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Safety and Efficacy of Feed Additives in Animal Production

Edited by
Lubomira Gresakova and Emilio Sabia

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About the Editors

Ľubomíra Grešáková

Ľubomíra Grešáková, Ph.D., is a senior researcher at the Department of Digestive Tract Physiology, at the Institute of Animal Sciences, Centre of Biosciences of the Slovak Academy of Sciences in Košice, Slovak Republic. Her research focuses on studying trace elements in animal nutrition and trace mineral metabolism in livestock, focusing on the transport mechanisms and bioavailability of trace elements.

Emilio Sabia

Emilio Sabia: works at the School of Agriculture, Food, Forestry and Environment (University of Basilicata, Italy). He obtained his PhD from the University of Basilicata (Italy) in 2009 and has carried out research on agriculture, the environment, animal nutrition, and animal welfare. In recent years, he has mainly focused on the environmental sustainability of agricultural production systems, using and further developing life cycle assessment (LCA) methosd to quantify greenhouse gas emissions and the other environmental impacts of agriculture and to investigate mitigation strategies in the livestock sector, for example, through changes in feeding and breeding.

Preface to "Safety and Efficacy of Feed Additives in Animal Production"

Due to the challenge of reducing the environmental impact of animal production, as well as maintaining animal health and sustainable livestock productivity, it is necessary to change the feeding strategy of livestock. Feed supplementation with safe and efficient nutrient additives should provide optimal animal performance and maximize livestock productivity, as well as reducing environmental pollution from animal production.

This book consists of one review and 14 articles written by research experts in their topic of interest, concerning the use of nutritional feed additives in animal nutrition to improve livestock health and productivity with respect to the environment. Several novel feed supplements that improve nutrient utilization and micronutrient bioavailability in animals, and provide safe and functional foods, were introduced.

Lubomira Gresakova and Emilio Sabia
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Article

Dietary Supplementation of Some Antioxidants as Attenuators of Heat Stress on Chicken Meat Characteristics

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Abstract: The study evaluated the effect of dietary chromium and vitamin C, Zinc, and sorrel wood powder supplements on chicken health and the nutritional, textural, and sensorial quality of chicken meat. A total of 120 Cobb 500 chickens (heat stress, 32 °C) were assigned into four treatments: control diet (C) and three test diets including 200 µg/kg diet chromium picolinate and supplemented with: 0.25 g vitamin C(VC)/kg diet (E1), 0.025 g Zn/kg diet (E2), and 10 g creeping wood sorrel powder (CWS)/kg diet (E3). Crude protein concentration increased in the breast meat from the E3 group; crude fat decreased in E1 and E3 compared to those fed the C diet. Dietary combinations of CrPic with VC, Zn, and CWS increased redness and decreased the luminosity parameter of breast meat compared with the C group. Dietary combinations of CrPic with VC and CWS lowered the hardness of breast meat. Significant positive correlation was found between hardness–gumminess (r = 0.891), gumminess–cohesiveness (r = 0.771), cohesiveness–resilience-EE (r = 0.861; r = 0.585), ash-L* (r = 0.426), and a negative one between ash–a* (r = 0.446). In conclusion, a dietary combination of CrPic with VC, Zn, and CWS as antioxidant sources could have a beneficial effect on quality without affecting sensory attributes.

Keywords: meat; antioxidants; chicken; sensory; texture; heat stress

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1. Introduction

Chicken meat is the most accessible protein source for humans in most countries. However, the poultry sector may be limited by a series of problems, including high or low environmental temperature, high stocking density, etc. Heat stress is one of the main problems encountered within the poultry sector [1,2]. Heat stress affects nutritional quality, resulting in a lower protein content and higher fat deposition, etc. [3], and sensorial quality such as low water-holding capacity, higher brightness, and lower redness of meat, etc. [4]. In recent years, much attention has been paid to the inclusion of phytochemicals (vitamins, minerals, phytogenic feed additives) with antioxidant properties in the feed mixture, which would overcome the effects of heat stress on poultry health and on the quality of the meat they produce [5]. Some authors have stated that dietary Cr supplementation had a positive effect on meat quality by decreasing the fat content of the carcass, [6] and on carcass traits of broiler chicks in natural [7,8] or heat stress conditions [9]. Moreover, it has been suggested that combinations between Cr and other antioxidants (Zn, vitamin C) might have a synergistic action [2,10].

Vitamin C is a renowned water-soluble vitamin, with undeniable antioxidant activity. In heat-stressed broilers, dietary vitamin C supplementations have improved performance and humoral immunity [11].

Zinc is one of the most important components of the poultry diet, being a cofactor for enzymes with implication in the antioxidant defense system [12]. It was reported

that dietary Zn supplementation in broiler chickens improved broiler carcass quality by reducing the percentage of abdominal fat, suppressing lipid peroxidation of chicken meat [13].

Creeping wood sorrel (*Oxalis corniculata*) is a little-known plant, which contains an important amount of vitamin C [14] and phenolic compounds with strong free radical scavenging activity [15]. Previous studies demonstrated that including creeping wood sorrel in chicken diet (1%) and Cr picolinate (0.2 mg/kg diet) could counteract the effects of heat stress, resulting in unaffecting growth performance, improving lactobacilli populations and reducing pathogenic bacteria [14].

However, dietary supplementation with Cr together with other minerals such as Zn, vitamins (C, E), or phytogenic feed additives may potentate the antioxidant effect. Therefore, the objective of this study was to assess the effect of dietary inclusion of chromium and vitamin C, Zinc, and sorrel wood powder supplements on chicken health and nutritional, textural, and sensorial quality of chicken meat.

2. Materials and Methods

2.1. Birds, Diets, and Treatments

A 6 week feeding trial (0–42 d) was conducted on 120 unsexed Cobb 500 chickens (1 day of age), weighed and randomly divided into four homogenous groups (C, E1, E2, and E3) with 30 chickens/group. Each group consisted of 6 replicates, with 5 chickens per replicate (1 replicate/chickens/cage). The feeding trial was conducted in an experimental hall of the National Institute for Animal Nutrition (Ilfov, Romania) according to experimental protocol approved (case no. 4775/02.08.2019) by the Ethics Commission of the Institute (Ethical Committee no. 52/30.07.2014). The chicks were housed in three-tiered digestibility cages (cage dimensions 65 × 75 × 45 cm, one cage per replicate) During the experimental period, the chickens were reared under controlled environmental conditions and monitored by a Viper Touch computer (temperature 32 ± 0.5 °C, humidity 36 ± 1.4%, with 0.38 ± 0.01% ventilation/broiler, and 899 ± 0.2 ppm CO_2 emission). The light regimen was 23 h light/1 h darkness. Compared with the control diet (C group), the experimental diets (E1, E2, and E3 group) included 200 µg/kg diet chromium picolinate, CrPic (Table 1). Additionally, the experimental diets contained 0.25 g vitamin C (VC)/kg diet (E1), 0.025 g Zn/kg diet (E2), and 10 g creeping wood sorrel powder (CWS)/kg diet (E3). The chickens had free access to feed and water. Creeping wood sorrel was analyzed, and the data was published previously by Saracila et al. [14]. The analysis revealed a concentration of 15.44% CP, 2.83% EE, 4.96 mg/g GAE total polyphenols, 31.60 mmol ascorbic acid equivalent antioxidant capacity and 11.77 mg/100 g vitamin C.

2.2. Sample Collection and Analysis

At the end of experiment (42 days), from each group with homogenous weights, 6 chickens/group were randomly selected (1 chicken/replicate). From each group selected, blood samples were collected aseptically from the sub-axial region into heparinized test tubes. The blood samples were centrifuged (775× *g* for 25 min at 4 °C), and the serum obtained was analyzed using an automatic BS-130 chemistry analyzer (Bio-Medical Electronics Co., Ltd., Shenzhen, China) in order to determine the biochemical parameters (serum glucose, cholesterol, triglycerides, phosphorus, calcium, iron, alanine aminotransferase, and aspartate aminotransferase).

2.3. Sampling

After collecting blood samples, according to the approved working protocol, the chicks (6 chicks/group) were electrically stunned and subsequently slaughtered by cervical dislocation. After bleeding and evisceration, thigh and breast meat samples were collected (6 breast and 6 thigh meat samples per group). From each of the samples collected, samples were taken to determine the proximate composition (dry matter—DM, crude protein—CP,

ether extractives—EE, ash) and to determine the color, texture, and pH parameters. Until analysis, the samples were stored in plastic bags at −20 °C.

Table 1. Nutrient composition of experimental basal diets (%).

Ingredient	Starter (0–14 d)				Grower(14–28 d)				Finisher (28–42 d)			
	C	E1	E2	E3 [1]	C	E1	E2	E3 [1]	C	E1	E2	E3 [1]
Corn	32.73	32.73	32.73	31.73	36.63	36.63	36.63	35.63	40.64	40.64	40.64	39.64
Wheat	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Corn gluten	2.00	2.00	2.00	2.00	4.00	4.00	4.00	4.00	6.00	6.00	6.00	6.00
Soybean meal	36.17	36.17	36.17	36.17	30.2	30.2	30.2	30.2	23.95	23.95	23.95	23.95
Creeping wood sorrel (CWS)	-	-	-	1.00	-	-	-	1.00	-	-	-	1.00
Oil	3.85	3.85	3.85	3.85	4.3	4.3	4.3	4.3	4.72	4.72	4.72	4.72
Monocalcium phosphate	1.68	1.68	1.68	1.68	1.52	1.52	1.52	1.52	1.43	1.43	1.43	1.43
Calcium carbonate	1.50	1.50	1.50	1.50	1.38	1.38	1.38	1.38	1.31	1.31	1.31	1.31
Salt	0.39	0.39	0.39	0.39	0.38	0.38	0.38	0.38	0.33	0.33	0.33	0.33
Methionine	0.33	0.33	0.33	0.33	0.25	0.25	0.25	0.25	0.21	0.21	0.21	0.21
Lysine	0.3	0.3	0.3	0.3	0.29	0.29	0.29	0.29	0.36	0.36	0.36	0.36
Choline	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
A1 Premix	1.00	1.00 [2]	1.00 [3]	1.00 [4]	1.00	1.00 [2]	1.00 [3]	1.00 [4]	1.00	1.00 [2]	1.00 [3]	1.00 [4]
Total	100	100	100	100	100	100	100	100	100	100	100	100
Chemical analysis—theoretical												
ME, Kcal/kg	3039.79				3128.99				3217.72			
CP, %	23.00				21.50				20.00			
EE, %	5.48				6.01				6.49			
CF, %	3.77				3.57				3.36			
Ca., %	0.96				0.87				0.81			
P, %	0.77				0.70				0.65			
P. available, %	0.48				0.43				0.41			
Lysine, %	1.44				1.29				0.16			
Methionin, %	0.69				0.61				0.32			
Tryptophan, %	0.25				0.22				1.19			

1 kg of A1 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 Vit. B1; 400 mg/kg vitamin B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg vitamin B6; 4 mg/kg Vit. 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. C: control diet; E1: experimental diet supplemented with 200 µg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 µg/kg diet CrPic +0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 µg/kg diet CrPic +10 g creeping wood sorrel powder (CWS)/kg diet. [1] Diet structure published previously by Saracila et al. [14]. [2] A1 premix + 20 mg CrPic/kg premix + 25 g vit. C/kg premix; [3] A1 premix + 20 mg CrPic/kg premix + 2.5 g Zn/kg premix; [4] A1 premix + 20 mg CrPic/kg premix + 1% creeping wood sorrel powder.

2.4. Analysis

The proximate composition of the thigh and breast was determined according to the chemical methods specified by AOAC [16]. Dry matter (ISO 6496/2001) and Ash (ISO 2171/2010) were determined by gravimetric method, crude protein (ISO 5983-2/2009) was analyzed by Kjeldahl method, and ether extractives were performed by extraction in organic solvents (SR ISO 6492/2001).

Chicken thigh and breast color analyses were performed using a Konica Minolta CR-400 (Tokyo, Japan) colorimeter and the CIELAB trichromatic system, which determines lightness (L*), saturation index in green/red (a*), and saturation index in blue/yellow (b*) values. The analyses were performed according to the method described by Panaite [17] and Vlaicu et al. [18]. Each analysis was performed in triplicate to obtain an average colorimetric value.

Firmness was determined by a Warner–Bratzler shear test using a Perten TVT 6700 texturometer (Perten Instruments, Hägersten, Sweden). The principle of this test is the measurement of the force expressed in Newtons (N) necessary to shear a piece of meat. Sample cuts (three rectangular slices/group with 2.0 cm long × 1.0 cm wide × 1.0 cm high) were made parallel to the direction of the muscle fibers. Firmness was calculated from the maximum point of the curve obtained from the test.

The texture profile analyses (TPA) were performed by a double cycle compression using a Perten TVT 6700 texturometer (Perten Instruments, Hägersten, Sweden), equipped with a Compression Platen cell. Four portions of cylindrical form (15 mm high and 20 mm wide) were cut out from each meat sample. The double compression cycle test was applied using an aluminum cylinder probe of 20 mm diameter and was performed up to 50%

compression of the original portion height previously prepared. The variables analyzed were: hardness, which is the maximum force needed to compress the sample; springiness, which represents the ability of a sample to recover to its original form after removal of the compressing force; resilience, which is the ratio of the negative force input to positive force input during the first compression; cohesiveness, which is a ratio between the total energy required for the first and second compression; and gumminess, which is defined as the product of springiness, hardness, cohesiveness. Each analysis was carried out in triplicate.

The pH values of the thigh and breast samples were measured 24 h postmortem according to SR ISO 2917: 2007 using a Hach HQ30d pH-meter (Hach Company, Loveland, CO, USA). An aqueous homogenate (meat: distillated water, 1:1) was prepared and filtered according to the method described by Turcu et al. [19]. The measurements were performed in triplicate.

Principal component analysis (PCA) was performed with the proximate compositions and color and textural parameters of meat using Graph-Pad Prism v. 9.02 (San Diego, CA, USA) software package for Windows. Principal Component Analysis (PCA) was applied to assess the relationships between meat characteristics. The similarities and differences between measured variables can be seen in the loading plot. Close variables suggest direct correlations, whereas the opposed variables indicate indirect relationships between them.

2.5. Sensory Analysis of Meat

Consumer acceptance tests were performed at the Faculty of Food Engineering, "Ștefan cel Mare" University of Suceava. An acceptance test with hedonic scale was used for the sensorial evaluation, using a 5-point scale (5 = extremely like and 1 = extremely dislike). The raw breast and thigh meat (n = 6 samples/group) were rated for sensory attributes by a semi-trained panel of 13 members selected from the University community. The study was conducted at room temperature of 22 °C under normal daylight. The samples were evaluated for muscle fiber appearance, appearance and characteristics of fat, flavor, firmness, juiciness, and tenderness.

2.6. Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA), and the means were compared applying Tukey's multiple range test using StatView program for Windows. The following statistical model was used:

$$Yi = Ti + ei$$

where Yi was the dependent variable, Ti is the treatment and ei is the error.

Significance was set at $p < 0.05$. The graphs highlighting the sensory attributes of chicken meat (breast and thigh) were plotted using the same statistical model with Graph-Pad Prism v. 9.02 (San Diego, CA, USA).

3. Results

3.1. Biochemical Measurements

At the beginning of the trial, the mortality rate was 2.5% (starter phase), while at the end of the experiment no mortality was recorded (0%). Table 2 revealed the biochemical measurements performed on serum samples. Serum glucose was significantly lower (by 19.72%) in E2 group compared to E1 and insignificant ($p > 0.05$) compared to the other groups (C, E3). Diets did not affect ($p > 0.05$) the other parameters such as cholesterol, triglyceride, phosphorus, calcium, iron, AST, ALT, gamma GT.

Table 2. Biochemical parameters determined in blood serum samples.

Variable	UM	C	E1	E2	E3	SEM	*p*-Value
Energy profile							
Glucose	mg/dL	247.1 ^{ab}	265.6 ^{a}	213.2 ^{b}	246.2 ^{ab}	8.076	0.0452
Cholesterol	mg/dL	144.8	139.6	132.9	146.4	3.783	0.6604
Triglyceride	mg/dL	37.10	40.89	42.65	51.87	2.694	0.3277
Mineral profile							
Phosphorus	mg/dL	5.7	5.1	5.6	5.3	0.134	0.4812
Calcium	mg/dL	8.3	8.7	8.5	8.8	0.119	0.4164
Iron	ug/dL	80.8	87.1	77.3	76.6	2.664	0.585
Enzyme profile							
ALT	U/L	3.4	4.5	3.6	4.8	0.300	0.3276
AST	U/L	453.0	399.5	309.2	300.4	31.410	0.9381
Gama GT	U/L	15.2	19.9	16.4	20.3	1.264	0.4135

^{a,b} Means within a column with no common superscript differ ($p < 0.05$). C: control diet; E1: experimental diet supplemented with 200 µg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 µg/kg diet CrPic + 0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 µg/kg diet CrPic + 10 g creeping wood sorrel powder (CWS)/kg diet; ALT—Alanine aminotransferase; AST—Aspartate aminotransferase.

3.2. Proximate Composition and Physicochemical Properties of Chicken Meat

Data on proximate composition of breast and thigh samples collected at the end of the experiment are presented in Table 3.

Table 3. Proximate composition of thigh and breast meat samples.

Variable	C	E1	E2	E3	SEM	*p*-Value
Thigh meat						
DM (%)	26.17	26.36	26.97	27.22	0.596	0.9879
CP (%)	18.61	18.51	18.41	19.12	0.444	0.9516
EE (%)	6.47 ^{a}	6.52 ^{ab}	7.43 ^{b}	6.83 ^{ab}	0.171	0.0228
Ash (%)	1.08	1.08	1.07	1.10	0.025	0.9888
Breast meat						
DM (%)	25.50	25.61	25.76	25.86	0.207	0.9409
CP (%)	22.21 ^{a}	22.68 ^{ab}	22.44 ^{ab}	23.42 ^{b}	0.204	0.0335
EE (%)	2.40 ^{a}	2.18 ^{b}	2.57 ^{a}	1.65 ^{b}	0.019	0.0100
Ash (%)	1.21 ^{a}	1.32 ^{b}	1.28 ^{a}	1.37 ^{b}	0.019	0.0100

^{a,b} Means within a column with no common superscript differ ($p < 0.05$). Where: C: control diet; E1: experimental diet supplemented with 200 µg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 µg/kg diet CrPic + 0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 µg/kg diet CrPic + 10 g creeping wood sorrel powder (CWS)/kg diet; DM = dry matter; CP = crude protein; EE—ether extractives; Ash.

In thigh meat, the concentrations of DM, CP and Ash did not differ ($p > 0.05$) between control group and experimental groups (E1, E2 and E3). However, the concentration of EE recorded significant differences ($p < 0.05$), being higher in E2 (CrPic + Zn) compared to C group.

In the analyzed chicken breast meat, there were significant increases ($p < 0.05$) in terms of CP and Ash and decreases in the EE. Thus, the dietary supplementation with CrPic + CWS determined the increase in crude protein concentration in breast meat compared to the C group; otherwise, the other groups had a statistically similar protein with group C. Additionally interesting is that CrPic + VC and CrPic + CWS caused a significant reduction in the concentration of crude fat in chicken breast compared to group C. The breast from E2 group had a significantly higher concentration of crude fat than E1 and E3, but similar to group C. Ash concentration was higher ($p < 0.05$) in groups E1 and E3

than in C. The group E2 had a significantly lower Ash content than in E1 and E3, but at a comparable level to C.

3.3. Color Parameters of Chicken Samples (Thigh and Breast Meat)

Table 4 shows the effects of diets on the color of thigh and breast muscle (pectoralis major). Supplementation of chicken diets with CrPic + VC (E1), CrPic + Zn (E2) and CrPic + CWS (E3) did not influence the value of the L* parameter of thigh meat. In contrast, the value of a* was significantly lower in the thigh collected from E1 than from E3 group. Yellowness parameter (b*) was also lower in the thigh samples from C group than in those from E1 group, while no difference was recorded in the E2 and E3 groups.

Table 4. Color parameters of the chicken meat.

Variable	C	E1	E2	E3	SEM	*p*-Value
			Thigh meat			
L*	49.04	49.87	50.26	50.07	0.236	0.2771
a*	1.90 ^{ab}	1.45 ^{a}	1.97 ^{ab}	2.52 ^{b}	0.194	0.032
b*	12.51 ^{a}	13.39 ^{b}	13.19 ^{ab}	13.02 ^{ab}	0.163	0.015
			Breast meat			
L*	54.03 ^{c}	50.81 ^{b}	51.38 ^{b}	46.93 ^{a}	0.330	<0.0001
a*	−1.26 ^{a}	0.80 ^{b}	0.62 ^{b}	1.49 ^{b}	0.169	<0.0001
b*	15.15 ^{c}	13.61 ^{b}	14.40 ^{bc}	12.13 ^{a}	0.182	<0.0001

$^{a–c}$ Means within a column with no common superscript differ ($p < 0.05$). C: control diet; E1: experimental diet supplemented with 200 µg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 µg/kg diet CrPic + 0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 µg/kg diet CrPic + 10 g creeping wood sorrel powder (CWS)/kg diet; L*: luminosity parameter; a*: redness; b*: yellowness.

Regarding the chicken breast, the L* parameter was significantly lower in the groups that included CrPic + VC, Cr + Zn, Cr + CWS in the diet than in the C group. Moreover, the values of a* parameter were higher in E1, E2 and E3 groups compared with the C group. The breast of chickens fed E1, E3 diets had a significant lower yellowness (b*) ($p < 0.05$) than those fed C diet. Notably is that the lowest value of b* parameter was recorded in the breast samples collected from E3 diet.

3.4. Meat Texture Parameters Determined by Shear with a Single Cycle and Double Cycle Compression

The chicken thigh meat recorded significant differences in the shear force parameter ($p < 0.05$) between experimental groups (E1, E2, and E3) and C group (Table 5). The thigh meat collected from E1 was firmer compared to those collected from E2 and E3. The combined effect of chromium and vitamin C led to an increase in the firmness of the meat, the cutting force registering the lowest value (30.88 N). The thigh meat of chicken from group E3 was firmer compared to meat from group E2, but the differences were not statistically significant.

The analysis of the texture profile (TPA) of thigh samples resulting from the application of the double compression test shows that the parameters of hardness, elasticity, cohesiveness, and gumminess did not register significant ($p > 0.05$) differences between groups. Cohesiveness shows how well the sample retains its structure after compression and includes adhesive and cohesive forces as well as viscosity and elasticity. Gumminess, a secondary parameter in the analysis of the texture profile, is determined as a product between firmness and cohesiveness. The resilience of chicken thigh was significantly lower in the group that included CrPic + Zn (E2), CrPic + CWS (E3) in the diet compared to group C and E1. The lowest resilience value was obtained in group E2 (diet supplemented with CrPic + Zn). The pH values were lower ($p < 0.05$) in E2 and E3 compared to C group. The dietary inclusion of CrPic+ VC did not have a significant effect on the pH of the thigh meat.

