBN 978-3-7258-3778-6