Table 5. Texture parameters of thigh meat.

Variable	C	E1	E2	E3	SEM	*p*-Value
Shear with a single cycle						
Firmness (N)	47.46 [a]	30.88 [b]	37.01 [c]	35.88 [c]	0.938	<0.0001
Double cycle compression						
Hardness (N)	27.23	26.36	23.27	25.25	0.874	0.4070
Springiness (%)	99.46	99.54	99.62	99.62	0.054	0.6681
Resilience (adm)	3.63 [a]	3.52 [a]	2.87 [b]	2.93 [b]	0.102	0.056
Cohesiveness (adm)	0.47	0.51	0.48	0.47	0.015	0.0158
Gumminess (N)	13.50	14.82	15.24	13.28	0.580	0.0086
pH	6.57 [a]	6.53 [a]	6.40 [b]	6.36 [b]	0.014	<0.0001

[a–c] Means within a column with no common superscript differ ($p < 0.05$). C: control diet; E1: experimental diet supplemented with 200 µg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 µg/kg diet CrPic + 0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 µg/kg diet CrPic + 10 g creeping wood sorrel powder (CWS)/kg diet.

The shear force showed inconsistent patterns concerning the supplemented diets (Table 6). It is observed that the breast meat obtained from groups E2 and E3 was characterized by a lower hardness ($p < 0.05$) compared to that from groups C and E1. The springiness of chicken breast collected from E2 and E3 groups did not differ significantly ($p > 0.05$) from that obtained for group C. Statistically significant differences were obtained between C and E1, the breast meat in group E1 showing a significantly lower elasticity (99.42%) compared to that of group C (99.74%). The resilience of chicken breast meat in groups E2 and E3 differs significantly ($p < 0.05$) from the values obtained in group E1. There were no differences between group C and the other groups. However, the lowest resilience value of breast meat (2.16 adm) was recorded in E2 group, which included CrPic + Zn in the diet. The cohesiveness and gumminess attributes of the chicken breast did not differ between groups. The inclusion of CrPic + VC in the chicken diet led to an increase in the pH of breast meat compared to C group. There were no significant differences in pH between C and E2 and E3. Diet supplementation with CrPic + Zn, and CrPic+ CWS respectively led to a significant decrease in pH value, compared to that of samples from E1.

Table 6. Texture parameters of breast meat.

Variable	C	E1	E2	E3	SEM	*p*-Value
Shear with a single cycle						
Firmness (N)	25.83	23.71	23.56	23.84	0.533	0.4033
Double cycle compression						
Hardness (N)	22.24 [a]	18.99 [a]	11.43 [b]	12.76 [b]	0.985	<0.0001
Springiness (%)	99.74 [a]	99.42 [b]	99.63 [a]	99.63 [a]	0.034	0.0052
Resilience (adm)	2.48 [ab]	2.96 [a]	2.16 [b]	2.21 [b]	0.096	0.0158
Cohesiveness (adm)	0.38	0.42	0.37	0.39	0.010	0.4132
Gumminess (N)	8.19	8.36	8.00	6.30	0.524	0.5484
pH	6.37 [b]	6.53 [a]	6.40 [b]	6.36 [b]	0.019	0.0041

[a,b] Means within a column with no common superscript differ ($p < 0.05$). C: control diet; E1: experimental diet supplemented with 200 µg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 µg/kg diet CrPic + 0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 µg/kg diet CrPic + 10 g creeping wood sorrel powder (CWS)/kg diet.

3.5. Relationship between Meat Characteristics

Figure 1 shows the PCA plot for chemical characteristics, L*, a*, b* color parameters, textural parameters, and some sensorial characteristics.

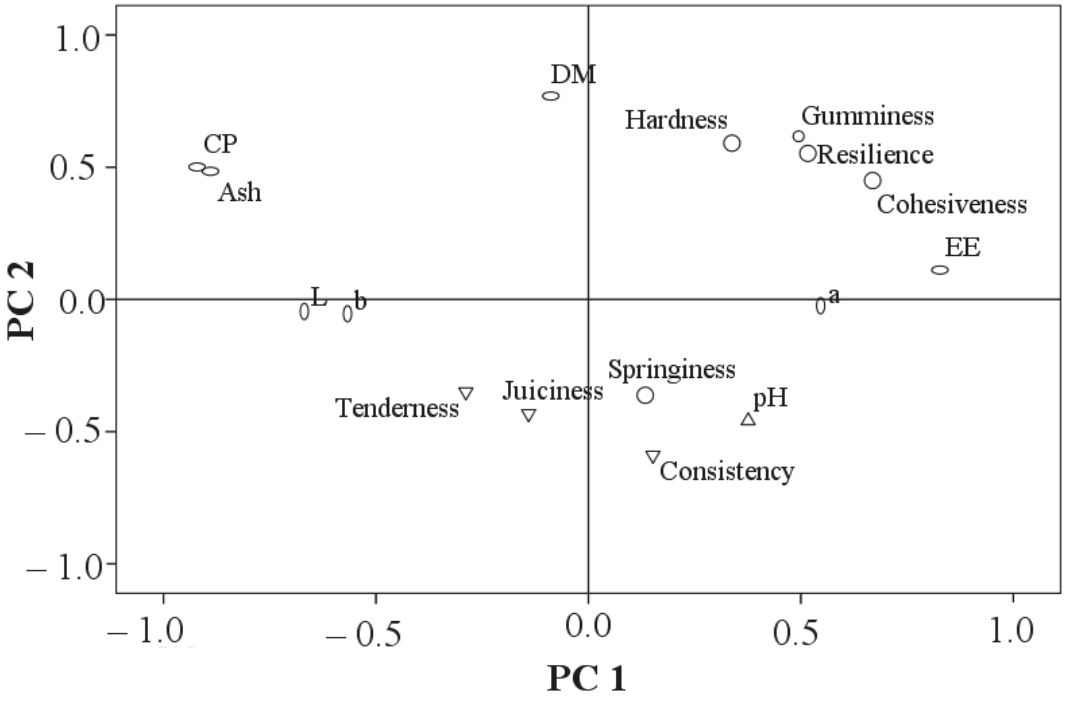

Figure 1. Principal component loading based on the chemical composition, L*, a*, b* color parameters, textural parameters, and sensory characteristics. L*: luminosity parameter; a*: redness; b*: yellowness; DM: dry matter; CP: crude protein; EE: ether extractives.

The first two PCs explain 50.81% of the total variance (PC1 = 32.30%, PC2 = 18.51%) for the evaluated characteristics. Some sensory characteristics such as muscle fiber appearance, appearance and fat characteristics, and smell were eliminated from the PCA because they have a low adequacy for PCs. The first principal component, PC1 was associated with chemical composition (CP, EE, and Ash) and L*, a*, b* color parameters. The second component, PC 2 was characterized by dry matter (DM), textural parameters (hardness and gumminess), and sensory characteristics (consistency, juiciness, and tenderness). Meat tenderness shows a weak negative correlation with EE (r = −0.342), which may be related to the intramuscular fat content that affects tenderness. Significant ($p < 0.01$) positive correlation was found between hardness and gumminess (r = 0.891), gumminess and cohesiveness (r = 0.771), between cohesiveness and resilience (r = 0.861), and EE (r = 0.585). A positive correlation was also found between Ash and L* (r = 0.426), and a negative one between Ash and a* (r = 0.446).

As Figures 2 and 3 show, the panelists indicated similar sensory attributes for chicken breast and thigh among groups. Thus, the dietary supplementation had not changed the consumer acceptance criteria.

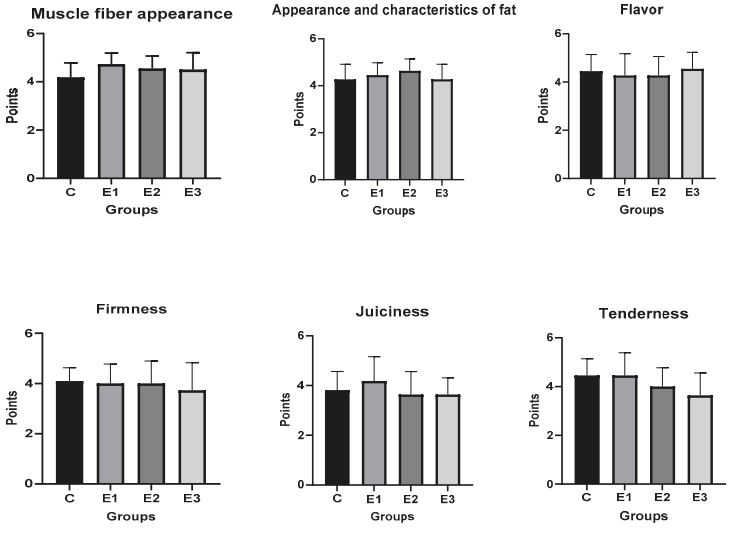

Figure 2. Sensory characteristics of chicken's breast (mean of points). Main effects of diets are presented in each graph (Prism Graph 9.02). Data are presented as mean ±SEM (n = 6 samples/group). No superscript denotes any statistical significance ($p > 0.1234$). C: control diet; E1: experimental diet supplemented with 200 μg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 μg/kg diet CrPic + 0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 μg/kg diet CrPic + 10 g creeping wood sorrel powder (CWS)/kg diet.

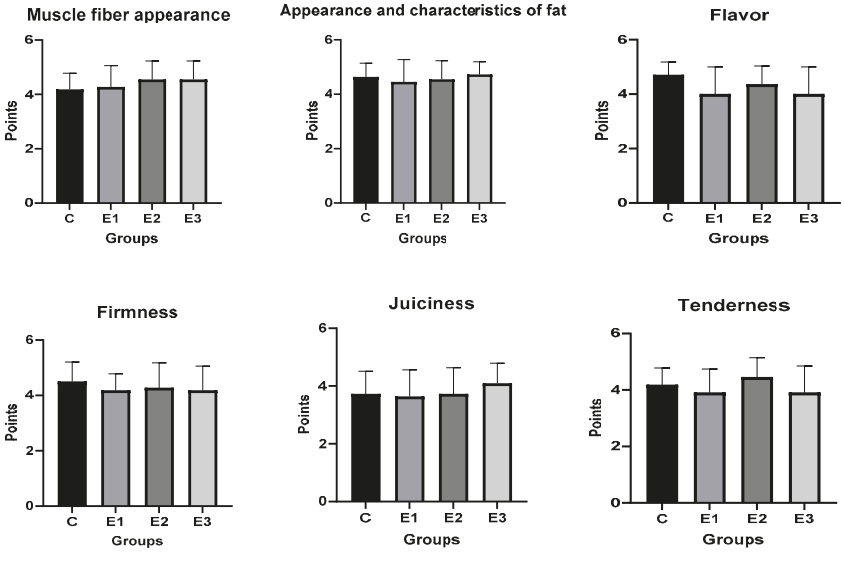

Figure 3. Sensory characteristics of chicken's thigh (mean of points) Main effects of diets are presented in each graph (Prism Graph 9.02). Data are presented as mean ± SEM of points (n = 6 samples/group). No superscript denotes any statistical significance ($p > 0.1234$). C: control diet; E1: experimental diet supplemented with 200 μg/kg diet CrPic + 0.25 g vitamin C (VC)/kg diet; E2: experimental diet supplemented with 200 μg/kg diet CrPic + 0.025 g Zn/kg diet; E3: experimental diet supplemented with 200 μg/kg diet CrPic + 10 g creeping wood sorrel powder (CWS)/kg diet.

4. Discussion

The implication of Cr in reducing glucose levels in heat-stressed broilers was well documented [20–22]. However, the literature is limited in studies regarding the effect of combinations of Cr with other antioxidant compounds. In this study, dietary supplementation with CrPic + Zn had a lowering effect in glucose level compared with CrPic + vitamin C. Nevertheless, there is evidence confirming that dietary Zn can decrease glucose level in heat-stressed broilers [23]. Contradictory results were reported by Abuajamieh et al. [24], showing that dietary organic zinc supplementation (50% and 100% of the Zn level from basal diet) in HS chicks increased blood glucose and additionally decreased blood calcium. Conversely, Saleh et al. [25] showed that Zn methionine supplementation (25, 50 and 100 mg/kg) in heat-stressed broiler diet significantly decreased plasma triacylglycerol, total cholesterol concentrations as compared to the control group. AST and glucose parameters did not record any difference. In this study, dietary supplementation with CrPic + VC or CrPic + CWS did not have any effect on serum biochemical parameters tested. In contrast, some researchers reported that combination of Cr with vitamin C has synergistic action and decreased glucose and cholesterol [10], while data using CrPic + CWS were not found. Perai et al. [26] showed that under stressful condition caused by transport, broiler chickens fed diet enriched in Cr + vit. C had a lower triglyceride level and a higher glucose level than before transport.

The dietary supplementation with CrPic + CWS determined an increase in crude protein concentration in breast meat compared to those fed C diet; otherwise, the other groups had a similar protein content with group C. Untea et al. [27] observed the positive influence of dietary chromium supplements (200, 400 μg/kg) on crude protein concentrations of breast meat in a study on broiler chickens raised under normal temperature conditions. Nevertheless, it is interesting to note that CrPic + VC and CrPic + CWS caused a significant reduction in the concentration of crude fat in chicken breast compared to group C. This reduction in crude fat is a good achievement because a high content of fat led to lipid oxidation, a key factor that negatively affects meat color and texture. Additionally, some researchers [28,29] have reported higher protein and lower fat content in breast meat, when chickens fed diets supplemented with 200 and 400 ppb Cr3+. The probable explanation for fat reducing effect could be the inhibitory potential on lipogenic activity in chick adipose tissue. Toghyani et al. [30] showed that under heat stress, broilers fed a diet enriched in Cr (500, 1000, 1500 ppb Cr nicotinate) recorded increases in crude protein of breast meat.

In this study, it was observed that the use of dietary VC, Zn, and creeping wood sorrel changed the meat color. The redness value (a*) of the thigh collected from E1 group was significantly lower than from E3 group. Therefore, the combination of CrPic + CWS had a favorable effect on thigh meat color compared to that of CrPic + VC. Nevertheless, the redness parameter was higher in E1, E2, and E3 groups compared to the C group. Definitely, those results showed that CrPic in combination with VC, Zn, and CWS improved the meat color. Moreover, the breast from E3 group had the highest concentration of crude protein and the highest redness. Myoglobin is the main heme protein in muscle tissue; perhaps an increase in myoglobin concentration has led to an increase in the redness of chicken breast color. According to Sałek et al. [31], Zn binds myoglobin and increase its oxygenation, which maintain the meat color. Consumers associate the increase in the redness parameter with a better quality of the meat [32]. Meat color is influenced by animal diet [33], its heme components concentration consisting of myoglobin, haemoglobin, cytochrome C [34,35], their oxidation-reduction state, chemical reactions, etc. [36]. The explanation of increasing the meat redness might be the antioxidant activity of supplements, being well-known that vit. C and Zn are involved in the redox reactions, delaying the meat oxidations processes. On the other hand, creeping wood sorrel contain phenols, but also large amounts of vitamin C, which could contribute to maintain the pigment stability of the meat. The luminosity parameter was significantly lower in the breast meat collected from groups that included CrPic + VC, CrPic + Zn, CrPic + CWS in the diet than in the C group. The lightness of the breast ranged from 46.93–51.38; redness from 0.61–1.49 and yellowness from

12.13–14.40. The breast meat of chickens fed E1, E3 diets recorded a significant decrease in the yellowness parameter than those fed the C diet. Notably, the lowest value of b* parameter was recorded in the breast samples collected from E3 diet. Similar results were reported by Huang et al. [9] who studied the effect of three sources of Cr added in the diet of broiler chickens (Cr propionate, CrPro; Cr picolinate, CrPic; Cr chloride, CrCl3) and two concentrations of added Cr (0.4, or 2.0 mg of Cr/kg) on meat quality of broilers raised under heat stress. The authors reported that broilers supplemented Cr had decreased b* values of meat color in breast muscle. Peña et al. [37] showed no differences on breast color when included ascorbic acid (250, 500, and 1000 g/ton) + citric flavonoids in the diet of heat-stressed chickens.

Results from our study revealed that the dietary supplementation with combinations of CrPic with VC, Zn, and CWS had a beneficial effect on the mechanical strength of the muscle fiber. From a textural point of view, Tudoreanu [38] has found that meat firmness depends mainly on the structure of myofibrin and connective tissue, while Astruc [39] has shown that it depends on the amount of fat and collagen. The texture profile analysis (TPA) of thigh samples resulting from the application of the double compression test shows that the parameters of hardness, elasticity, cohesiveness, and gumminess did not register significant ($p > 0.05$) differences between groups. The resilience of chicken thigh was significantly lower in the group that included CrPic+ Zn (E2), CrPic + CWS (E3) in the diet compared to group C and E1. The lowest resilience value was obtained in group E2 diet was supplemented with CrPic + Zn. This observation could be related with the increase in EE in E2 than in C. The pH values of thigh meat were lower in E2 and E3 compared to C group. Normally, postmortem, the muscle undergoes various reactions, through which it is transformed into meat. For example, muscle glycogen is denatured by glycolysis, forming lactic acid. This reaction results in an increase in the acidity of meat. Heat stress can affect the pH of meat, increasing it, as a consequence of glycogen consumption in reserves and thus the production of lactic acid is reduced [40]. According to Listrat et al. [33], in poultry, the texture is strongly affected by the postmortem acidification kinetics of muscle.

The breast meat obtained from groups fed combination of CrPic + Zn and CrPic + CWS had a lower hardness ($p < 0.05$) compared to that from groups C and E1. This indicates that the caused an increase in the tenderness of the chicken breast compared to the control diet and the combination of CrPic + VC. Chromium, in combination with Zn or CWS may lead to a decrease in the mechanical strength of muscle fiber, having an effect of increasing its firmness. The breast meat in group E1 had a significantly lower elasticity (99.42%) compared to that of group C (99.74%). This result suggests that dietary supplementation with CrPic + VC leads to a significant decrease in the ability of the chicken breast meat to return to its original shape after compression. Nevertheless, the lowest resilience value of breast meat (2.16 adm) was recorded in E2 group, which included CrPic + Zn in the diet. This achievement could be correlated with the low hardness of the chicken breast meat in E2. The inclusion of CrPic + VC in the chicken diet led to an increase in the pH of breast meat compared to C group. pH is considered one of the crucial variables determining meat quality [41]. According to Berri et al. [42] the normal pH values of chicken meat at 15 min postmortem are around 6.2 to 6.5, whereas normal ultimate pH values are around 5.8 [43].

The results of PCA revealed that the assessed meat characteristics were clustered in function of similarity. The first component, PC1 underline a close association between EE, cohesiveness and a* parameters, variables which are negatively correlated with CP, Ash, L* and b*. The second component, PC2 distinguished between DM and a* parameters and between textural parameters, hardness and springiness, this fact being expectable. Additionally, an opposite relation can be observed between cohesiveness and springiness, meat springiness showing a direct relation with sensorial parameters, consistency, juiciness, and tenderness.

Sensory attributes of meat such as tenderness, juiciness, muscle appearance, are among the most critical attributes in consumer preferences [44]. In this regard, several studies conducted on consumers have shown that these attributes could be influenced

by supplementation of broiler diets. For example, Velasco and Williams [45] showed that chicken meat quality could be improved by adding natural antioxidants. Some selected panelists have indicated an improvement in the flavor of thigh meat, reduced the metallic taste and the overall aftertaste as consequence of supplementing chicken diets with faba bean compared to a soy-based diet [46]. However, Suliman et al. [47] evaluated the sensory attributes of meat obtained from broilers fed diet supplemented with 1, 2, 3, 4, 5, and 6% clove seeds. The authors showed that the sensory attributes were not significantly different between the treatment groups except tenderness. However, in our study, according to panelists, the experimental diets had no influence on sensory attributes tested compared to the conventional diet.

Taken together, the achievement such as improvement of meat color could be probably attributed to the antioxidant potential of the tested supplements being involved in the redox reactions. However, the improvement of breast meat tenderness observed in this study as consequence of supplementing the broiler chicken diet with antioxidants is an important result as it strongly influences consumer satisfaction. Nevertheless, the influence of dietary antioxidants on the regulatory mechanisms that define metabolic and physiological changes in muscle tissue is complex, poorly understood, and further studies are needed to investigate it.

5. Conclusions

The dietary supplementation with CrPic + creeping wood sorrel determined an increase of crude protein concentration in breast meat, and CrPic + vitamin C and CrPic + creeping wood sorrel reduced the concentration of crude fat compared to those fed the C diet. Dietary supplementation with combinations of CrPic with vitamin C, Zn, and creeping wood sorrel improved the breast meat color, increasing the redness parameter and decreasing the luminosity parameter. Conversely, dietary supplementation with combinations of CrPic with vitamin C and creeping wood sorrel had a beneficial effect on textural parameters of chicken breast meat, increasing the tenderness. Furthermore, there was a clear relationship among proximate composition, color, and texture attributes of chicken meat. Taken together, this study revealed that a dietary combination of CrPic with vitamin C, Zn, creeping wood sorrel, respectively, as sources of antioxidants could attenuate the negative effects of heat stress on nutritional quality and texture without affecting the sensorial parameters of chicken meat.

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Data Availability Statement: All data is contained within the article.

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Effects of Rocket Seed Oil, Wheat Germ Oil, and Their Mixture on Growth Performance, Feed Utilization, Digestibility, Redox Status, and Meat Fatty Acid Profile of Growing Rabbits

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Abstract: Vegetable oils are a source of natural antioxidants, including tocopherols, sterols, phenolic compounds, coenzymes, and polyunsaturated fatty acids that provide nutritional value, organoleptic properties, and significantly delay or prevent lipid oxidation. Eighty-four V-line rabbits at 5 weeks of age with an initial body weight (BW) of 535.60 ± 13.48 g were assigned randomly to four experimental groups (seven replicates in each group with three rabbits each). The first group served as a control and received 0.3 mL/kg BW of distilled water (CON), while the second and third groups received 0.3 mL/kg BW of rocket seed oil (RSO) and wheat germ oil (WGO), respectively. The fourth group received a mixture of oils consisting of 0.15 mL of RSO and 0.15 mL of WGO/kg BW (MOs). The experiment lasted 7 weeks. The study investigated the effects of RSO, WGO, and their mixture on growth performance, feed utilization, antioxidant status, and immune response of growing rabbits. The results indicated that the rabbits that were administered orally with RSO and WGO or their mixture had higher ($p \leq 0.05$) final BW, weight gain, and average daily gain when compared to the control group. In addition, the feed conversion ratio improved significantly with RSO, WGO, and MOs treatments. Different oil treatments improved nutrient digestibility, nutritive value, and nitrogen balance. Moreover, the rabbits that received RSO, WGO, and their mixture had an improvement the meat fatty acid composition compared to the control rabbits. Oral administration of RSO, WGO, and their mixture significantly improved serum protein fractions, decreased blood urea nitrogen, and had a positive effect on serum total lipids, HDL-c, and LDL-c. Furthermore, the treatments of RSO, WGO, and MOs had a significant improvement in the antioxidative status and immune response.

Keywords: rocket seed oil; wheat germ oil; growing rabbits; growth performance; antioxidant status; digestibility

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1. Introduction

Phytogenic feed additives have been recognized as antimicrobials, antioxidants, antitoxigenic, anti-coccidiosis, and antiparasitic [1–3]. In addition, phyto-feed additives improve the palatability and digestibility of feed, enhance the absorption of nutrients, as well as manipulate the microbial habitat and gut functions of domestic animals [4,5]. Moreover, they protect the feed lipids from oxidative damage and improve the antioxidant and immune status of the animal. Furthermore, phyto-feed supplementations are natural additives, less poisonous, residue-free, with more integrity and perfect as feed additives for poultry when compared to antibiotics [1]. Consequently, they can be considered as an important tool in poultry nutrition for enhancing growth performance, feed efficiency and reproductive performance, and reducing the incidence of diseases and the house emissions

of poultry [1,4]. The inclusion of phytogenic feed additives in the diet can improve the nutritional value of meat and tissue composition [6].

As one of the phytogenic feed additives, vegetable oils, such as olive oil, rice bran oil, corn germ oil, and wheat germ oil are commonly used as food supplements in the human diet [7]. Vegetable oils are natural, healthy, and nutritious due to their high content of unsaturated fatty acids and functional molecules, and their high energy value [8].

Rocket (*Eruca sativa* Mill.) belongs to the large family of Brassicaceae (also called Cruciferae or the mustard family). The rocket is an annual or biannual herb that originated in the Mediterranean region and has spread through the world [9]. *E. sativa* species are widely used in folklore and traditional medicine for their therapeutic properties as digestive, astringent, laxative, emollient, depurative, diuretic, rubefacient, stimulant, and tonic [10]. The composition of rocket seeds has shown the presence of many active compounds, such as glucosinolates (glucoerucin and glucoraphanin), flavonoids (quercetin, kaempherol, and isohamnetin), carotenoids, and vitamin C, which are ascribed to antioxidant activity [10]. Rocket seeds contain up to 25–35% of oil [11] and rocket seed oil (RSO) has about 18% of the total saturated fatty acids and 82% of the total unsaturated fatty acids. Rocket seed oil prompts the regeneration of hepatic tissue, decreases hepatic lipid levels, and possesses potent free radical scavenging [12], as well as inhibits melanoma tumor growth in mice [13].

Additionally, rocket seed oil inhibits the growth of some Gram-positive and Gram-negative bacteria and has approximately the same efficiency as the broad-spectrum antibiotic Gentamicine [14]. Moreover, RSO ameliorated the harmful effect of aflatoxin on rabbit blood, semen, and pathological changes in the liver, kidney, and testes [15]. Furthermore, *E. sativa* improved significantly the final body weight, average daily gain, feed intake, and feed conversion ratio of rabbits [16]. The dietary supplementation of 1 g RSO/kg diet alone or with 1 g onion seed oil/kg diet in the growing rabbit's diet for 12 weeks under heat stress improved growth performance, carcass weight, and nutrient digestibility as well as enhanced immunity [17]. In this vein, Alagawany et al. [18] found that dietary supplementation with 0.5–2 g/kg diet of watercress oil alone or in combination with coconut oil for 8 weeks in intensive rabbit production improved growth performance, feed utilization, antioxidant status, and immunity, as well as reduced pathogenic cecal bacteria. On the other hand, the addition of high levels of RSO (1–3 mL/kg body weight) to the rabbits for 2 weeks resulted in a reduction of the body weight with an increasing RSO oil dose [19].

Wheat (*Triticum aestivum* L.) germ is produced during wheat milling and is used worldwide as a diet supplement in the feed formulation of farm animals [20]. Wheat germ oil (WGO) represents about 10–15% of the whole wheat germ [21]. In addition, it contains tocopherol derivatives and tocotrienols [22], n − 3 fatty acids, especially alpha-linolenic acid [23], fat-soluble carotenoids [24], phytosterols, especially D5-avenasterol [25] and phenolic compounds [26]. Moreover, wheat germ oil has an anti-inflammatory effect and strong antioxidant effects [21,26]. Whereas, it reduces O_2-production and NADPH oxidase activity, and thereby, decreases oxidative stress [23]. WGO manages the serum lipid profile and prevents hypercholesterolemia and atherosclerosis in male albino rabbits fed high cholesterol diet [27]. Other benefits of wheat germ and its derivatives are lowering cholesterol absorption, retarding platelet aggregation, delaying ageing, improving physical endurance, enhancing fertility [25], as well as preventing and curing carcinogenesis [28]. Furthermore, dietary WGO supplementation increased the body weight of male broilers [29].

Taking previous knowledge into account, the present study aimed to investigate the effects of RSO, WGO, and their mixture on growth performance, feed utilization, nutrient digestibility, carcass characteristics, meat fatty acid profile, and redox and immune status of growing rabbits.

2. Materials and Methods

2.1. Animal Management and Feeding

Eighty-four V-line rabbits at 5 weeks of age (after weaning) with an initial BW of 535.60 ± 13.48 g were assigned randomly into four experimental groups (seven replicates in each group, three rabbits in each replicate). The first group served as the control and received 0.3 mL/kg BW of distilled water (CON), the second group received 0.3 mL/kg BW of rocket seed oil (RSO), the third group received 0.3 mL/kg BW of wheat germ oil (WGO), and the fourth group received a mixture of oils consisting of 0.15 mL of RSO and 0.15 mL of WGO/kg BW (MOs). The oils of wheat germ and rocket seeds were obtained from El Madina Factory for natural seed extract in Borg El Arab, Alexandria, Egypt.

Rabbits were given oils once daily via gavage (oral administration) for 7 weeks from 28 May to 15 July. The basal ration was formulated and pelleted to meet the nutrient requirements of rabbits, according to the NRC [30]. The rations were offered to rabbits *ad libitum*. The ingredients and chemical composition of the pelleted rations are shown in Table 1. The rabbits were offered free access to freshwater.

Table 1. The ingredients and chemical analysis of the experimental ration.

Ingredients	**(g/kg)**
Berseem hay	280.00
Barley	173.00
Corn yellow	179.00
Wheat bran	120.00
Soybean meal 44%	200.00
Molasses	30.00
Di-Ca-Ph	10.00
Salt	3.00
Vitamin premix [1]	3.00
Lysine	1.00
Methionine	1.00
Chemical analysis	**(g/kg dry matter)**
Organic matter	912.50
Ash	87.50
Crude protein	174.04
Ether extract	84.09
Crude fiber	127.49
Nitrogen-free extract	526.88
Neutral detergent fiber	373.00
acid detergent fiber	210.59
Hemicellulose	162.41
Energy value	
Gross energy (kcal/kg)	3940.87
Digestible energy (kcal/kg)	2528.56

[1] It provides the following nutrients (unit/kg diet): Vitamin A, 12,000 IU; vitamin D3, 2000 IU; vitamin E, 11 IU; vitamin K, 2 mg; pantothenic acid (d-Ca pantothenate), 10 mg; folic acid, 1 mg; choline (choline chloride), 250 mg; Mn (manganous oxide), 60 mg; Fe (ferrous sulfate), 30 mg; Zn (zinc oxide), 50; Cu (copper sulfate), 10 mg; iodine (ethylenediamine dihydroiodide), 1 mg; cobalt (cobalt sulphate heptahydrated), 0.1 mg; and Se (sodium selenite), 0.1 mg.

All the rabbits were kept under similar management, as well as hygienic and environmental conditions. Freshwater was automatically available all the time through stainless steel nipples that were fixed in each cage. The rabbits were housed in galvanized wire cages (dimensions: 40 × 50 × 65 cm) located in a well-ventilated building. The daily photoperiod is a 16:8 h light-dark cycle. This study was conducted at the Rabbit Research Laboratory, Department of Animal and Fish Production, Faculty of Agriculture (Saba Basha), Alexandria University. All the protocols applied in the present experiment have been approved by the Alexandria University, Animal Care and Use Committee with approval no. AU: 19/21/03/25/3/16.

2.2. Body Weight and Feed Intake

The rations were removed at night before the days of rabbit weight. The growing rabbits were weighed weekly in the morning before being given a feed. The average daily gain (ADG) and weight gain percentage were calculated. The feed intake was recorded biweekly, then daily feed consumption was calculated by dividing the weekly feed intake by 14 days. The feed conversion ratio (FCR) was calculated by dividing the daily feed intake by the average daily gain.

2.3. Digestibility Trial

At 10 weeks of age, sixteen male rabbits were randomly taken to determine the nutrient digestion coefficients of the experimental diets. The rabbits were allocated to four different treatments (four rabbits in each group). The rabbits within each treatment were housed individually in metabolic cages that enabled the separation of urine and feces. The preliminary period was 2 days to adapt rabbits to the new cages and then followed by 5 days as a collection period for feces and urine. During the collection period, the total excreted feces and urine of each rabbit are collected daily in buckets before offering a morning meal and weighing them.

Representative samples (10%) of the total quantity of feces from each rabbit were oven-dried daily at 70 °C for 48 h to determine the total dry matter (DM) of the feces and to calculate the quantity of feces on a DM basis. At the end of the collection period, the faecal samples from each rabbit were mixed thoroughly, and representative samples (10%) of the mixtures were ground through a 1-mm screen on a Wiley mill grinder and then stored frozen at −20 °C prior to the chemical analysis.

Nutritive values in terms of total digestible nutrients (TDN) and digestible crude protein (DCP) were calculated according to the classic formula [31] as follows:

$$\text{TDN\%} = \text{DCP\%} + \text{DCF\%} + \text{DNFE\%} + (\text{DEE\%} \times 2.25) \quad (1)$$

where DCP is the digestible crude protein, DCF is the digestible crude fiber, DNFE is the digestible nitrogen-free extract, and DEE is the digestible ether extract.

$$\text{NR: Nutritive value} = (\text{TDN (\%)}/\text{DCP (\%)}) - 1 \quad (2)$$

Digestible energy (DE) was calculated using the equation according to [32], as follows:

$$\text{DE (kcal/kg diet)} = 44.3 \times \text{TDN\%} \quad (3)$$

2.4. Lipid Content and Fatty Acid Profile of Rabbit Meat

The lipid content and fatty acid profile of rabbit meat were determined in the musculus semitendinosus of three slaughtered rabbits per group. Total lipids were extracted with chloroform:methanol (2:1 v/v) from 0.8 g of meat, according to the procedure of Folch et al. [33].

Lipid extraction from the meat samples was performed according to the procedure of Pearson [34]. About 10 g of the sample was weighed in a 250 mL centrifuge bottle. The total volume was completed to 16 mL with distilled water, then 40 mL of methanol and

20 mL of chloroform were added and macerated for 2 min. After that, 20 mL of chloroform was added and macerated for 30 s, then 20 mL of water was added and macerated again for 30 s. The mixture was centrifuged for 10 min at 2000–2500 rpm. The bottom layer of chloroform was removed and filtered through a coarse filter paper into a dry-weight flask or beaker. Then, the chloroform was evaporated to dryness.

Preparation of fatty acid methyl esters from the total lipids of the sample was performed according to the procedure of Radwan [35]. A sample of total lipids (50 mg) was transferred into a Screw-Cap flask, then 2 mL of benzene and 5 mL of methanolic sulphuric acid (1 mL of conc sulphuric acid and 100 mL of methanol) were added. The vial was covered under a stream of nitrogen gas, then placed in a water bath at 90 °C for 90 min. The flask was cooled, then 10 mL of distilled water was added and the methyl esters in each flask were extracted with 5 mL of petroleum ether three times. The petroleum ether extracts were combined and concentrated to their minimum volume using a stream of nitrogen. The analysis of fatty acids was carried out by gas-liquid chromatography (HP, Hewlett Packard 6890 GC model) equipped with a flame ionization detector (FID). Separation was achieved in a column HP-INWAX (cross linked polyethylene glycol, 60 m, 0.25 mm ID, 0.25 μm film thickness) under the following conditions: Detector temperature, 250 °C; injector temperature, 220 °C; injection volume, 3 μL; split ratio, 50:1; carrier gas, nitrogen; gas flow, 1.5 mL/min. Before running the samples, a standard mixture of methyl esters was analyzed under identical conditions. The retention times of the unknown sample of methyl esters were compared with the standard. The proportions of methyl esters were calculated by the triangulation method.

2.5. Serum Biochemical Parameters

Before slaughter, 4 mL of blood sample was taken with a sterile syringe from the ear vein of five growing rabbits from each group. The blood sample was placed into a sterile vacutainer tube without an anticoagulant for the serum biochemical analysis.

The serum total protein, lipid profile, and urea were estimated colorimetrically using commercial kits produced by Bio Diagnostic Co., Giza, Egypt. The serum total protein and albumin were determined according to Doumas et al. [36]. The serum globulin concentration was calculated by the difference between the total protein and albumin [37].

Total lipids were estimated by the reaction with sulphuric and phosphoric acids and vanillin to form a pink chromophore [38]. Triglycerides were measured colorimetrically using the quadruple enzymatic reaction [39]. Cholesterol was determined after enzymatic hydrolysis and oxidation as described by Allain et al. [40]. High-density lipoprotein-cholesterol (HDL-c) was determined according to the methods of Grove [41]. Low-density lipoprotein-cholesterol (LDL-c) was determined using the following calculation according to Warnick et al. [42] using the following equation:

$$\text{LDL-c} = \text{cholesterol-} (\text{HDL-c} + \text{vLDL-c}). \quad (4)$$

The very low-density lipoprotein-cholesterol (vLDL-c) was calculated by dividing the value of TG by a factor of 5 according to the method of Warnick, Benderson, and Albers [42]. Serum urea was assayed according to Chaney and Marbach [43].

The triiodothyronine (T3) and thyroxine (T4) hormones were determined in the serum by a direct radioimmunoassay technique. Kits from the Diagnostic Products Corporation (Los Angles, CA, USA) with ready, antibody-coated tubes were used based on the manufacturer's instructions, according to Kubasik et al. [44].

2.6. Antioxidant Assays

Thiobarbituric acid reactive substances (TBARs) were measured colorimetrically according to Tappel and Zalkin [45]. The catalase activity (U/mL serum; EC 1.11.1.6, CAT) was measured according to Luck [46]. The superoxide dismutase activity (U/mL serum; EC 1.15.1.1, SOD) was evaluated according to Misra and Fridovich [47]. The total antioxidant

capacity (TAC) was estimated colorimetrically using commercial kits produced by Bio Diagnostic, Egypt according to the method of [48].

2.7. Antibody Titers against SRBCs

The primary and secondary immune response was assayed by measuring antibody titer against sheep red blood cell counts (SRBCs), as the agglutination titer described by Wegmann and Smithies [49]. The agglutination titer was calculated as the log of the reciprocal of the highest serum dilution for the whole agglutination [50].

2.8. Chemical Analysis

Chemical analyses of the experimental rations and feces samples were carried out according to AOAC [51] for crude protein (CP, method 968.06), ether extract (EE, method 920.39), crude fiber (CF, method 932.09), and ash (method 967.05). The nitrogen-free extract (NFE) was calculated according to the next equation:

$$\text{NFE (\%)} = 100 - (\text{CP\%} + \text{EE\%} + \text{CF\%} + \text{Ash\%}). \quad (5)$$

Organic matter (OM) was calculated as the difference between 100% DM and ash. Gross energy (GE, kcal/kg) of the experimental diets was calculated based on 5.64, 4.11, and 9.44 kcal GE/g CP, NFE, and EE, respectively NRC [52].

Digestible energy (DE) of the experimental diets was calculated according to the equation described by Cheeke [53], as follows:

$$\text{DE (kcal/kg)} = 4.36 - 0.0491 \times \text{NDF\%} \quad (6)$$

$$\text{whereas, NDF\%} = 28.924 + (0.657 \times \text{CF\%}) \text{ and ADF\%} = 9.432 + 0.912\ (\text{CF\%}) \quad (7)$$

The concentration of hemicellulose was estimated as the difference between NDF and ADF. Nitrogen in urine was determined by the micro-Kjeldahl method [51].

2.9. Statistical Analysis

Data of the experiment were analyzed statistically using the one-way analysis of variance (ANOVA) with the SPSS 11.0 statistical software [54]. Differences among means were determined using the Duncan test [55]. Data were analyzed using the following model:

$$Y_{ij} = U + A_i + E_{ij} \quad (8)$$

where U is the overall mean, A_i is the effect of wheat germ oil, rocket seed oil, and their mixture treatments; and E_{ij} is the random error.

3. Results

3.1. Body Weight, Feed Intake, and Feed Conversion Ratio

The feed intake of rabbits that were administered orally with RSO and WGO or their mixture is presented in Table 2. The final BW increased significantly ($p = 0.001$) when rabbits were administered orally with RSO and WGO or their mixture compared to the control group. Weight gain and ADG increased significantly ($p = 0.001$) with WGO and MOs treatments compared to the control group. The highest value of final BW, weight gain, and ADG was recorded in the MOs treatment. However, vegetable oil treatments did not affect the average weight gain percentage, total feed intake, and daily feed intake. Meanwhile, FCR improved significantly with RSO and WGO or their mixture treatments compared to the control group.

Table 2. Growth performance, feed intake, and feed conversion ratio of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture.

Items	Control	Rocket Seed Oil	Wheat Germ Oil	Mixture Oils	*p*-Value
Initial body weight (g/rabbit)	525.12 ± 10.17	540.43 ± 38.96	541.05 ± 26.33	535.81 ± 30.52	0.977
Final body weight (g/rabbit)	1995.71 ± 28.86 ^{c}	2104.29 ± 27.42 ^{b}	2237.57 ± 19.98 ^{a}	2233.81 ± 43.72 ^{a}	0.001
Weight gain (g/rabbit)	1470.60 ± 31.80 ^{c}	1563.86 ± 30.76 ^{b}	1696.52 ± 24.33 ^{a}	1698.00 ± 32.43 ^{a}	0.001
Average daily gain (g/day)	30.01 ± 0.65 ^{c}	31.91 ± 0.63 ^{b}	34.62 ± 0.50 ^{a}	34.65 ± 0.66 ^{a}	0.001
Weight gain percent (%)	280.99 ± 9.72	300.02 ± 24.83	318.15 ± 16.21	322.97 ± 19.10	0.372
Feed intake (g/experimental period)	5541.45 ± 25.16	5531.86 ± 14.09	5516.33 ± 11.23	5568.77 ± 21.40	0.275
Daily feed intake (g/day)	113.09 ± 0.51	112.89 ± 0.29	112.58 ± 0.23	113.65 ± 0.44	0.277
Feed conversion ratio	3.78 ± 0.08 ^{a}	3.55 ± 0.07 ^{b}	3.26 ± 0.04 ^{c}	3.29 ± 0.07 ^{c}	0.001

^{a,b,c} Means with a different superscript in the same row are significantly different ($p \leq 0.05$).

Results in Figure 1 indicate that there was no significant difference between the treatments in body weights in the first 3 weeks of the present study. However, body weights increased ($p \leq 0.05$) with RSO and WGO or their mixture treatments from the 4th week to the 7th week of the experiment compared to the control group.

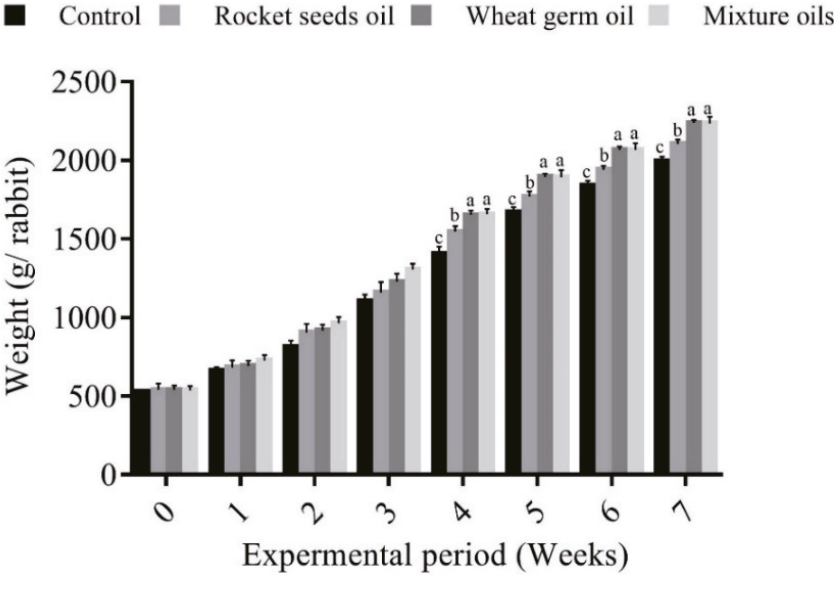

Figure 1. Body weight of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture. Columns marked with different superscripts are significantly different at $p \leq 0.05$.

3.2. Digestion Coefficients of Nutrient, Nutritive Values, and Nitrogen Balance

The different experimental treatments with oils had a significant ($p \leq 0.05$) improvement in the digestibility of dry matter, organic matter, crude protein, ether extract, and fiber fraction compared to the control group (Table 3). The results illustrated in Figure 2 show that the oral administration of RSO, WGO, and their mixture had a significant effect on the total TDN, DCP, and DE. The data presented in Table 3 showed that there was a significant ($p \leq 0.05$) improvement in nitrogen intake, absorbed nitrogen, NB, NB as % of N-intake, and NB as % of absorbed-N in rabbits that received Mos, followed by those of rabbits that received RSO and WGO compared to the control group. The results showed

that there were no significant differences in fecal nitrogen and urinary nitrogen among the experimental diets.

Table 3. Digestion coefficients of nutrient and nitrogen balance of experimental diets of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture.

Items	Control	Rocket Seed Oil	Wheat Germ Oil	Mixture Oils	*p*-Value
Digestion coefficient (%)					
Dry matter	59.80 ± 1.09 ^{c}	62.28 ± 0.91 ^{b}	64.46 ± 0.04 ^{b}	67.79 ± 0.45 ^{a}	0.001
Organic matter	63.58 ± 1.10 ^{c}	65.72 ± 0.86 ^{bc}	67.64 ± 0.18 ^{b}	70.57 ± 0.40 ^{a}	0.001
Crude protein	69.01 ± 0.13 ^{b}	70.39 ± 0.77 ^{b}	70.84 ± 0.47 ^{b}	73.21 ± 1.05 ^{a}	0.008
Ether extract	52.16 ± 1.26 ^{d}	58.77 ± 0.77 ^{c}	62.47 ± 1.02 ^{b}	67.11 ± 0.44 ^{a}	0.001
Crude fiber	57.60 ± 0.64	58.69 ± 2.63	59.78 ± 0.53	62.12 ± 1.77	0.293
NDF	54.17 ± 0.84 ^{d}	57.93 ± 0.87 ^{c}	60.89 ± 0.21 ^{b}	64.97 ± 0.45 ^{a}	0.001
ADF	53.32 ± 0.91 ^{d}	58.29 ± 0.82 ^{c}	61.56 ± 0.55 ^{b}	65.88 ± 0.39 ^{a}	0.001
Hemicellulose	55.27 ± 1.04 ^{c}	57.47 ± 0.95 ^{c}	60.02 ± 0.28 ^{b}	63.80 ± 0.59 ^{a}	0.002
Nitrogen free extract	65.50 ± 2.00 ^{b}	66.98 ± 1.02 ^{b}	69.09 ± 0.48 ^{ab}	71.89 ± 0.40 ^{a}	0.012
Nitrogen balance, NB					
N intake (g/day)	2.97 ± 0.03 ^{c}	3.15 ± 0.03 ^{b}	3.20 ± 0.03 ^{b}	3.35 ± 0.03 ^{a}	0.001
Feces N (g/day)	0.92 ± 0.01	0.93 ± 0.02	0.94 ± 0.02	0.90 ± 0.03	0.670
Absorbed N (g/day)	2.05 ± 0.02 ^{c}	2.21 ± 0.03 ^{b}	2.27 ± 0.02 ^{b}	2.46 ± 0.04 ^{a}	0.001
Urine N (g/day)	0.53 ± 0.02	0.46 ± 0.02	0.48 ± 0.02	0.48 ± 0.04	0.315
N balance (g/day)	1.52 ± 0.02 ^{c}	1.76 ± 0.02 ^{b}	1.79 ± 0.03 ^{b}	1.98 ± 0.07 ^{a}	0.001
NB intake (%)	51.17 ± 0.46 ^{b}	55.86 ± 0.74 ^{a}	55.90 ± 0.81 ^{a}	58.95 ± 2.13 ^{a}	0.006
NB absorption (%)	74.15 ± 0.78 ^{b}	79.37 ± 0.92 ^{a}	78.90 ± 0.86 ^{a}	80.45 ± 1.83 ^{a}	0.012

^{a,b,c,d} Means with a different superscript in the same row are significantly different ($p \leq 0.05$). NDF: Neutral detergent fiber; ADF: Acid detergent fiber; NB: Nitrogen balance.

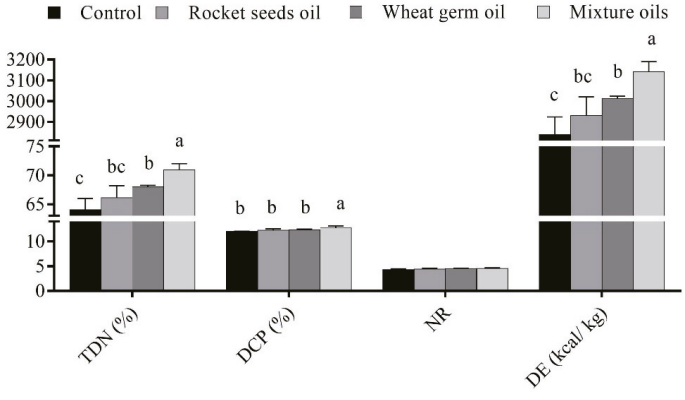

Figure 2. Nutritive value and digestible energy of experimental diets of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture. Columns marked with different superscripts are significantly different at $p \leq 0.05$. TDN: Total digestible nutrients; DCP: Digestible crude protein; NR: Nutritive value; DE: Digestible energy.

3.3. Lipid Content and Fatty Acids Profile of Rabbit Meat

The results of the lipid content and the fatty acid composition of the rabbit meat were influenced by the oil administration (Table 4 and Figure 3). It was observed that rabbits that received RSO, WGO, and MOs had a significant ($p = 0.001$) decrease in the meat content of lipids compared to the control rabbits. In addition, rabbits that received RSO and WGO had a significant ($p = 0.001$) increase in the meat content of linolenic c18:3 n-3 compared to the control rabbits. Moreover, TUFA in meat increased significantly ($p = 0.050$) with MOs treatment and insignificantly with RSO and WGO treatments compared to the control group. There was a significant ($p = 0.050$) reduction in the palmitic c16:0 and oleic c18:1 concentration in the muscles of rabbits that received a mixture of oils and WGO compared to the control rabbits. Furthermore, there was a significant ($p = 0.050$) reduction in SFA concentration in the muscle of rabbits that received a mixture of oils compared to the control rabbits. On the other hand, oral administration of RSO, WGO, and their mixture had no effect on the meat content of linoleic c18:2, PUFA, and omega-6.

Table 4. Meat fatty acid profile of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture.

Items	Control	Rocket Seed Oil	Wheat Germ Oil	Mixture Oils	*p*-Value
Palmitic (c16:0,%)	33.54 ± 0.56 ^{a}	32.45 ± 1.74 ^{ab}	30.79 ± 1.04 ^{ab}	27.03 ± 2.47 ^{b}	0.050
Stearic (c18:0,%)	6.78 ± 0.20	6.45 ± 0.15	6.49 ± 1.12	7.19 ± 1.08	0.902
Palmitoleic (c16:1,%)	4.42 ± 0.56	4.43 ± 0.83	3.18 ± 0.64	4.65 ± 0.29	0.372
Oleic (c18:1,%)	31.35 ± 0.43 ^{a}	30.21 ± 0.96 ^{ab}	28.30 ± 0.42 ^{b}	28.64 ± 0.85 ^{b}	0.050
Linoleic (c18:2; n-6,%)	19.31 ± 1.52	20.65 ± 3.20	24.13 ± 1.99	24.03 ± 1.08	0.330
Linolenic (c18:3; n-3,%)	3.12 ± 0.07 ^{c}	4.32 ± 0.25 ^{b}	5.29 ± 0.35 ^{a}	5.59 ± 0.12 ^{a}	0.001

^{a,b,c} Means with a different superscript in the same row are significantly different ($p \leq 0.05$).

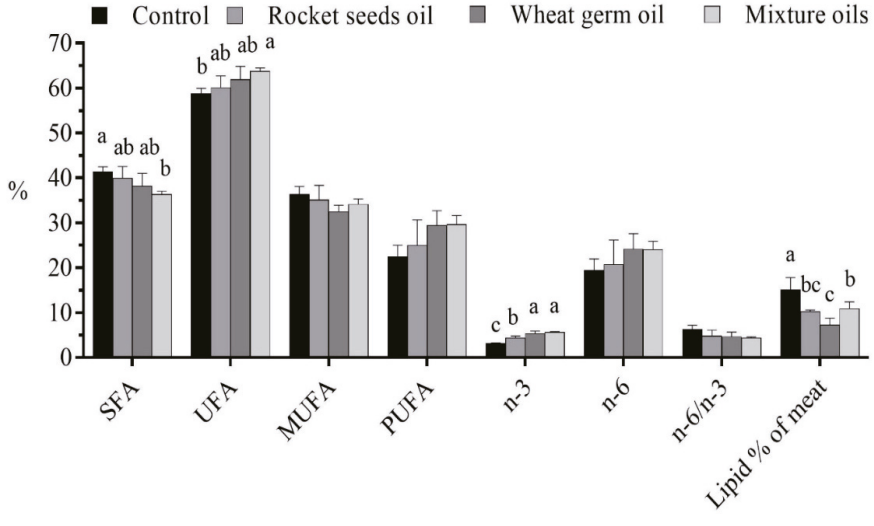

Figure 3. Meat fatty acid profile and lipids content of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture. Columns marked with different superscripts are significantly different at $p \leq 0.05$. SFA: Saturated fatty acids; UFA: Unsaturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; n-3: Omega-3; n-6: Omega-6.

3.4. Serum Biochemical Parameters

The changes in the serum biochemical parameters due to the oral administration of growing rabbits with RSO and WGO or their mixture are presented in Table 5. The serum total protein increased significantly ($p = 0.019$) by oral administration of MOs compared with the control group. However, serum albumin tended to increase in groups that received RSO and MOs than in the control group. As well as serum globulin tended to increase in the rabbits that received WGO and MOs than in the control group. In comparison with the control group, oral administration of RSO and WGO or a mixture of them caused ($p = 0.050$) a decrease in serum blood urea nitrogen.

Table 5. Serum biochemical constituents of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture.

Items	Control	Rocket Seed Oil	Wheat Germ Oil	Mixture Oils	*p*-Value
Protein profile					
Protein (g/dL)	5.37 ± 0.09 ^{b}	5.39 ± 0.15 ^{b}	5.70 ± 0.20 ^{ab}	5.97 ± 0.05 ^{a}	0.019
Albumin (g/dL)	3.06 ± 0.14 ^{ab}	3.36 ± 0.09 ^{a}	3.01 ± 0.06 ^{b}	3.38 ± 0.12 ^{a}	0.041
Globulin (g/dL)	2.31 ± 0.22 ^{ab}	2.03 ± 0.21 ^{b}	2.69 ± 0.19 ^{a}	2.59 ± 0.16 ^{a}	0.050
A/G ratio	1.41 ± 0.22	1.56 ± 0.20	1.15 ± 0.10	1.34 ± 0.13	0.215
Kidney function					
Urea (mg/dL)	49.50 ± 1.70 ^{a}	44.30 ± 1.22 ^{b}	45.70 ± 0.89 ^{ab}	45.40 ± 1.12 ^{b}	0.050
Lipid profile					
Total lipid (mg/dL)	433.00 ± 11.45 ^{b}	470.00 ± 3.85 ^{a}	424.70 ± 6.55 ^{b}	424.50 ± 4.26 ^{b}	0.001
TG (mg/dL)	61.60 ± 2.93	65.10 ± 4.89	69.10 ± 2.41	71.10 ± 5.67	0.411
Cholesterol (mg/dL)	63.00 ± 3.74	61.40 ± 4.48	62.00 ± 2.02	60.40 ± 1.89	0.955
HDL-c (mg/dL)	29.40 ± 1.29 ^{b}	32.50 ± 0.92 ^{ab}	34.00 ± 0.71 ^{a}	33.20 ± 1.16 ^{a}	0.033
LDL-c (mg/dL)	21.28 ± 3.40 ^{a}	15.88 ± 3.74 ^{b}	14.18 ± 1.97 ^{b}	12.98 ± 2.00 ^{c}	0.001
vLDL-c (mg/dL)	12.32 ± 0.59	13.02 ± 0.98	13.82 ± 0.48	14.22 ± 1.13	0.412
HDL-c/LDL-c ratio	1.61 ± 0.39	2.50 ± 0.53	2.56 ± 0.30	2.78 ± 0.40	0.238
Hormone assay					
T4 (ng/mL)	2.83 ± 0.10	3.48 ± 0.10	3.15 ± 0.15	3.46 ± 0.30	0.070
T3 (ng/mL)	1.04 ± 0.07 ^{b}	1.34 ± 0.02 ^{a}	1.36 ± 0.04 ^{a}	1.42 ± 0.16 ^{a}	0.035

^{a,b,c} Means with a different superscript in the same row are significantly different ($p \leq 0.05$). A/G ratio: Albumin/globulin ratio; HDL-c: High-density lipoprotein-cholesterol; LDL-c: Low-density lipoprotein-cholesterol; vLDL-c: Very low-density lipoprotein-cholesterol; T3: Thyroxine; T4: Triiodothyronine.

In terms of lipid profile, it has been observed that oral administration of RSO ($p = 0.001$) increased total lipids compared to other groups. Serum blood HDL-c was higher ($p = 0.033$) in the WGO and MOs groups than in the RSO and the control groups. Whereas, all the treated rabbits with vegetable oils had ($p = 0.001$) a decrease in serum LDL-c compared to the control group. Moreover, there is no significant ($p > 0.05$) effect between oil treatments and the control group in serum blood TG, cholesterol, and vLDL-c and HDL-c/LDL-c ratio. The treatments of RSO, WGO, and a mixture of them had a positive effect on thyroid hormone secretion.

3.5. Antioxidant Status

Oral administration of RSO and MOs resulted in a significant ($p = 0.050$) decrease in blood serum lipid peroxidation (i.e., TBARs) (Figure 4). Rocket seed oil and MOs reduced serum TBARS concentration by about 9.43% of the control group. However, there is a significant increase in serum antioxidant activities as measured by CAT ($p = 0.021$) and SOD ($p = 0.001$) by oral administration of RSO and WGO or their mixture when compared to the control group, while serum TAC activity increased significantly ($p = 0.017$) by oral administration of RSO and MOs when compared to the control group.

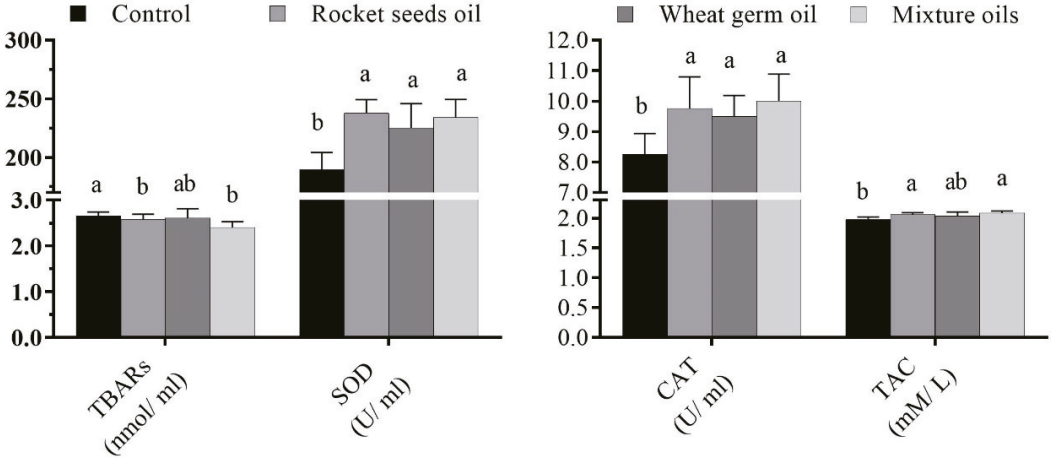

Figure 4. Serum thiobarbituric acid reactive substances and antioxidants status of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture. Columns marked with different superscripts are significantly different at $p \leq 0.05$. CAT: Catalase; SOD: Superoxide dismutase; TAC: Total antioxidant capacity; TBARs: Thiobarbituric acid reactive substances.

3.6. Antibody Titers against SRBCs

The humoral immune response includes the response of natural antibodies to SRBCs, which were measured at 9, 10, and 11 weeks of age, as shown in Table 6. Oral administration of RSO, WGO, and their mixture caused an improvement ($p = 0.010$) in the antibody titers against SRBCs of growing rabbits at 9 weeks of the rabbit age compared to the control group. However, the antibody titers against SRBCs of growing rabbits at 10 weeks of rabbit age significantly increased ($p = 0.044$) in the group that orally received WGO compared to the control.

Table 6. Antibody titers against SRBCs of V-line growing rabbits (5–12 weeks of age) that were administered orally with rocket seed oil, wheat germ oil, and their mixture.

Items	Control	Rocket Seed Oil	Wheat Germ Oil	Mixture Oils	*p*-Value
SRBCs at 9 weeks of age	0.74 ± 0.04^{b}	0.84 ± 0.02^{a}	0.87 ± 0.02^{a}	0.84 ± 0.02^{a}	0.010
SRBCs at 10 weeks of age	0.76 ± 0.03^{b}	0.84 ± 0.03^{ab}	0.85 ± 0.02^{a}	0.76 ± 0.03^{b}	0.044
SRBCs at 11 weeks of age	0.71 ± 0.04	0.76 ± 0.11	0.88 ± 0.02	0.71 ± 0.03	0.201

^{a,b} Means with a different superscript in the same row are significantly different ($p \leq 0.05$). SRBCs: Sheep red blood cell counts.

4. Discussion

4.1. Growth Performance and Feed Utilization

Vegetable oils, such as olive oil, rice bran oil, corn germ oil, rocket seed oil, and wheat germ oil have been used as a source of energy, essential polyunsaturated fatty acids and fat-soluble vitamins with great nutritional and health benefits [56]. Rocket, *E. sativa* is considered an important leafy vegetable crop that is high in antioxidant molecules, and its seeds contain oil up to 25–35% [11]. Wheat has been called the staff of life, and people who have many health concerns have used wheat germ oil [57]. Wheat germ oil represents about 10–15% of the whole wheat germ, which is reported to be one of the potential resources for beneficial molecules [21].

In the current study, the final BW, weight gain, and ADG increased significantly in rabbits that were administered orally with RSO and WGO or their mixture compared to the control group. In the same vein, the RSO treatment significantly increased the final BW and daily gain of rabbits [16]. Moreover, the final BW, ADG, and total gain of growing rabbits were higher significantly with the supplementation of RSO, and the mixture of RSO and onion oil [17] or with the supplementation of watercress oil and the mixture of watercress oil and coconut oil [18]. Furthermore, dietary supplementation of WGO increased the BW of male broilers [29]. In conflict with our results, the addition of RSO (1–3 mL/kg BW) in rabbit's diet for 2 weeks tended to decrease BW as the dependent dose [19].

In the current study, all the groups were fed the same diet (isocaloric), but the groups receiving the supplemental oils (0.3 mL/kg BW) had more caloric value by ≈3 kcal/kg BW as an energy intake, representing a 0.7% increase in the daily gross energy intake. This increase is not significant, and it could not affect the obtained results of growth, feed utilization, physiological performance, and digestibility. Previous studies revealed that ADG, slaughter live BW, and carcass weight were not affected either by the fat source or fat level [58]. In this study, the difference in gross energy was 3.44%, which was obtained using different fat sources (linseed and black soldier fly). In addition, the increase in fat levels by 3% did not affect the growth rate of rabbits due to the decreased feed intake, but it increased energy digestibility and feed efficiency [59,60]. Moreover, rabbits fed three diets with different energy levels (2707, 2436, and 2276 kcal DE/kg) did not affect live BW, weight gain, FCR [61]. However, the increase in gross energy by 8.79% could affect the rabbit BW [62].

Accordingly, the improvement in BW and BWG with vegetable oil supplementation in the present study could be attributed to the content of active molecules in rocket seeds, such as carotenoids, vitamin C, glucoerucin, and flavonoids, which are health-promoting agents [63]. In addition, the volatile oil of rocket seeds contains isothiocyanates, which have antimicrobial, antioxidant, and anticarcinogenic activities [64]. Moreover, WGO is a natural source of α-tocopherol, which increases ($p \leq 0.05$) the BW and BWG of growing rabbits [65]. Mustacich et al. [66] supported these findings by stating that the natural α-tocopherol has higher natural activity than synthetic α-tocopherol. Furthermore, linoleic acid found in WGO operates as the precursor of cell membrane phospholipids [67], which could also participate in the growth improvement in the current study.

The oral administration of vegetable oils had no effect on daily feed intake and total feed intake during the whole experimental period in the present study. The route of administration in the present study was gavage, which could not affect the diet palatability and therefore maintained the normal feed intake. Meanwhile, FCR improved significantly with RSO and WGO or their mixture treatments compared to the control group. In accordance, the feed intake did not affect the 12-weeks aged rabbits that were given orally different levels of watercress oil plus coconut oil compared to the control group. However, the FCR of rabbits was improved with watercress oil or watercress oil plus coconut oil [18]. The improvement in the FCR of growing rabbits may be attributed to the properties of these oils that act as antibacterial, antiprotozoal, antifungal, and antioxidants [18,68,69]. Moreover, rocket seed cakes increased the FCR of male rabbits, which may be due to their beneficial effect on stimulating and activating the digestive system [16].

4.2. Digestion Coefficients of Nutrient, Nutritive Values, and Nitrogen Balance

The different experimental oil treatments had a significant ($p \leq 0.05$) improvement in nutrient digestibility compared to the control group. This improvement in nutrient digestion coefficients resulted in a significant increase in body weight gain and an improvement in the FCR (Table 2). Whereas, rocket seed cakes have a beneficial effect on stimulating and activating the digestive system [16]. In parallel to our results, using onion or moringa oils or a mixture of them significantly ($p \leq 0.05$) improved digestibility coefficients of CP and EE compared to the control group. Meanwhile, the digestibility of DM, OM, CF, and NFE was not affected significantly by the addition of different oils [70,71]. In this line, the addition of 1 g RSO/kg diet or 1 g onion seed oil/kg diet and their combination in the growing rabbit's diet increased ($p \leq 0.05$) the digestion coefficients of CP and EE compared to the control group [17]. In this regard, the addition of fennel seeds, oregano leaves only or the mixture to the rabbit's diet had a significant ($p \leq 0.05$) improvement in DM, OM, and CF digestibility [72].

Concerning the nutritive values and digestible energy of the experimental diets, the results showed that TDN, DCP, and DE increased significantly in RSO, WGO, and their mixture groups. In accordance with the current results, the fat-containing rabbit diets with or without a herbal mixture formulation containing fennel seeds and oregano leaves improved both TDN and DCP values [72]. Moreover, there was a significant ($p \leq 0.05$) improvement in nitrogen intake, absorbed nitrogen, and NB in rabbits that received Mos, followed by rabbits that received RSO and WGO compared to the control group. The improvement in N absorbed and NB of the experimental diets of rabbits that received vegetable oils resulted in a significant ($p = 0.050$) decrease in serum blood urea nitrogen in the current study (Table 5).

4.3. Lipid Content and Fatty Acid Profile of Rabbit Meat

Currently, consumers are increasingly praising foods that contain not only macronutrients, but also beneficial compounds for health and welfare [73]. Rabbit meat is a good source of protein with a low fat and cholesterol content, and it has a lower energetic value than red meat [74]. Moreover, the fatty acid composition of rabbit meat consists of a high polyunsaturated fatty acid content [74]. Several studies have suggested that n-3 fatty acids play an important role in human nutrition since they help reduce the incidence of lifestyle diseases such as coronary artery diseases, atherosclerotic diseases, hypertension and diabetes, as well as certain inflammatory diseases, such as arthritis and dermatitis [23]. The increase in the n-3 PUFA level in rabbit meat is feasible and could be achieved by feeding them n-3 PUFA rich diets. Furthermore, the supplementation of natural antioxidants in feed declines lipid peroxidation and enhances the stability of unsaturated fatty acids [29].

The current findings show that rabbits that received RSO, WGO, and their mixture had a significant ($p = 0.001$) decrease in the meat content of lipids and had a positive effect on meat fatty acid composition, including a significant increase in TUFA and linolenic c18:3 n-3 and had a significant reduction in the meat content of SFA, palmitic c16:0, and oleic c18:1 compared to the control rabbits.

These results are in agreement with other studies that have shown that dietary manipulations in monogastric animals, including rabbits, can change the quantity and chemical composition of the fatty acid. Whereas, vegetable oils can lower the PUFA/SFA ratio and increase the n-3 value in rabbit meat [6,75,76]. In addition, supplementing the rabbit feed with 8% linseed increased the C18:3 n-3 concentration and decreased the n-6/n-3 ratio in rabbit meat compared to the control group [77]. Daily linseed oil supplementation for 30 days increased the amount of ω-3 fatty acid in the muscular tissue lipids of bucks from 4.49 to 7.72%, i.e., by 1.72 times [78]. Moreover, the feeding of linseed oil rich in n-3 PUFA can be an effective method for increasing the tissue levels of these fatty acids in broiler chickens [79–81]. Blending different vegetable oils can modify the fatty acid composition

and raise levels of natural antioxidants and bioactive lipids in the mixes, thus, leading to an improvement in the nutritional value and oil stability [82].

4.4. Serum Biochemical Parameters

Oral administration of RSO, WGO, and their mixture improved the serum total protein as well as albumin and globulin levels compared to the control group. In agreement with the current results, the serum total protein as well as albumin and globulin levels improved significantly ($p \leq 0.05$) when the rabbits were given the mixture of moringa oil and RSO, followed by individual moringa oil and RSO supplementation or growing rabbits that received moringa oil alone or with onion oil [70,71]. The significant increase in serum total protein and albumin observed in rabbits that were administered orally with RSO, WGO, and their mixture indicated the ability of these oils to stimulate the regeneration of hepatic tissue, which increased protein synthesis in the liver and improved the functional status of the liver cells [12,83] or these oils had a positive effect on thyroid hormone secretion, which could affect the metabolism of nutrients (Table 5). In addition, the rocket seeds contain vitamin C and carotenoids [63], which play an important role in the protection against oxidative damage [84] as indicated in improving the serum antioxidant status of rabbits in the present findings (Figure 3). Moreover, WGO has a high content of other nutritional and health-benefit factors, such as vitamin E and phytosterol [85], which may be the reason for its improving effect on the blood protein profile. The increase in serum total protein and globulin is a general indication of the immune status of the animal, since the liver can synthesize enough globulin for immunologic action, as mentioned by Sunmonu and Oloyede [86]. These improvements signify better disease resistance and increase immune response and animal resistance against any physiological or physical stressors [87].

Oral administration of RSO and WGO or a mixture of them caused ($p = 0.050$) a decrease in serum blood urea nitrogen. This reduction in urea nitrogen may be due to the improvement of NB as indicated in the present results (Table 3). In terms of lipid profile, it has been observed that oral administration of RSO, WGO, and MOs had a positive effect on serum total lipids, HDL-c, and LDL-c compared to the other groups.

In agreement with the current results, the supplementation of 1–3 mL/kg BW of RSO to the rabbit's diet decreased ($p \leq 0.05$) the level of serum LDL-c and increased the serum HDL-c level compared to the control group [19]. In addition, Abozid et al. [88] discovered that RSO had a clear effect on improving the lipid profile due to its high concentration of plant sterols. Whereas, plant sterols reduce the incorporation of dietary and biliary cholesterol into micelles, leading to low cholesterol synthesis and cholesterol absorption as well as increasing LDL-c receptor activity, which eventually results in lower serum LDL-c concentration [89]. Moreover, total cholesterol, triglycerides, and LDL-c concentrations ($p > 0.05$) decreased as well as the HDL-c ($p \leq 0.05$) increased with the WGO treatment [90]. The lowering effect of WGO on triglyceride, cholesterol, and LDL-c may be attributed to the high content of vitamin E and phytosterol in WGO [85]. Furthermore, linoleic acid found in WGO leads to the abstraction of cholesterol and acts as the precursor of cell membrane phospholipids [67,91]. WGO contains policosanol, which can reduce the high concentration of blood total cholesterol [92].

4.5. Antioxidants

Oxidative stress is defined as an imbalance between pro- and anti-oxidant species, which leads to molecular and cellular damage [93]. The oxidative damages could be mitigated by endogenous defense systems, such as CAT, SOD, and GPX system, but this system is not completely efficient, especially under stress [94]. The determination of single components of this system or so-called TAC could reveal the efficiency of enzymatic and non-enzymatic antioxidative systems [95].

Vegetable oils, such as olive oil, rice bran oil, corn germ oil, and wheat germ oil have been used as a source of plant antioxidants [96,97]. In the current findings, the oral administration of RSO and WGO or their mixture showed a significant decrease in TBARs

and a significant increase in blood serum antioxidant properties (CAT, SOD, and TAC). In accordance with the present results, the treatments with RSO and WGO have a positive effect on the antioxidant status, as they are very efficient scavengers of free radicals [18,98]. Whereas, RSO decreases hepatic lipid peroxidation as a result of increasing hepatic SOD activity [12]. In addition, RSO has a high content of natural antioxidants, such as phenolic compounds [99].

Wheat germ oil has a positive effect on the antioxidant defense system [23] due to its content of antioxidant compounds, such as alpha, beta, and gamma-tocopherols, tocotrienols [22], and fat-soluble carotenoids, such as lutein, zeaxanthin, and beta-carotene [24]. Moreover, WGO has remarkable antioxidant effects owing to the individual and synergistic effects of fatty acids, antioxidants, vitamins, phytosterols, and phenolic compounds [24,100]. Another prospective interpretation is that WGO promotes the tocopherol-mediated redox system and prevents the synthesis of eicosanoids (prostaglandins) that stimulate the lipid peroxidation process [101].

4.6. Antibody Titers against SRBCs

Antibody titers against SRBCs of growing rabbits were improved significantly by oral administration of RSO, WGO, and their mixture. These results suggest that RSO, WGO, and their mixture might have synergistic effects on immune responses. In harmony with the current findings, the addition of 1 g RSO/kg diet or 1 g onion seed oil/kg diet and their combination in the growing rabbit's diet improved ($p \leq 0.05$) antibody titer against SRBCs compared to the control group [17]. Moreover, dietary supplementation with watercress oil plus coconut oil resulted in improvement in the immunity of growing rabbits and may be associated with a reduction in pathogenic cecal bacteria and enhancing the antioxidant status [18].

The improvement of antibody titer against SRBCs may be due to carotenoids found in Rocket, *E. Sativa*, which can conserve phagocytic cells from antioxidative defects, improve T and B lymphocyte proliferative responses, and ameliorate the production of inevitable interleukins [102]. Moreover, carotenoids raise plasma IgG levels [103]. WGO contains higher amounts of vitamin E (more than approximately 50 mg vitamin E/100 g oil), whereas dietary vitamin E combined with Se in diets caused an improvement in the antibody titers against SRBCs of growing rabbits [65]. Otherwise, WGO is rich in n-3 PUFA as reported in the current study (Table 3 and Figure 3), mainly alpha-linolenic acid, which recovers the immune system [104].

5. Conclusions

The treatment with rocket seed oil, wheat germ oil, and their mixture increased body weight, improved feed conversion ratio, nutrient digestibility, and nitrogen balance. In addition, it enhanced the fatty acid profile and lipid content of the rabbit meat, and improved the blood metabolites. The combination of rocket seed oil and wheat germ oil had a synergistic effect on minimizing lipid peroxidation in blood serum and maximizing the antioxidant defense system, which probably translates into enhancing growth performance, muscle integrity, immunity, and disease resistance in growing rabbits. Getting such positive results is vital, especially under commercial production conditions. However, further studies could be needed to validate the efficacy of incorporating the studied oils into the rabbit's diets rather than the oral gavage to facilitate animal husbandry under commercial farming.

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Data Availability Statement: The data that support the findings of this study are available from the authors upon reasonable request.

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Effects of Dietary Supplementation of Humic Substances on Production Parameters, Immune Status and Gut Microbiota of Laying Hens

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Abstract: Despite the fact that humic substances (HS) have been frequently studied in relation to their effects on livestock health, studies on their influence on egg production and quality, immunity, and intestinal microbiota of laying hens are limited. In this study, the influence of 0.5% HS supplementation on the specific production parameters of eggshell mineral quality, immune parameters (relative expression of IgA, IGF-2, MUC-2 gene in cecum; activity of phagocytes, percentage of selected lymphocyte subpopulations in the peripheral blood), and number of lactic acid bacteria and enterobacteria in the intestinal contents in laying hens was tested. The addition of 0.5% HS to the laying hen feed had a positive effect on egg laying rate, daily egg mass, egg weight, feed conversion and eggshell quality and also had an immunostimulatory effect manifested by increased phagocyte activity and B cell response. Concurrently, an increase in the number of enterobacteria in the intestinal contents and a decrease in the proportion of T lymphocytes ($p < 0.05$) was observed, which can be considered as a negative effect of HS. The results confirmed that HS can be used for the improvement of egg production and targeted immunostimulation, but their effect on the intestinal microbiota and T lymphocytes should be studied in more detail.

Keywords: humic substance; egg; immune answer; lymphocyte; intestinal microbiota

1. Introduction

Humic substances (HS) have a complex organic matter structure, which, among other things, reduces the toxic effect of herbicides, heavy metals, and soil-polluting radionuclides [1–4]. Moreover, it has been shown that HS transfer micronutrients, especially iron from soil to plants, and improve microbial populations in soils [5]. Active components of HS consist of humic acid, humus, ulmic acid, fulvic acid, humin, and certain microelements [6,7]. HS have primarily been used to stimulate plant growth [8], but they were also successfully applied in poultry nutrition, e.g., in various forms (natural and acidified HS) and concentrations (0.25% of HS extract, 0.6%, 0.8%, and 1.0% HS) [9–12].

Recently, trends in agriculture and animal feed applications are aimed at studying the impacts of various feed supplements targeting the enhanced health and productivity of the

animals to produce healthy and safe food [13]. Egg quality and production are the most important economic factors in layer hen industry because worldwide egg consumption has been increasing in last decade [14]. Laying hen nutrition could be considered as one of the critical factors for optimizing egg quality in terms of nutritional composition or the economic aspects of whole egg production [15]. Novel nutritional approaches in poultry nutrition represent appropriate strategies for the improvement of animal feed conversion, mainly via feed ingredients or additives. Natural nutritional substances, such as antioxidants, vitamins, trace elements, etc., are well known for their positive impact on health and disease prevention in laying hens [16], while the use of HS in the diets of laying hens as feed additive is substantially new issue [17]. The productive performance and egg quality in layers could be affected by hen nutrition [18], and because of that, we assumed the improvement of the observed variables with HS supplementation in the diets of laying hens.

Nagaraju et al. [19] presented that the addition of humic acids to antibiotic-free feed improved the performance and immune status of broilers. Specifically, modulation of immune functions by means of HS also enables the support of health and production parameters. The effect of HS on the body's immune system is related to the ability of these substances to form relatively strong complexes with carbohydrates [20]. Consequently, these complexes make it possible to produce glycoproteins in the body, which are defined by the ability to bind to NK cells and T lymphocytes in order to act as modulators and to ensure subsequent communication between these cells. Thus, the ability of HS to affect the immune system lies in the regulation of immune activity and the prevention of excessive activity [21].

In addition, HS are also able to support the formation of a protective mucous film on the intestine epithelium, which has an impact on gut health [22]. Similarly, Islam et al. [23] reported positive effect of HS on farm animal health through the modification of the ecosystem in the intestine. HS allows for the better utilization of nutrients and improves not only gut health but also the quality of laid eggs [24]. Arpášová et al. [24] observed that the addition of humic acid in 0.5% concentration led to significantly higher egg production with an increased egg albumen index. Moreover, HS with supplementation with probiotic products based on lactobacilli significantly increased the egg albumen index and Haugh units. Sopoliga et al. [25] reported increased hatchability in pheasant laying hens after the intake of humic substances in comparison to hens fed with the conventional feed mixture.

However, scientific studies on the effect of HS supplementation in the diets laying hens on egg production and quality that are also supported by the immune parameters are limited. For this reason, our study was focused on the influence of 0.5% HS supplementation on the production parameters of eggshell mineral quality, immune parameters (relative expression of IgA, IGF-2, MUC-2 gene in cecum; activity of phagocytes, percentage of selected lymphocyte subpopulations (IgM+, CD3+, CD4+, CD8a+) in the peripheral blood), and the number of lactic acid bacteria and enterobacteria in the intestinal contents in laying hens.

2. Materials and Methods

2.1. Experimental Design

The animal protocol for this research was approved by the Ethical Committee for Animal Care and Use of University of Veterinary Medicine and Pharmacy in Košice (the Slovak Republic) and the Food Administration of the Slovak Republic (approved the experimental protocol number 3040/14-221). All procedures in this study were performed in accordance with the principles of the European Directive on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Parliament and Council, 2010). The experimental diets were composed of commercial basal feed mixture with naturally occurring biogenic and heterogeneous organic substances as a HS supplement in accordance with regulation 68/2013 from 16 January 2013 of the European Union Commission,

which states that the use of Leonardite is allowed as a source of HS as a feed component in animal diets.

A total of sixty laying hens of the Lohman Brown classic hybrid (Eggro-farm Ltd., Košická Polianka, Slovakia) in the 17th week of life were divided into two groups: the control (C) group and experimental humic (H) group. Each treatment group consisted of 30 birds (average weight 1.36 ± 0.15 kg). The control group (C) was fed with a basal feed mixture (De Heus, Bučovice, Czech Republic) without HS supplementation during the trial. The experimental group (H) was fed with basic feed mixture supplemented with 0.5% dietary natural HS (Humac Ltd. Košice, Slovakia). The diet of the experimental group (H) was prepared daily and was enriched with HS at a concentration of 0.5%. The supplement was applied to the surface of the basal diet. The nutritional composition of the basal feed mixtures is presented in Table 1. The dietary natural HS supplement used in the experiment was ground and physically purified Leonardite without chemical treatment. It contained natural HS with more than 65% of humic acid without acid salts [12]. Laying hens were housed in floor pens with deep wood untreated litter in the form of being free range in secure and controlled microclimate conditions according to the Lohmann Brown Classic Management Guide [26]. The diet of the laying hens was formulated according to the recommended nutrient content for Lohmann Brown Classic hens [26]. The chemical compositions of the fermented feed and diets were determined for dry matter, crude protein, crude fat, crude fibre, starch, and total phosphorus according to the EC Commission Regulation 152/2009 [27] and Semjon et al. [28].

Table 1. Components and nutritional composition of laying hen diet.

Component	**Diet**	
Corn grain, %	27.50	
Wheat grain, %	31.00	
Barley grain, %	7.50	
Soybean meal, %	11.00	
Corn gluten meal, %	1.65	
Rapeseed meal, %	1.70	
Sunflower cake, %	3.20	
Sunflower oil, %	3.00	
Wheat bran, %	5.00	
Limestone, %	6.00	
Monocalcium Phosphate, %	1.25	
Salt, %	0.20	
Premix of amino acids, vitamins, and minerals *, %	1.00	
Ingredients	**As Fed**	**Dry matter**
Dry matter, g·kg^{-1}	899.00	1000.00
Crude protein, g·kg^{-1}	157.50	175.19
Crude fat, g·kg^{-1}	44.23	49.20
Crude fiber, g·kg^{-1}	48.48	53.93
Ash, g·kg^{-1}	128.60	143.05
Starch, g·kg^{-1}	414.80	461.40
Calcium, g·kg^{-1}	38.00	42.27
Phosphorus, g·kg^{-1}	4.80	5.34
Sodium, g·kg^{-1}	1.50	1.67
Copper, mg·kg^{-1}	18.08	20.11
Zinc, mg·kg^{-1}	65.90	73.30
Manganese, mg·kg^{-1}	87.55	97.39

* Premix of amino acids, vitamins, trace elements (per kg): lysine 140 155 g; DL-methionine 180 g; vit. A 1,200,000 IU; D3 500,000 IU; E 2000 mg; pantothenic acid 1800 mg; niacin 6000 156 mg; choline 60 mg; B6 500 mg; B12 1.8 mg; folic acid 200 mg; copper 1100 mg; iron 8400 mg; zinc 8000 mg; 157 manganese 12,000 mg; iodine 110 mg; selenium 40 mg.

Laying hens were fed once a day with daily prepared diets. During weeks 17 and 18, they were fed 75 g; in week 19, they were fed 81 g; from week 20 to the laying phase, they were fed 93 g; and during the laying phase, they were fed 100 g of diet/layer/day. Access of the layers to water was ad libitum. The lightening program from the 17th week of age was set to a lighting period of at least 10 h, taking the natural day length into account, and it was increased by one hour every week up to 14 h until 21 weeks of age and remained stable from that point on [26]. Sufficient ventilation to ensure good litter condition was set. Their health and weight were monitored continuously. The experiment was finished when the laying hens were 29 weeks of age, at which point twelve hens were randomly selected from both groups. These animals were used for blood collection, and after euthanization by cervical dislocation, they were carcassed and sampled for further laboratory tests.

2.2. Production Parameters Screening

Daily egg production, egg weight and feed intake were recorded daily throughout the trial. The feed conversation ratio (FCR) was expressed as each kilogram of feed consumed per kilogram of egg produced by the batch. The laying rate (%) was calculated as the number of laid eggs to number of laying hens by batch per day. For the presented experiment, the number of eggs produced and the consumption of feed per animal, egg weight (g), FCR, and laying rate in week 29 were recorded.

2.3. Egg Shell Analysis

The mineral composition of the eggshells was analyzed according to the procedure of Skalická et al. [29]. Eggshell samples were immediately frozen and stored at −20 °C until they were analyzed. The samples were digested in a MLS 1200 MEGA (Milestone Microwave Laboratory System, Shelton, CT, USA) microwave oven using a mixture of 5 mL HNO3 and 1 mL HCl per 1 g of sample. The digested samples were analyzed for the presence of Ca, Mg, K, Na, Cu, Zn, and Mn using an atomic absorption spectrometer (Unicam Solar 939, Cambridge, UK). The phosphorus (P) content in the eggshell samples was determined spectrophotometrically [30]. A total of 18 egg samples from the C and H experimental groups were collected in week 29 and were subjected to eggshell analysis. Eggshell analysis was performed in triplicate and was expressed as the mean and the standard deviation

2.4. Homogenization of Cecum and Isolation of Total RNA of IgA, IGF-2 and MUC-2 Genes

Tissue samples (cecum) were cut into approximately 20 mg pieces and were immediately placed in RNA Later solution (Qiagen, West Sussex, UK). They were stored at −70 °C before RNA purification, as described in Karaffová et al. [31]. A total of 12 cecum samples from each experimental group of laying hens were collected after slaughtering and, the samples were subjected to analysis.

2.5. Relative Expression of IgA, IGF-2 and MUC-2 Genes in Quantitative Real-Time PCR (qRT-PCR)

The mRNA levels of selected genes were determined. In addition, the mRNA relative expression for reference gene coding GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was selected based on expression stability using BestKeeper software (Pfaffl, Germany). The primer sequences, optimal annealing temperature, and time for each primer used for qRT-PCR are listed in Table 2. All primer sets allowed DNA amplification efficiencies between 94% and 100%.

The amplification and detection of specific products were performed using the CFX 96 RT system (Bio-Rad, Hercules, CA, USA) and the Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA). Subsequent qRT-PCR to detect the relative expression of mRNA in the selected parameters was conducted over 38 cycles under the following conditions: initial denaturation at 94 °C for 3 min, subsequent denaturation at 93 °C for 45 s, and annealing (Table 2) and extension for 10 min at 72 °C. A melting curve from 50 °C to 95 °C with readings at every 0.5 °C was produced for each individual qRT-

PCR plate. Analysis was performed after every run to ensure a single amplified product for each reaction. All reactions for real-time PCR were done in duplicate, and the mean values of the duplicates were used for subsequent analysis. We also confirmed that the efficiency of the target gene amplification including GAPDH was essentially 100% in the exponential phase of the reaction, where the quantification cycle (Cq) was calculated. The Cq values of the studied genes were normalised to an average Cq value of the reference genes (ΔCq), and the relative expression of each gene was calculated as $2^{-\Delta Cq}$.

Table 2. List of primers used in RT-PCR for IgA, MUC-2, and IGF-2 mRNA detection in layer hens.

Primer	Sequence 5′–3′	Annealing/Temperature Time	References
IgA For	GTCACCGTCACCTGGACTACA	55 °C/30 s	[32]
IgA Rev	ACCGATGGTCTCCTTCACATC		
Muc 2 For	GCTGATTGTCACTCACGCCTT	54 °C/1 min	[33]
Muc 2 Rev	ATCTGCCTGAATCACAGGTGC		
IGF-2 For	CTCTGCTGGAAACCTACTGT	55 °C/30 s	[34]
IGF-2 Rev	GAGTACTTGGCATGAGATGG		
GAPDH For	CCTGCATCTGCCCATTT	59 °C/30 s	[35]
GAPDH Rev	GGCACGCCATCACTATC		

2.6. Phagocyte Activity Testing

The percentage of active phagocytes as well as the engulfing capacity of the phagocytes was determined using a commercial Phagotest® assay (Glycotope Biotechnology, Heidelberg, Germany). The test was performed according to the manufacturer's instructions and were performed using fresh heparinized blood [36].

2.7. Identification of Lymphocyte Subpopulations

For the identification of selected lymphocyte subpopulations, mononuclear cells were isolated from 600 µL of heparinized blood diluted 1:1 with phosphate buffer saline (PBS; MP Biomedicals, Illkirch, France), which was carefully overlaid on the 2.5 mL of separation solution LSM 1077 (PAA Laboratories GmbH, Pashing, Austria). Mononuclear cells were obtained from the interphase between the separation solution and the plasma after centrifugation at 600× *g* for 30 min. The obtained cells were washed twice with PBS through centrifugation at 250× *g* for 5 min. The concentration of the mononuclears was determined after staining with Türck's solution in a Bürker chamber and was adjusted to 5×10^5 cells in 50 µL.

To identify selected subpopulations of lymphocytes, direct immunostaining using two combinations of conjugated mouse anti-chicken monoclonal antibodies (Southern Biotech, Birmingham, AL, USA): CD4/CD8a/CD45 and CD3/IgM was used according to the specifications given in Table 3. The cells were incubated with antibodies for 20 min in the dark at laboratory temperature. The cells were then washed twice with 1 mL PBS (250× *g* for 5 min) and were resuspended in 100 µL of PBS for subsequent cytometric analysis.

Table 3. Specification and amounts of used mouse anti-chicken monoclonal antibodies.

Type	Fluorochrome	Clone	Isotype	Concentration	Amount/5×10^5 cells
anti-CD3	FITC	CT-3	IgG1 κ	0.5 mg·mL^{-1}	2 µL
anti-CD4	FITC	CT-4	IgG1, κ	0.5 mg·mL^{-1}	2 µL
anti-CD8a	R-PE	CT-8	IgG1, κ	0.1 mg·mL^{-1}	1 µL
anti-CD45	APC	LT-40	IgM, κ	0.1 mg·mL^{-1}	5 µL
anti-IgM	R-PE	M-1	IgG2b κ	0.1 mg·mL^{-1}	1 µL

2.8. Flow Cytometric Analysis

Phagocytic activity analysis as well as the identification of lymphocyte subpopulations was performed on a six colour BD FACSCanto™ flow cytometer (Becton Dickinson

Biosciences, San Diego, CA, USA) using BD FACS Diva™ software. The position of the analysed cells was gated in FSC vs. SSC dot plots. Granulocytes and monocytes were gated for phagocytic activity analysis. Bacterial aggregates were excluded from further analysis based on the low DNA content in the red fluorescence histogram (FL-2). The percentage of active phagocytes and the mean fluorescence intensity were determined in the green fluorescence histogram (FL-1).

Gated lymphocytes were used for the identification of lymphocyte subpopulations, while contaminating chicken thrombocytes were differentiated from lymphocytes based on their higher side scatter profiles [37]. CD3+ lymphocytes represent T lymphocytes, and IgM+ cells are a subpopulation of B lymphocytes. CD4+CD8a- and CD4+CD8a$^{low/mid}$ subpopulations were counted together as a representative of the T helper lymphocytes. The CD4-CD8a+ subpopulation was evaluated as T cytotoxic cells. Proportions of lymphocytes are expressed in percentage.

2.9. Intestinal Bacteria Analysis

In the contents of the small intestine and caecum were analysed to determine the number of lactic acid bacteria and enterobacteria using the plate count method after a 10-fold dilution in saline. MRS agar plates (HiMedia, Karnataka, India) that had been anaerobically incubated (GasPak system, Becton Dickinson, San Diego, CA, USA) for 48 h at 37 °C were used to determine the number of lactic acid bacteria. Enterobacteria were counted on Endo agar plates (HiMedia, Karnataka, India) after a 24 h incubation period at 37 °C under aerobic conditions. The bacterial counts are expressed in $\log_{10}$ of colony forming units per gram of content ($\log_{10}$ cfu·g^{-1}) ± standard deviation.

2.10. Statistical Analysis

The results obtained in this experiment were expressed as the means of the relevant units and the standard deviation (SD). Data were evaluated using the unpaired Student's T-test with the statistical software GraphPad Prism 8.3 (GraphPad Software, San Diego, CA, USA). A significance level of $p < 0.05$ was set prior to the data analysis.

3. Results

3.1. Production Parameters

Dietary supplementation with 0.5% HS additives had a significant effect on the following laying performance variables recorded in week 29: laying rate, daily egg mass, egg weight, and FCR, compared to the control group ($p < 0.05$) (Table 4).

Table 4. The results of layer production indices in week 29 (means ± SD).

Variable	C	H	*p*-Value
Laying rate, %	84.29 ± 7.87 ^{b}	95.91 ± 6.98 ^{a}	0.013
Daily egg mass, g/hen/day	47.50 ± 4.37 ^{b}	57.06 ± 4.05 ^{a}	0.001
Egg weight, g	56.36 ± 0.39 ^{b}	59.50 ± 0.67 ^{a}	<0.001
Feed consumption, g/hen/day	115.86 ± 2.27	116.00 ± 1.53	0.892
Feed conversion ratio	2.46 ± 0.22 ^{b}	2.04 ± 0.14 ^{a}	0.001

C: control group; H: layers fed a diet enriched with supplementation of 0.5% HS. ^{a,b} Means not sharing the same superscripts in the same row are significantly different.

3.2. Eggshell Mineral Analysis

When analyzing experimental eggshell samples, higher concentrations of minerals were determined, compared to the results from the control group. A significant increase was observed in the contents of copper ($p < 0.001$), phosphorus ($p < 0.01$), calcium, manganese, and zinc ($p < 0.05$) (Table 5).

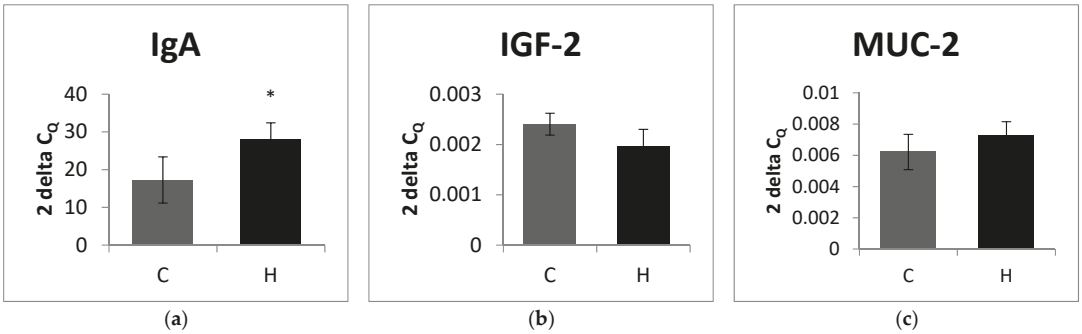
Table 5. The results of eggshell mineral analysis (means ± SD).

Variable	C	H	*p*-Value
Calcium, g·kg^{-1}	57.66 ± 0.27 ^{b}	59.22 ± 1.09 ^{a}	0.014
Magnesium, g·kg^{-1}	4.40 ± 0.25	4.67 ± 0.46	0.281
Phosphorus, g·kg^{-1}	2.69 ± 0.35 ^{b}	4.77 ± 1.14 ^{a}	0.005
Sodium, g·kg^{-1}	0.96 ± 0.04	0.91 ± 0.09	0.276
Potassium, g·kg^{-1}	0.61 ± 0.06	0.55 ± 0.10	0.306
Copper, mg·kg^{-1}	36.68 ± 4.22 ^{b}	46.69 ± 1.90 ^{a}	0.001
Zinc, mg·kg^{-1}	21.14 ± 6.59 ^{b}	31.96 ± 8.09 ^{a}	0.049
Manganese, mg·kg^{-1}	26.36 ± 2.09 ^{b}	30.09 ± 1.57 ^{a}	0.013

C: control group; H: layers fed a diet enriched with supplementation of 0.5% HS. ^{a,b} Means not sharing the same superscripts in the same row are significantly different.

3.3. The Relative Expression of Selected Genes

The relative expression for the IgA gene was significantly upregulated in the humic group compared to the control group ($p < 0.05$). Similarly, MUC-2 gene expression was upregulated, but it was not significant in H group, compared to the control. On the contrary, the relative expression for the IGF-2 gene was not influenced by the addition of HS (Figure 1).



(**a**) (**b**) (**c**)

Figure 1. Relative expression of IgA (**a**), IGF-2 (**b**), and MUC-2 (**c**) genes in cecum of laying hens treated with 0.5% HS. Results at each time point are the median of $2^{-\Delta Cq}$. C: control group; H: layers fed a diet enriched with supplementation of 0.5% HS. Columns marked with stars are significantly different from control: * $p < 0.05$.

3.4. Cellular Immune Response

In testing the effect of HS on cellular immunity, the phagocyte activity, which represents the innate component of the immune response and the representation of the selected lymphocyte population in the blood as a parameter of the acquired immune response, was monitored.

The addition of HS to the feed of laying hens significantly increased the percentage of active phagocytes as well as the engulfing capacity (Figure 2).

The results from the phenotyping of the blood lymphocytes showed that HS stimulate the differentiation of the B lymphocytes when a significantly higher proportion of IgM+ lymphocytes was recorded (Figure 3a). This finding is also supported by the increased expression of IgA genes in the gut. In contrast, the proportion of total T cells (CD3+) was thus reduced (Figure 3b). There was no statistically significant effect of HS on the T cell subpopulations; either CD4+ (Figure 3c) or CD4-CD8+ (Figure 3d) was observed.

Figure 2. Influence of 0.5% HS on phagocyte activity in layer blood evaluated as (**a**) percentage of active phagocytes (phagocytic activity) and (**b**) engulfing capacity of the phagocytes (expressed as mean fluorescence intensity—MFI). C: control group; H: layers fed a diet enriched with supplementation of 0.5% HS. Columns marked with stars are significantly different from control: * $p < 0.05$; *** $p < 0.001$.

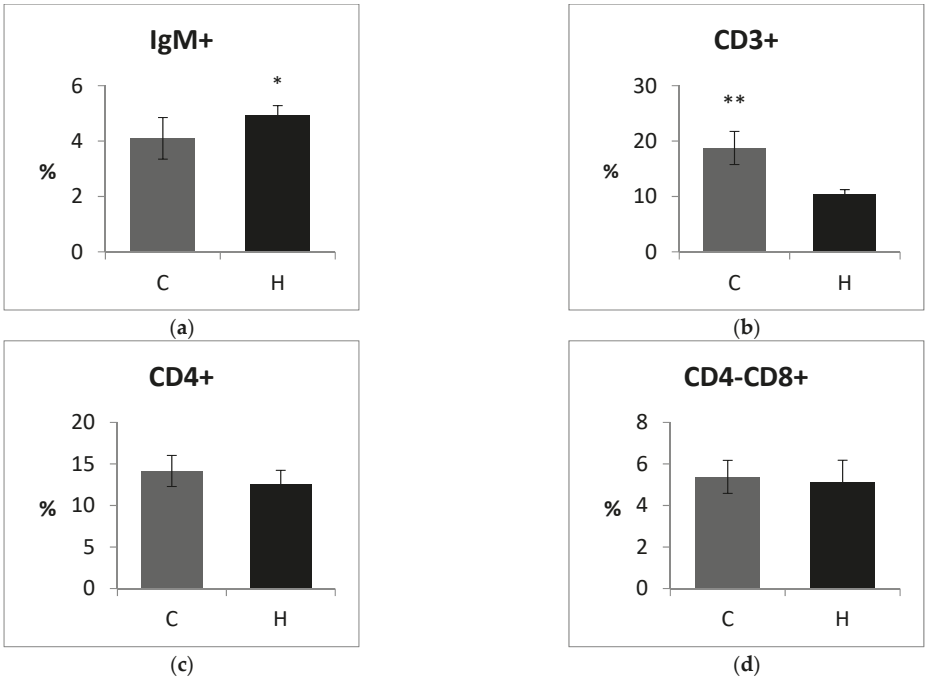
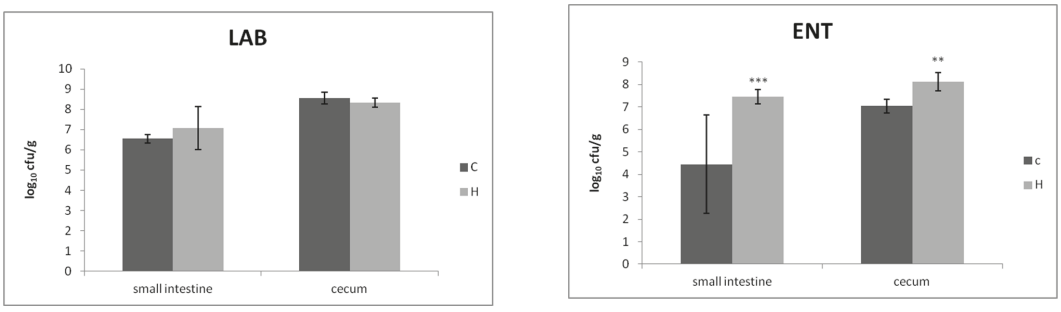


Figure 3. Percentage of (**a**) IgM+, (**b**) CD3+, (**c**) CD4+, and (**d**) CD4-CD8+ lymphocytes in the blood of laying hens receiving 0.5% HS. C: control group; H: layers fed a diet enriched with supplementation of 0.5% HS. Columns marked with stars are significantly different from control: * $p < 0.05$; ** $p < 0.01$.

3.5. Intestinal Microbiota

The effect of HS on the intestinal microbiota was evaluated on the basis of the presence of lactic acid bacteria and enterobacteria in the contents of the small intestine and the cecum. The results showed that the application of HS did not affect the numbers of LAB in any of the monitored sections of the intestine (Figure 4a) but did significantly increase the numbers of enterobacteria, both in the small intestine and in the cecum (Figure 4b).



(**a**) (**b**)

Figure 4. The influence of 0.5% HS on the counts of (**a**) lactic acid bacteria (LAB) and (**b**) enterobacteria (ENT) in the contents of the small intestine and the caecum. C: control group; H: layers fed a diet enriched with supplementation of 0.5% HS. Columns marked with stars are significantly different from control: ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

HS are a heterogeneous group of natural organic molecules that are part of soil and water organic matter. These are substances with a whole range of positive health effects. In our work, we focused on the effect of the administration of HS on cellular immune response, the protection of intestine in poultry as well as on specific productive parameters.

Dietary supplementation with HS additives had a significant effect on the laying rate, daily egg mass, egg weight, and feed conversion, which indicate the ability of HS to stabilize the intestinal microbiota, thus ensuring improved nutrient utilization in feed [38,39]. These findings are consistent with our results. Similarly, the beneficial effect of feeding humates in a 0.25% concentration during broiler fattening was observed in Kocabagli et al. [40]. In this study, the supplementation of humates at the time of fattening (day 22–42) significantly improved feed conversion as well as increased the body weight of the broilers compared to the control group and broilers supplemented with HS over the time period of 0–21 days. However, some studies suggest conflicting results. For example, a study in 2006 by Rath et al. [41] reported that HS in 0.5 and 1% concentrations have a negative effect on the broiler growth performance.

The importance of minerals on eggshell quality is unquestionable. The eggshell consists of 94.85% of minerals, of which calcium carbonate, magnesium carbonate, and calcium phosphate have the highest proportions. Therefore, phosphorus and calcium are the most important elements for the formation of the outer shell of an egg. In addition, eggshell contains 4.15% organic matter and water in a proportion of less than 2% [42]. According to the achieved results, the increase in the mineral content in the eggshell was favorably influenced by the supplementationHS in the diet of laying hens. Moreover, the administration of 0.5% HS product (Humac Natur AFM) contains up to 42,278 mg·kg^{-1} of calcium and 5111 mg·kg^{-1} of magnesium [20]. The increase in the mineral content may also be related to the relationships among the elements or the HS components. The absorption of calcium, manganese, iron, and phosphorus decreases with higher levels of HS in the diet [41]. Reasons for this could be the chelating effects of HS that are influenced by their large number of carboxylic acid side chains [43]. Moreover, HS have the ability to bind to elements from the environment and can also release these elements after changes in external conditions. This property, in the case of action in the gastrointestinal tract, affects the eggshell quality [44]. Interestingly, the results obtained by Ergin et al. [45] suggested that addition of humic acids (30 mg·kg^{-1} diet) enhanced eggshell strength without affecting feed efficiency and egg production. Likewise, Tancho [46] concluded that eggshell thickness was increased in hens fed with humates at levels of 1 and 2 g·kg^{-1}.

In order to achieve maximum production, good health is essential, which is conditioned by the state of the immune system. Generally, the largest immune organ is the

gut-associated lymphoid tissue (GALT), and the first line of defense is non-specific barriers, including mucin and other components. The mucus layer also acts as a medium for molecule transport between luminal content and enterocytes. We observed a non-significant increase in relative mRNA expression for MUC-2 but a significant increase in IgA gene expression, which is consistent with the finding that humic acids may aid in the formation of a protective film on the mucus epithelium of the gastrointestinal tract, which protects against infectious agents and toxins, thereby also improving animal feed utilization [23]. In addition, the main function of IgA is to neutralize antigens on the mucosal surfaces. On the other hand, our results showed that HS in a 0.5% concentration did not affect IGF-2 gene expression. This fact is interesting from the point of view that IGF-2 plays a very important role in the postnatal development of the organism and mediates most of growth effects; thus, it has significant effects on the skeletal and muscular systems. In our previous study, we observed the same effect of 0.8% HS on the relative expression of IGF-2 in broilers [20]. On the contrary, Weber et al. [47] reported an increase of serum IGF-1 in pigs treated with Menefee humate (0.25% of humic acid). In tune with previous studies and our knowledge, we suppose that the influence of HS on the expression of growth factors is dependent on the concentration of the HS that is administered.

As in our previous study, where we administered 0.8% HS to broilers, even now, after the application of 0.5% HS to laying hens, we observed a significant increase in both the percentage of active phagocytes and their engulfing capacity [20]. Sanmiguel and Rondón [48] suggested that this effect on phagocytes is time dependent. They administered 0.1% and 0.2% HS to laying hens and found that the phagocytic index was elevated after 8 and 30 days of application, but subsequently (on 60th day), phagocytic index significantly decreased and was lower than in the control group. Although the mode of action of HS on phagocytosis has not been fully explained, studies with human neutrophils have shown that HS stimulate their adhesion abilities and superoxide production, and they are able to mediate intracellular signal transduction leading to NF-κB induction, which is crucial for the transcription of many proinflammatory genes (e.g., IL-2, IL-8, MCP-1, TNF-α, GM-CSF) [49]. Similarly, Riede et al. [50] confirmed the stimulatory effect of HS on the oxidative burst of human neutrophils but without activating chemotaxis. The authors hypothesize that HS contain chemical structures that can be recognized by the neutrophils and can activate them.

Because works on the effect of HS on individual lymphocyte subpopulations are rare, we examined the proportions of IgM+ lymphocytes, T lymphocytes, and T helper and T cytotoxic cells in our study. According to a study by Luthala [51], in chickens, the CD8αα homodimer may be expressed on peripheral CD4+ cells, but expression of the CD8αβ heterodimer only occurs on cytotoxic T cells. In this type of expression, chicken and mammalian lymphocytes differ significantly. Functionally, a subpopulation of CD4+CD8+ T cells in chickens is reported as T helper and/or regulatory lymphocytes. These findings were confirmed by both the expression of the CD25 molecule (IL-2 receptor—typical for regulatory cells), which was detected on part of the cells from the CD4+CD8- as well as the CD4+CD8+ subpopulations and by their cytokine profiles [52]. For these reasons, even in our study, CD4+CD8+ lymphocytes with low and medium expression of the CD8 molecule were included in the T helper cell subpopulation.

We found that the addition of 0.5% HS to the feed of laying hens increased the proportion of IgM+ lymphocytes and thus decreased the proportion of T lymphocytes, while the proportion of T helper and T cytotoxic cells was not affected. The increased percentage of the IgM+ lymphocyte subpopulation is consistent with the finding of increased gene expression for IgA in the gut. Zhang et al. [38] (2020) also noted significantly elevated serum IgM as well as IgG levels after the administration of 0.1 and 0.5% HS to laying hens, which confirms the activation of B cells. Similarly, Salah et al. [53] reported an increase in the serum gamma globulin fraction containing most of immunoglobulins after 5 days of humic acid application to broiler chickens. Interestingly, when we administered 0.8% HS to broiler chickens from day 11 of life to the end of fattening (day 38) in the previous

experiment, we observed a significantly higher percentage of T cells as well as T helper cells (CD4+), but the gene expression for IgA was not affected [20]. Based on the above results, we assume that the obtained cellular immune response depends not only on the used concentration and the duration of HS application but also depends on the category of poultry to which they are applied.

The exact mechanism of action by which HS affect specific subpopulations of immune cells has not yet been elucidated, but their effect on cytokine production has been confirmed. Vetvicka et al. [54] reported a significant increase in the production of IL-5 and IL-6 in mice intraperitoneally treated with HS. These cytokines stimulate B lymphocyte differentiation and growth and increase the secretion of immunoglobulins. The theory that explains the immunomodulatory potential of HS is that humates are able to form complexes with carbohydrates, amino acids, and peptides. Currently such complexes are considered to be biologically active and can subsequently bind to the surface of T lymphocytes and NK cells, thereby regulating their functions, including cytokine production. Subsequently, cytokines influence further immune reactions [50,55].

Regarding the effect of HS on the intestinal microbiota, the results of different authors vary considerably. While some have reported a decrease in enterobacteria [20,39], others have observed an increase [56]. Similarly, different results were observed for other bacterial species. In our case, there was an increase in the numbers of *E. coli* in the intestine and in the cecum, but the number of lactic acid bacteria was not affected. Such a trend can be considered as negative. These results are consistent with in vitro microbiological tests that have shown that various species of *Lactobacillus* spp. and different strains of *E. coli* also respond differently to HS (unpublished data). Similar results were obtained by Buzoleva and Sidorenko [57], who found that the multiplication of enterobacteria in the presence of HS depends on both the type of bacterium and the HS composition.

5. Conclusions

We can conclude that the addition of 0.5% HS to laying hen feed had a positive effect on laying rate, daily egg mass, egg weight, feed conversion, and eggshell quality and also had an immunostimulatory effect manifested by the increasing activity of the phagocytes and the B cell immune response. On the other hand, there was an observed increase in the number of enterobacteria in the intestinal contents, and the proportion of T lymphocytes in the blood was significantly reduced. The presented results confirm that HS can be used for the improvement of egg production and quality and for activation of phagocytosis and specific antibody immunity, but their influence on the intestinal microbiota will need to be further studied with respect to a wider range of microbial species inhabiting the digestive tract of laying hens.

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Article

Feeding Laying Hens a Diet Containing High-Oleic Peanuts or Oleic Acid Enriches Yolk Color and Beta-Carotene While Reducing the Saturated Fatty Acid Content in Eggs

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Abstract: We investigated the dietary effects of high-oleic peanuts (HOPN) or oleic fatty acids (OA) on older production hen performance, egg mass and quality, and lipid composition. A total of 99 laying hens were divided between three treatments and fed ad libitum for 8 weeks: (1) Conventional diet; (2) HOPN diet; (3) OA diet. Body weight (BW) was measured at weeks 1 and 8, and feed, egg weights (EW), and egg quality parameters were collected. Data was analyzed by analysis of variance at $p < 0.05$ significance level. There were no treatment differences in 8 week BW, feed conversion ratio, or average weekly egg quality parameters. The 8 week average EW of eggs from the HOPN group had reduced EW relative to the other treatment groups ($p = 0.0004$). The 8-week average yolk color score ($p < 0.0001$) was greater in eggs from the HOPN group relative to the other treatments. Overall, the β-carotene ($p < 0.006$) and OA content ($p < 0.0001$) was greater in eggs from the HOPN group, with reduced saturated fats in eggs from the HOPN group relative to the other treatments. These results suggest that HOPN and/or OA may be a useful layer feed ingredient to enrich eggs, while significantly reducing egg size in older production hens.

Keywords: alternative feed ingredients; high-oleic peanuts; laying hens; shell eggs

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1. Introduction

For decades, soybean meal has been utilized globally in poultry diets, with maize providing the primary source of dietary energy [1]. Yet these feed ingredients are often inaccessible to developing countries for poultry production due to high cost and availability of soybean and corn, with the United States, Brazil, and Argentina being the largest producers and exporters of soybeans [1]. Hence, other protein sources like canola meal, peanut meal, fishmeal, and blood meal are also utilized.

In India, Ghana, and Nigeria, peanut meal is commonly utilized as a protein-rich poultry feed ingredient [2–6], yet in the US, 85% of peanut production is for peanut butter and snacks, with the remaining 15% crushed for oil [7]. Early poultry feeding trials established that peanut meal prepared from conventional normal-oleic peanuts (22–30% protein and 44–56% total fat, with a total fatty acid profile of 52% oleic acid and 27% linoleic acid) is a reasonable poultry feed ingredient [8,9]. Additionally, limited studies have investigated the use of high-oleic peanuts (22–30% protein and 44–56% total fat) with an 80% oleic fatty acid and 2% linoleic fatty acid profile as an alternative poultry ration. Toomer et al. [10] reported that eggs produced from Leghorns in peak egg production (40 weeks of lay) fed high-oleic peanuts had increased β-carotene and oleic fatty acid content, with increased yolk color compared to conventional eggs, with no significant differences in hen performance (with the exception of egg mass) or egg quality between

treatment groups. However, egg weights produced from hens in peak egg production (40 weeks of lay) fed a high-oleic peanut diet were significantly smaller in mass relative to conventional eggs at all time points measured [10]. Hence, the primary aim of this project was to determine the effect of feeding a high-oleic peanut or an oleic acid diet on the size/mass of the eggs produced by older production hens (57 weeks of lay). The establishment of a feeding regimen to reduce the production of oversized eggs commonly seen with increasing production age is of great commercial interest [11]. Based on our previous findings [10], we conjecture that eggs produced from hens fed a high-oleic peanut or oleic acid diet will have enhanced β-carotene and unsaturated fatty acid content, with smaller egg size (mass), as compared to eggs produced from hens fed a conventional diet. Moreover, we aimed to investigate the effects of a high-oleic peanut diet on the sensory attributes of the eggs produced.

2. Materials and Methods

All animal research procedures used in these feeding trials were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #17-001-A).

2.1. Experimental Design, Animal Husbandry, Dietary Treatments, and Hen Performance

All experimental diets were formulated in Concept 4 (level 2, version 10.0) software to be isonitrogenous (18% protein) and isocaloric (3080 kcal/kg metabolizable energy), with an estimated particle size between 800 and 1000 µm (Table 1). The control diet (Treatment 1) was prepared as a conventional layer diet with defatted soybean meal and corn, while Treatment 2 was prepared using aflatoxin-free whole non-roasted unblanched high-oleic peanuts. Peanuts were crushed using a Roller Mill to form crumbles, prior to inclusion in Treatment 2. Treatment 3 was prepared by supplementing the control diet with 2.64% (% by weight) of food-grade oleic fatty acid oil (Millipore Sigma, Burlington, MA, USA). Each of the experimental diets were supplemented with vitamin, mineral, and selenium premixes manufactured at the NC State University Feed Mill (Raleigh, NC, USA) to meet and/or exceed poultry requirements for vitamins, minerals, and selenium. All experimental diets were analyzed by the North Carolina Department of Agriculture and Consumer Services and the Food and Drug Protection Division Laboratory (Raleigh, NC, USA) for aflatoxin and microbiological contaminants.

Brown Leghorn hens were selected for use in this study from the University Flock, NC State University (Raleigh, NC, USA). In total, 99 Brown Leghorn hens (57 week of lay) were assigned to three dietary treatment groups for 8 weeks: (1) Conventional diet; (2) HOPN diet; (3) OA diet. There were three replicates per treatment, with hens individually housed in battery cages (each cage measured 12 inches wide × 18 inches deep × 18 inches height) in one room at the Chicken Education Unit, NC State University (Raleigh, NC, USA). Hens were provided feed and water ad libitum and 14 L:D for 8 weeks. Body weights were recorded for each individual hen at week 1 and week 8, with feed weights recorded weekly. Shell eggs were collected, enumerated, and weighed daily. Total number of eggs produced per replicate and per treatment was calculated for each experimental week and for the total 8 week feeding trial. The average feed conversion ratio (FCR) was calculated as total feed consumed over the 8-week feeding (kg)/dozens of eggs produced for each treatment group over the 8-week feeding trial.

2.2. Egg Quality and Grading

Bi-weekly (0-week, 2-week, 4-week, 6-week, and 8-week), 36 eggs were randomly selected with 12 shell eggs per treatment (4 eggs randomly selected from each replicate) for quality assessment and USDA grading. Fresh shell eggs were collected on the day of quality assessment and USDA grading. Shell eggs were analyzed for DSM yolk color score, vitelline membrane strength, Haugh unit, and shell strength by the Laying Hen and Small Flock Management Lab, Prestage Department Poultry Science, NC State University.

Haugh unit values were determined using methods described by Haugh [12] and were recorded with the Technical Services and Supplies (TSS) QCD system (Dunnington, York, United Kingdom). The QCD system was calibrated to the DSM Color Fan, consisting of a series of 15 colored plastic tabs with a range of yolk colors from light yellow to orange red (color index 1 to 15), defined by Vuillemier [13]. In general, a texture analyzer (TA.XTplus) was used to measure the shell strength and vitelline membrane strength by the breaking strength using a 5-kg load cell per the manufacturer's instructions (Stable Micro Systems, Surrey, United Kingdom), with measurements in grams of force. Vitelline membrane strength was determined using methods described by Jones et al. (2005), with a 2 mm/second test speed and 0.0001 kg trigger force [14]. Modified methods of Jones et al. (2002) were used to measure shell strength with a 2 mm/second test speed and a 0.001 kg trigger force [15].

Table 1. Composition of formulated experimental laying hen diets [1].

Ingredients	Treatments [2]		
	Control	HOPN	OA
		% (by weight)	
Yellow Corn	46.4	39.0	52.3
Corn Gluten Meal	5.0	10.4	5.0
High-Oleic Peanut [3]	0.0	20.0	0.0
Soybean Oil	7.8	0.0	0.0
Soybean Meal	21.4	0.0	20.4
Wheat Bran	6.0	16.8	6.0
Oleic Acid Oil	0.0	0.0	2.6
Calcium Carbonate	10.9	10.8	11.3
Dicalcium Phosphorus	1.6	1.4	1.5
Sodium Chloride	0.3	0.3	0.3
L-Lysine	0.0	0.5	0.0
DL-Methionine	0.1	0.1	0.1
L-Tryptophan	0.0	0.03	0.0
L-Threonine	0.0	0.13	0.0
Choline Chloride	0.2	0.2	0.2
[4] MYC-Out™	0.1	0.1	0.1
Mineral Premix [5]	0.2	0.2	0.2
Vitamin Premix [6]	0.1	0.1	0.1
Selenium Mix [7]	0.1	0.1	0.1
Metabolizable Energy (kcal/kg)	3080	3080	3080

[1] Three isocaloric, isonitrogenous (18% protein) formulated diets were fed to Brown Leghorn (57 week of lay) hens for 8 weeks. [2] Treatments: control = conventional soybean meal and corn mash diet, HOPN = unblanched high-oleic peanut crumbles (20%) and corn mash diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil. [3] High-Oleic Peanuts = unblanched raw whole high-oleic peanut crumbles. [4] MYC-Out™ = mycotoxin binder and feed antioxidant manufactured Adisseo (Alpharetta, GA, USA). [5] Mineral premix, manufactured by NCSU FeedMill, supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt. [6] Vitamin premix, manufactured by NCSU FeedMill, supplied the following per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid. [7] Selenium premix, manufactured by NCSU FeedMill, = 1 mg Selenium premix provided 0.2 mg Se (as Na_2SeO_3) per kg of diet.

2.3. β-Carotene, Lipid Content, and Fatty Acid Analysis

All experimental diets were analyzed for lipid content, fatty acid, and β-carotene content in triplicate by an external vendor ATC Scientific (Little Rock, AR, USA), using AOAC approved methods. Gross energy analysis of feed samples was performed by ATC Scientific using an adiabatic oxygen bomb calorimeter with standard methods. Bi-weekly, a total of 45 eggs were randomly selected, with 15 eggs per treatment (5 eggs per replicate) for lipid content, β-carotene, and fatty acid analysis by ATC Scientific using AOAC approved methods. Each egg sample was mixed for homogeneity in a whirl-pak® (Millipore Sigma, St. Louis, MO, USA) bag for 3 min using a Smasher™ Lab Blender (Weber Scientific, Hamilton, NJ, USA). Subsequently, all egg samples were frozen at −20 °C and

stored frozen until chemical analysis within 2 weeks of collection. Frozen homogenous egg samples were shipped on dry ice overnight to vendor for analysis within 2 weeks of collection. Lipid (total cholesterol, crude fat) and fatty acid analysis of homogenous egg samples and feed samples were analyzed using direct methylation methods, as described by Toomer et al. [10]. Total cholesterol was measured as mg cholesterol/100 g sample weight (feed or egg), while crude fat was measured as a percentage of gram crude fat/gram sample weight (feed or egg). Fatty acid content was measured as a percentage of gram of fatty acid/gram total lipid content of a sample (feed or egg). Methods used to determine β-carotene content in eggs are detailed in the AOAC 958.05 [16] color of egg yolk method. Egg fat hydrolysis methods were determined using the AOAC method 954.02 [17].

2.4. Cooking Methods and Consumer Acceptance Testing of Scrambled Eggs

The Sensory Service Center, in the Food, Bioprocessing, and Nutrition Sciences Department, NC State University (Raleigh, NC, USA), performed all sensory testing and data analysis of egg samples. The sensory protocol was reviewed and deemed exempt by the NC State University Institutional Review Board for human subjects. Scrambled egg samples were prepared following safe food handling practices. During preparation and service, all team members wore gloves, hairnets/hats, and lab coats. Whole shell eggs were received and refrigerated at 4 °C upon arrival. On the day of testing, preparation of raw eggs was completed on a separate table from the cooking/serving areas of scrambled eggs to prevent cross contamination of any microbial hazards, and gloves were changed and hands were frequently washed during any transition from raw to cooked product.

On the day of the sensory evaluation, approximately 150 shell eggs per treatment were cooked. In total, 10 sets of 15 eggs/treatment were cracked into a bowl and beat together until homogenous. A large non-stick pan was heated over medium heat for approximately 1 min. The homogenous egg mixture was added to the heated pan and stirred slowly with a wooden spatula, bringing in the mixture from the edges of the pan for 3.5 min and subsequently removing the eggs from the pan. Scrambled egg samples from each of the three treatments were placed in labeled aluminum pans, covered with aluminum foil, and held in a heated holding cabinet at 180 °F to maintain quality. Scrambled egg portions were dispensed into lidded soufflé cups with three-digit codes to identify treatment for consumer testing.

Self-reported scrambled egg consumers (n = 109) were recruited from the NC State University staff and student population. Consumer panelists confirmed no egg food allergies and/or sensitivities prior to participation. Consumer panelists were disqualified if they were younger than 17 years of age, older than 65 years of age, or if they only consumed scrambled eggs once a month. Upon completion of the test, consumer panelists were compensated with a $5 gift card to a local store. Compusense20 Cloud (Guelph, ON, Canada) was used for data collection and analysis. Samples were presented monadically with a 2-min enforced rest period between egg samples. Consumers evaluated various aroma, flavor, and texture liking attributes using a 9-point hedonic scale, where 1 = dislike extremely and 9 = like extremely. Consumers used a 5-point anchored Just About Right (JAR) scale to evaluate flavor and color attributes. Consumers were provided with spring water and unsalted crackers for palate cleansing.

2.5. Statistical Analysis-Laying Hen Performance and Egg Lipid and Fatty Acid Content

Each hen served as the experimental unit for all performance data. All performance data was evaluated for significance by one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$ using SAS statistical software (version 9.4). If ANOVA results were significant ($p < 0.05$), Tukey's multiple comparisons t-test was conducted to compare the mean of each treatment group with the mean of every other treatment at $p < 0.05$ significance level. Comparisons were made between body weights (33 birds/treatment), feed intake (33 birds/treatment), feed conversion ratio (33 birds/treatment), and egg weights (total # of eggs collected over the 8-week feeding trial).

In total, 12 eggs per treatment (4 eggs per replicate randomly selected) were statistically analyzed by one-way ANOVA ($p < 0.05$) using SAS. Means were separated by least squares means, with Tukey-Kramer adjustment for multiple comparisons ($p < 0.05$) for treatment differences in egg quality parameters (Haugh unit, vitelline membrane strength, shell strength, yolk color score) at each bi-weekly experimental time-point (0-week, 2-week, 4-week, 6-week, 8-week). Additionally, eggs were statistically analyzed for treatment differences in egg quality parameters in all eggs collected over the 8-week feeding trial (180 total, 60 eggs per treatment). In total, 15 homogenous egg samples (5 per replicate) were statistically analyzed by one-way ANOVA ($p < 0.05$) using SAS. Means were separated by least squares means with the Tukey-Kramer adjustment for multiple comparisons ($p < 0.05$) for treatment differences in egg β-carotene content and egg lipid and fatty acid content (45 total egg samples at each time-point) weekly (2-, 3-, 4-, 5-, 6-, 7-, and 8-week).

2.6. Statistics-Sensory Evaluation

Statistical analysis was conducted using XLSTAT software (version 2016; Addinsoft, New York, NY, USA). Descriptive analysis results and consumer liking scores were analyzed by ANOVA, with Fisher's least significant difference test at a significance level of $p < 0.05$. Principal component analysis was applied to descriptive analysis to determine how products were differentiated relative to one another. Consumer Just About Right (JAR) scores were evaluated by chi-squared analysis, and purchase intent was evaluated using a Kruskal-Wallis test with Dunn's post hoc test, using methods previously described by Jo et al. [18].

3. Results and Discussion

3.1. Feed Analysis

While all experimental diets were formulated to be isocaloric (3080 kcal/kg metabolizable energy) and isonitrogenous (18% crude protein), chemical analysis was conducted to determine the fatty acid profile of the experimental diets. There were no differences in the levels of crude total fat or gross energy between experimental feed samples (Table 2). In contrast, the control diet had significantly greater amounts of stearic acid and palmitic acid ($p < 0.01$) levels in comparison to the other treatment groups (Table 2). Moreover, the control diet had the greatest percentage of total cholesterol in comparison to the other dietary treatment groups. The HOPN diet contained the highest percentage of oleic fatty acid content of all the diets, followed by the OA diet, with the control diet having the lowest oleic fatty acid content ($p < 0.05$; Table 2).

3.2. Hen Performance

There were no mortalities over the 8-week feeding trial, and all laying hens were healthy and exhibited normal behavior. There were no significant treatment differences in body weights at the onset or termination of the 8-week feeding trial (Table 3). Interestingly, while there were no significant treatment differences in average egg weights at 4-weeks or 8 weeks of the study, the 8-week average egg weights produced from hens fed the HOPN dietary treatment were significantly smaller than the controls or OA group ($p = 0.0004$, Table 3). At 2 weeks and 6 weeks, the egg weights from hens fed the HOPN diets produced eggs less than egg weights from the control group, while egg weights were similar between HOPN and OA treatment groups (Table 3). This parallels results by Toomer et al. [10] and Van Elswyk et al. [19], demonstrating a reduction in egg mass when laying hens are fed diets rich in unsaturated fatty acids relative to conventionally produced eggs.

Table 2. Lipid content and fatty acid analysis of dietary treatments [1].

	Treatments			
Item Measured	**Control**	**HOPN**	**OA**	***p*-Value**
Crude Fat [2] (%)	10.0 ± 0.2	12.7 ± 0.2	6.2 ± 0.2	0.07
Palmitic Acid [2] (%)	22.6 ± 0.005 [a]	7.5 ± 0.005 [c]	14.7 ± 0.005 [b]	0.001
Stearic Acid [2] (%)	5.4 ± 0.002 [a]	1.9 ± 0.002 [c]	3.8 ± 0.002 [b]	0.001
Oleic Acid [2] %	38.4 ± 0.01 [c]	74.2 ± 0.01 [a]	43.8 ± 0.01 [b]	0.02
Trans-Elaidic [2] (%)	2.8 ± 0.02 [b]	0.9 ± 0.02 [c]	5.8 ± 0.02 [a]	0.01
Linoleic Acid [2] (%)	21.7 ± 0.002 [a]	8.8 ± 0.002 [b]	20.7 ± 0.002 [a]	0.03
Linolenic Acid [2] (%)	1.0 ± 0.002	0.4 ± 0.002	1.7 ± 0.002	0.07
Omega 3 [2] (%)	1.0 ± 0.001	0.4 ± 0.001	1.7 ± 0.001	0.07
Omega 6 [2] (%)	21.7 ± 0.001 [a]	8.8 ± 0.001 [b]	1.3 ± 0.001 [c]	0.03
β-Carotene (ppm)	<5.0	<5.0	<5.0	0.07
Total Cholesterol (mg/100 g)	50.4 ± 0.01 [a]	8.4 ± 0.01 [b]	3.6 ± 0.01 [c]	0.0
Gross Energy (kcal/kg)	4129 ± 0.06	4205 ± 0.06	3802 ± 0.06	0.07

[1] Treatments: control = conventional soybean meal and corn diet, HOPN = unblanched high-oleic peanut crumbles (20%) and corn diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil. Lipid (crude fat, total cholesterol), fatty acid, and beta-carotene analysis was performed by an AOAC-certified lab, ATC Scientific (Little Rock, AR, USA) food-grade oleic fatty acid oil. [2] Crude Fat content = g crude fat/g total sample weight ∗ 100, Fatty acid content = g of fatty acid/g total lipid content ∗ 100. Total cholesterol (mg/100 g dry weight) and lipid analysis was determined by direct methylation methods. Gross energy analysis was determined using an adiabatic oxygen bomb calorimeter and standard methods. Each value represents the mean ± the standard error for each triplicate sample. [a,b,c] Means within the same row with differing superscripts are significantly different ($p < 0.05$).

Table 3. Performance of laying hens fed a diet of high-oleic peanuts or oleic acid.

Variable	**Treatments** [1]			***p*-Value**
	Control	**HOPN**	**OA**	
Body Weight (g)				
Week 1	2059 ± 61.9	2009 ± 61.9	1989 ± 61.9	0.50
Week 8	2257 ± 53.5	2128 ± 53.5	2185 ± 53.5	0.06
Egg Weights (g) [2]				
Week 2	67.3 ± 1.4 [a]	62.4 ± 1.4 [b]	64.7 ± 1.4 [ab]	0.006
Week 4	66.6 ± 1.7	65.6 ± 1.7	66.3 ± 1.7	0.83
Week 6	66.6 ± 1.4 [a]	62.0 ± 1.4 [b]	65.3 ± 1.4 [ab]	0.005
Week 8	68.0 ± 1.7	65.7 ± 1.7	64.9 ± 1.7	0.18
8week Ave Egg Weight (g)	67.1 ± 0.6 [a]	63.9 ± 0.6 [c]	65.3 ± 0.6 [b]	0.0004
8 week FCR [3]	2.4±0.09	2.2±0.09	2.1±0.09	0.07

In total, 99 brown Leghorn (57 week of lay) hens were fed one of three isonitrogenous (18% crude protein) diets ad libitum for 8 weeks. Body weights were collected at week 1 and week 8 of the study (33 hens per treatment, 3 replicates, with 11 birds per replicate). [1] Treatments: control = conventional soybean meal and corn mash diet, HOPN = 20% unblanched high-oleic peanut + corn diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil. [2] Weights (g) of eggs were determined daily and weekly for each treatment group. Data represents the bi-weekly (2, 4, 6, and 8 weeks) averages ± standard error for each time point for each treatment group. [3] Feed conversion ratio (FCR) = kg and total feed intake over the 8-week/total dozen eggs produced over 8 weeks for each treatment group. Total number of eggs produced: Control = 1598 eggs, HOPN = 1617 eggs (≈49 eggs/hen), and OA = 1665over the 8-week feeding tria; Each value (body weights and egg weights) represents the mean ± the standard error. [a,b,c] Means within the same row lacking a common superscript differ significantly ($p < 0.05$).

Egg size has been shown to be greatly influenced by body weight and age [20], nutrition [21], and pullet management [22]. Egg size has been shown to be directly influenced by body weight; for every 45 g of body weight increase, there is a 0.5-g increase in egg size from 18 weeks of age in laying hens [22]. Hence, as the laying hen ages in the production cycle and increases in body weight, there is a proportionate increase in egg size [22]. The United States Department of Agriculture egg classification system [23–25] categorizes eggs by minimum weight per dozen eggs: jumbo 30 ounces (>63 g content per egg), extra-large 27 ounces (63–56 g content per egg), large 24 ounces (56–50 g content per egg), medium 21 ounces (50–44 g content per egg), small 18 ounces (44–38 g content per egg), peewee 15 ounces (<38 g content per egg) [25]. While there were no treatment differences in the total number of eggs produced, with 1598 eggs (≈48 eggs/hen) produced from the

control group, 1617 eggs (≈49 eggs/hen) produced from the HOPN group, and 1665 eggs (≈50 eggs/hen) produced from the OA group over the 8-week feeding trial, the control fed hens produced 1246 total jumbo eggs and 352 x-large size eggs, and the OA fed hens produced 1299 jumbo eggs and 366 x-large eggs, respectively. Conversely, hens fed the HOPN diet produced a total of 711 jumbo eggs and 906 x-large eggs over the 8-week feeding trial.

Therefore, in this study, jumbo eggs (≈63 g content per egg) were the predominate egg classification for most eggs produced from older production hens (57 week of lay) in all treatment groups. In contrast to our previous high-oleic peanut layer feeding trial with younger age production hens (40 weeks of lay), extra-large eggs were the predominate egg classification for most eggs produced [10], which parallels other studies demonstrating a trend of increased egg size with hen production age [26]. Nonetheless, in this study, eggs produced from hens fed the HOPN diet had significantly smaller egg size/mass relative to the other treatment groups ($p < 0.001$) at 57 weeks of lay, suggesting that a diet rich in unsaturated fats may be an effective commercial feeding regimen to manage the production of oversized eggs in older production hens.

While the feed conversion ratio (calculated as the total kg of feed intake over 8 weeks/total # dozen eggs produced over 8 weeks) of the control group (2.4 kg/dozen eggs) was higher than the other treatment groups (2.2 = HOPN, 2.1 = OA), there was no significant treatment differences between the feed conversion ratio (FCR) between the treatment groups (Table 3). FCR is a very important production parameter within the commercial egg industry, as a predictor of profitability utilizing the cost of kg of feed consumed [27] per total number of eggs produced. Thus, FCR is a measure of how efficiently an animal utilizes incoming dietary feed nutrients to generate the desired product of meat and/or eggs [27]. Typically, within the US commercial egg production industry, the typical FCR for each hen is about 2 kg of feed consumed/kg of egg produced, with each hen producing about 330 eggs per year [28].

3.3. Egg Grading, Quality, and Production

All eggs produced in this feeding trial were graded as USDA Grade AA of superior quality, with thick, firm egg whites and defect-free egg yolks. Additionally, the shells were clean and without defects. There were minimal numbers of blood spots or meat spots, with no statistical difference at the 95% confidence interval between eggs produced between the treatment groups (data not shown). The Haugh Unit (HU), first defined by Raymond Haugh [12], is commonly used to measure albumen (egg white) quality from the height and thickness of the albumen. Hence, fresher, higher-quality eggs have thicker egg whites and thus higher HU values. In this study, there were no significant differences in the average weekly HU between the treatment groups (Table 4). The vitelline membrane is a two-layer transparent casing enclosing the yolk separating the yolk from the egg albumen [29]. Vitelline membrane strength is an important physical attribute key to processing shell eggs and the separation of egg yolk from the albumen. Vitelline membrane strength is often used as a measurement of freshness since the vitelline membrane strength is time-dependent and reduced with increased time and storage handling conditions [29]. There were no significant differences in the average weekly shell strength or vitelline membrane strength between the treatment groups (Table 4).

However, the average weekly egg yolk color of eggs produced from hens fed the HOPN diet were significantly higher compared to eggs produced from hens fed the control and OA diets ($p < 0.0001$, Table 4). Moreover, upon visual observation, egg yolks from hens fed the HOPN diets were a visibly darker yellow/orange color intensity in comparison to the egg yolks produced from hens fed the control and OA diets (Figure 1).

Table 4. Egg quality of eggs produced from laying hens fed a diet of high-oleic peanuts or oleic acid.

Item	Treatments [1]			*p*-Value
	Control	HOPN	OA	
Shell Strength (g force)				
8 week ave	4833.6 ± 101.8	5079.6 ± 100.5	4945.5 ± 100.5	0.23
Vitelline Membrane Strength (g force)				
8 week ave	0.23 ± 0.004	0.24 ± 0.004	0.24 ± 0.004	0.36
Haugh Unit (HU)				
8 week ave	92.7 ± 0.71	93.6 ± 0.70	92.9 ± 0.69	0.60
Yolk Color Roche (1–15)				
8 week ave	4.9 ± 0.10 [c]	6.7 ± 0.10 [a]	5.4 ± 0.10 [b]	<0.0001

In total, 99 brown Leghorn (57 week of lay) hens were fed one of three isonitrogenous (18% crude protein) diets ad libitum for 8 weeks. Egg quality (Haugh unit, yolk color score, vitelline membrane strength, and shell strength) was determined bi-weekly (2, 4, 6, and 8 weeks) using the Technical Services and Supplies (TSS) QCD system, with calibration with the DSM Color Fan for yolk color. [1] Treatments: Control = conventional soybean meal and corn mash diet, HOPN = 20% unblanched high-oleic peanut + corn diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil. Eggs were collected weekly and analyzed for quality. Yolk color = Roche Color Fan color index 1–15 (lightest to darkest color intensity). Each value represents the 8-weekly average ± the standard error with 12 eggs/treatment (3 eggs/replicate), N = 36 total measured bi-weekly for 8 weeks. [a,b,c] Means on the same row lacking a common superscript differ significantly, ($p < 0.05$).

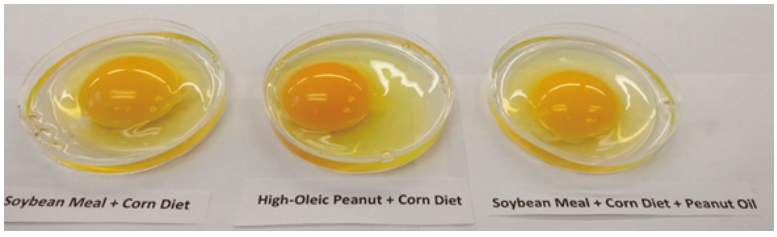

Figure 1. Representative images of yolk color within whole egg samples from each treatment group at week 8 of the feeding trial. Ninety-nine brown Leghorn (57 week of lay) hens were fed one of three isonitrogenous (18% crude protein) diets ad libitum for 8 weeks. At 8-weeks, one whole egg was randomly selected for this photograph as a representative of yolk color observations seen on the day of egg processing with 12 eggs per treatment. This image is not representative of any other egg quality parameters measured. Treatments: Control = conventional soybean meal and corn mash diet, HOPN = 20% unblanched high-oleic peanut + corn diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil.

Similarly, in the yolk color score, the β-carotene content in eggs produced from hens fed the HOPN diet was significantly greater than the β-carotene content in eggs produced from hens fed the control or OA diets at all time points measured (Figure 2; Week 1 $p < 0.01$, Week 2 $p < 0.01$, Week 3 $p < 0.001$, Week 4 $p < 0.01$, Week 5 $p < 0.0001$, Week 6 $p < 0.01$, Week 7 $p < 0.01$, Week 8 $p < 0.01$). β-carotene is a carotenoid, which is a lipid soluble antioxidant found abundantly in plants, is responsible for the rich yellow and deep orange colors in plants, and is a precursor to vitamin A [30]. Conventional commercial eggs are rich in lutein and zeaxanthin [31]. However, yolk lutein and zeaxanthin are highly subjective to oxidation during egg processing, storage, transport, and/or cooking [31].

There were no treatment differences in total cholesterol content between eggs produced from the three treatment groups over the course of the study (Table 5). Similarly, there were no significant treatment differences in total crude fat content in eggs produced from the three treatment groups at weeks 2, 4, 5, 6, 7, or 8 (Table 5). But at week 3 of the 8-week feeding trial, eggs produced from hens fed the OA diet had significantly reduced levels of crude fat relative to the other treatments ($p < 0.01$, Table 5). At week 2 and week 5, there were no significant treatment differences in palmitic acid content between eggs

produced from hens fed the three treatment groups (Table 5). At week 3, 4, 6, 7, and 8, eggs produced from hens fed the HOPN diet had significantly less palmitic acid levels in comparison to eggs produced from the other treatment groups (Table 5). While there were no significant treatment differences in stearic acid levels in eggs produced from the three treatment groups at week 2, eggs produced from hens fed the HOPN and OA diets had significantly lower levels of stearic acid, relative to eggs produced from the control group at experimental weeks 3, 4, 6, 7, and 8 of the study (Table 5). Stearic acid content was similar between eggs produced from hens fed the HOPN and OA diets at weeks 3, 4, 6, 7, and 8 (Table 5). Stearic acid content was lowest in eggs produced from laying hens fed the HOPN treatment group relative to the other treatments only at week 5 of the study ($p < 0.05$).

Monounsaturated OA content was similar between eggs produced from the three treatment groups at week 2 (Table 5). OA content was significantly different between eggs produced from each of the treatment groups, with the highest oleic acid content in eggs produced from hens fed the HOPN diet and lowest in control eggs at week 3 ($p < 0.0001$), week 4 ($p < 0.0001$), week 6 ($p < 0.001$), week 7 ($p < 0.0001$), and week 8 ($p < 0.0001$). At week 5, OA content was significantly greater in eggs produced from hens fed the HOPN diet, while OA levels were similar between eggs produced from hens fed the control and OA diets ($p < 0.01$, Table 5). Total linoleic acid content was significantly greater in eggs produced from control fed hens at all time points (exception of week 4) relative to eggs produced from hens fed the HOPN diet, while levels were similar between eggs produced from hens fed the HOPN and OA diets at week 2, 5, 6, 7, and 8 (Table 6). Regardless, there were no significant treatment differences between the total linoleic content in eggs produced from hens fed the three treatment groups at week 4 only (Table 6).

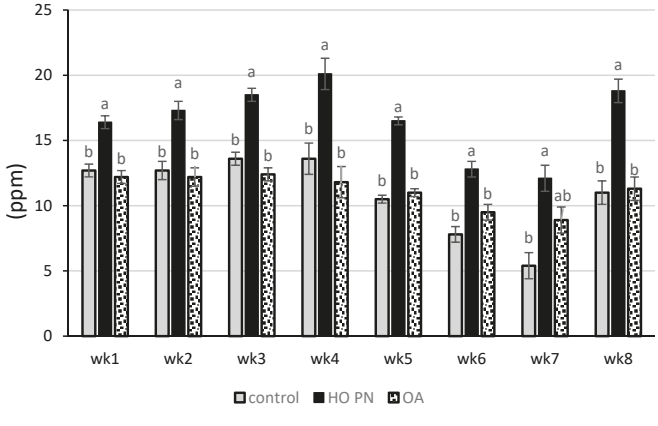

Figure 2. Effect of diet on the β-Carotene content of eggs produced. In total, 99 brown Leghorn (57 weeks of lay) hens were fed one of three isonitrogenous (18% crude protein) diets ad libitum for 8 weeks. Treatments: Control = conventional soybean meal and corn mash diet, HOPN = 20% unblanched high-oleic peanut + corn diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil. Beta-carotene analysis was performed by an AOAC-certified lab, ATC Scientific (Little Rock, AR, USA). β-carotene content in eggs was determined using AOAC 958.05 methods [14]. *p*-values at the various sampling time-points were the following: Week-1 $p = 0.002$, Week-2 $p = 0.004$, Week-3 $p = 0.0003$, Week-4 $p = 0.006$, Week-5 $p < 0.0001$, Week-6 $p = 0.004$, Week-7 $p = 0.008$, Week-8 $p = 0.001$. Each bar column represents the average ± the standard error for each experimental time-point, with 15 egg samples/treatment group (5 egg samples per replicate, 3 replicates) and a total of 45 eggs analyzed at each time-point. [a,b] Bar columns with differing superscript are significantly different ($p < 0.05$).

Table 5. The effect of feeding laying hens a high-oleic peanut (HOPN) diet or an oleic fatty acid (OA) diet on the fatty acid profile of the eggs produced.

Week	Item Measured	Treatments [1]			*p*-Value
		Control	HOPN	OA	
2	Cholesterol (mg/100 g)	310.1 ± 16.3	312.0 ± 16.3	309.6 ± 16.3	0.99
	Crude Fat (%)	9.0 ± 1.0	7.5 ± 1.0	6.0 ± 1.0	0.19
	Palmitic (%)	2.0 ± 0.21	1.4 ± 0.21	1.3 ± 0.21	0.09
	Stearic (%)	0.7 ± 0.07	0.5 ± 0.07	0.4 ± 0.07	0.05
	Oleic (%)	3.9 ± 0.5	4.1 ± 0.5	2.7 ± 0.5	0.15
3	Cholesterol (mg/100 g)	318.7 ± 16.7	327.0 ± 16.7	279.5 ± 16.7	0.18
	Crude Fat (%)	10.0 ± 0.5 ^{a}	10.3 ± 0.5 ^{a}	6.2 ± 0.5 ^{b}	0.003
	Palmitic (%)	21.9 ± 0.2 ^{a}	18.7 ± 0.2 ^{b}	21.5 ± 0.2 ^{a}	0.0002
	Stearic (%)	7.6 ± 0.2 ^{a}	6.6 ± 0.2 ^{b}	6.8 ± 0.2 ^{b}	0.015
	Oleic (%)	42.4 ± 0.1 ^{c}	53.2 ± 0.1 ^{a}	46.0 ± 0.1 ^{b}	<0.0001
4	Cholesterol (mg/100 g)	330.2 ± 19.1	334.7 ± 19.1	294.9 ± 19.1	0.34
	Crude Fat (%)	8.2 ± 0.9	8.1 ± 0.9	8.1 ± 0.9	0.99
	Palmitic (%)	21.5 ± 0.2 ^{a}	18.3 ± 0.2 ^{b}	21.8 ± 0.2 ^{a}	<0.0001
	Stearic (%)	7.4 ± 0.1 ^{a}	6.4 ± 0.1 ^{b}	6.8 ± 0.1 ^{b}	0.002
	Oleic (%)	41.5 ± 0.4 ^{c}	53.9 ± 0.4 ^{a}	47.2 ± 0.4 ^{b}	<0.0001
5	Cholesterol (mg/100 g)	330.9 ± 9.5	325.6 ± 9.5	301.2 ± 9.5	0.14
	Crude Fat (%)	8.7 ± 1.0	7.5 ± 1.0	7.4 ± 1.0	0.64
	Palmitic (%)	20.7 ± 0.8	18.1 ± 0.8	20.4 ± 0.8	0.12
	Stearic (%)	7.2 ± 0.2 ^{a}	6.2 ± 0.2 ^{b}	7.2 ± 0.1 ^{a}	0.03
	Oleic (%)	41.4 ± 1.5 ^{b}	53.7 ± 1.5 ^{a}	44.7 ± 1.5 ^{b}	0.003
6	Cholesterol (mg/100 g)	307.6 ± 16.6	312.8 ± 16.6	311.6 ± 16.6	0.97
	Crude Fat (%)	5.8 ± 0.6	6.2 ± 0.6	6.3 ± 0.6	0.81
	Palmitic (%)	20.2 ± 0.21 ^{a}	17.9 ± 0.21 ^{b}	21.1 ± 0.21 ^{a}	0.01
	Stearic (%)	7.1 ± 0.1 ^{a}	6.5 ± 0.1 ^{b}	6.4 ± 0.1 ^{b}	0.007
	Oleic (%)	39.8 ± 0.9 ^{c}	52.3 ± 0.9 ^{a}	44.8 ± 0.9 ^{b}	0.0002
7	Cholesterol (mg/100 g)	305.9 ± 8.5	280.1 ± 8.5	312.9 ± 8.5	0.08
	Crude Fat (%)	6.3 ± 0.5	6.5 ± 0.5	6.1 ± 0.5	0.82
	Palmitic (%)	22.1 ± 0.2 ^{a}	18.8 ± 0.2 ^{b}	22.1 ± 0.2 ^{a}	<0.0001
	Stearic (%)	7.8 ± 0.2 ^{a}	6.4 ± 0.2 ^{b}	6.9 ± 0.2 ^{b}	0.003
	Oleic (%)	43.4 ± 0.4 ^{c}	54.5 ± 0.4 ^{a}	48.2 ± 0.4 ^{b}	<0.0001
8	Cholesterol (mg/100 g)	327.5 ± 13.2	351.2 ± 13.2	330.0 ± 13.2	0.43
	Crude Fat (%)	7.8 ± 0.7	6.6 ± 0.7	5.2 ± 0.7	0.12
	Palmitic (%)	21.6 ± 0.3 ^{a}	18.2 ± 0.3 ^{b}	21.7 ± 0.3 ^{a}	0.0003
	Stearic (%)	7.7 ± 0.1 ^{a}	6.4 ± 0.1 ^{b}	6.6 ± 0.1 ^{b}	0.0002
	Oleic (%)	42.2 ± 0.5 ^{c}	51.6 ± 0.5 ^{a}	46.7 ± 0.5 ^{b}	<0.0001

In total, 99 brown Leghorn (57 week of lay) hens were fed one of three isonitrogenous (18% crude protein) diets ad libitum for 8 weeks. Total cholesterol (mg/100 g weight sample) and lipid analysis was determined by direct methylation methods. Egg fat hydrolysis methods were measured using the AOAC method 954.02. [1] Treatments: Control = conventional soybean meal and corn mash diet, HOPN = 20% unblanched high-oleic peanut + corn diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil. Crude fat content = g crude fat/g total sample weight ∗ 100, fatty acid content = g of fatty acid/g total lipid content ∗ 100. Each value represents the mean ± the standard error for each experimental time-point with 15 egg samples/treatment group (5 egg samples per replicate, 3 replicates). In total, 45 eggs were analyzed at each time-point. ^{a,b,c} Means within the same row lacking a common superscript differ significantly ($p < 0.05$).

There were no significant differences in the total linolenic fatty acid content in eggs produced from hens fed the three treatment groups at week 2 (Table 6). Total linolenic fatty acid content was significantly lower in eggs produced from hens fed the HOPN diet in comparison to the total linolenic fatty acid content in eggs produced from hens fed the control and OA treatments at week 3 ($p < 0.0001$), week 4 ($p < 0.001$), week 5 ($p < 0.01$), week 7 ($p < 0.001$), and week 8 ($p < 0.001$). The total linolenic fatty acid content was similar between eggs in the control and OA treatment groups at week 3, 4, 5, and 6. Even so, the average total linolenic acid content in all egg samples from all treatment groups within the study were very low (≤0.2%, Table 6).

Table 6. The effect of feeding laying hens a high-oleic peanut (HOPN) diet or an oleic fatty acid (OA) diet on the unsaturated fatty acid profile of the eggs produced.

Week	Item (%)	Treatments [1]			*p*-Value
		Control	HOPN	OA	
2	Linoleic	1.1 ± 0.12 [a]	0.52 ± 0.12 [b]	0.56 ± 0.12 [b]	0.03
	Linolenic	0.02 ± 0.003	0.003 ± 0.003	0.003 ± 0.003	0.05
	Omega 3	0.17 ± 0.1	0.25 ± 0.1	0.29 ± 0.1	0.68
	Omega 6	1.3 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.05
	C22:6 n3 [¥]	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.63
3	Linoleic	11.4 ± 0.2 [a]	6.8 ± 0.2 [c]	8.6 ± 0.2 [b]	<0.0001
	Linolenic	0.20 ± 0.003 [a]	0.10 ± 0.003 [b]	0.20 ± 0.003 [a]	<0.0001
	Omega 3	3.4 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	0.15
	Omega 6	13.5 ± 0.2 [a]	9.0 ± 0.2 [c]	10.7 ± 0.2 [b]	<0.0001
	C22:6 n3 [¥]	3.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.3	0.11
4	Linoleic	7.6 ± 2.2	6.6 ± 2.2	8.2 ± 2.2	0.88
	Linolenic	0.20 ± 0.009 [a]	0.10 ± 0.009 [b]	0.20 ± 0.009 [a]	0.0004
	Omega3	4.9 ± 0.3	4.3 ± 0.3	4.1 ± 0.3	0.26
	Omega 6	9.7 ± 2.2	8.8 ± 2.2	10.2 ± 2.2	0.90
	C22:6 n3 [¥]	4.7 ± 0.3	4.2 ± 0.3	3.9 ± 0.3	0.31
5	Linoleic	9.6 ± 0.4 [a]	6.6 ± 0.4 [b]	7.5 ± 0.4 [b]	0.004
	Linolenic	0.17 ± 0.01 [a]	0.09 ± 0.01 [b]	0.14 ± 0.01 [a]	0.006
	Omega 3	6.6 ± 1.6	5.1 ± 1.6	6.9 ± 1.6	0.72
	Omega 6	11.5 ± 0.4 [a]	8.6 ± 0.4 [b]	9.3 ± 0.4 [b]	0.006
	C22:6 n3 [¥]	6.4 ± 1.67	5.0 ± 1.6	6.8 ± 1.6	0.73
6	Linoleic	9.1 ± 0.4 [a]	6.7 ± 0.4 [b]	7.7 ± 0.4 [ab]	0.008
	Linolenic	0.16 ± 0.01 [a]	0.10 ± 0.01 [b]	0.12 ± 0.01 [ab]	0.02
	Omega 3	8.0 ± 1.0	5.6 ± 1.0	6.3 ± 1.0	0.30
	Omega 6	11.1 ± 0.4 [a]	8.7 ± 0.4 [b]	9.8 ± 0.4 [ab]	0.02
	C22:6 n3 [¥]	7.8 ± 1.0	5.5 ± 1.0	6.1 ± 1.0	0.31
7	Linoleic	10.0 ± 1.1 [a]	4.8 ± 1.1 [b]	7.9 ± 1.1 [ab]	0.04
	Linolenic	0.20 ± 0.006 [a]	0.10 ± 0.006 [c]	0.13 ± 0.006 [b]	0.0002
	Omega 3	4.1 ± 0.4	3.8 ± 0.4	3.3 ± 0.4	0.38
	Omega 6	12.0 ± 1.1	6.9 ± 1.1	10.0 ± 1.1	0.05
	C22:6 n3 [¥]	3.9 ± 0.4	3.8 ± 0.4	3.2 ± 0.3	0.41
8	Linoleic	10.5 ± 0.3 [a]	7.2 ± 0.3 [b]	7.6 ± 0.3 [b]	0.0002
	Linolenic	0.18 ± 0.007 [a]	0.09 ± 0.007 [c]	0.13 ± 0.007 [b]	0.0004
	Omega 3	4.7 ± 0.4	5.6 ± 0.4	4.4 ± 0.4	0.22
	Omega 6	12.7 ± 0.3 [a]	9.7 ± 0.3 [b]	9.6 ± 0.3 [b]	0.0002
	C22:6 n3 [¥]	4.5 ± 0.4	5.5 ± 0.4	4.3 ± 0.4	0.19

In total, 99 brown Leghorn (57 week of lay) hens were fed one of three isonitrogenous (18% crude protein) diets ad libitum for 8 weeks. Fatty acid analysis of egg samples was determined using standard direct methylation methods. Egg fat hydrolysis methods were measured using the AOAC method 954.02. [1] Treatments: Control = conventional soybean meal and corn mash diet, HOPN = 20% unblanched high-oleic peanut + corn diet, OA = control diet supplemented with 2.64% food-grade oleic fatty acid oil. Fatty acid content = g of fatty acid/g total lipid ∗ 100. Each value represents the mean ± the standard error for each experimental time-point with 15 egg samples/treatment group (5 egg samples per replicate, 3 replicates). In total, 45 were eggs analyzed at each time-point. [¥] C22:6 n3 = polyunsaturated docosahexaenoic fatty acid. [a,b,c] Means the same row lacking a common superscript differ significantly ($p < 0.05$).

There were no significant treatment differences in the total omega 3 content in eggs produced from hens from the three treatment groups at week 2, 3, 4, 5, 6, 7, or 8 (Table 6). There were no significant treatment differences in the total omega 6 content in eggs produced from the three treatment groups at week 2, week 4, or week 7 (Table 6). At week 3, eggs produced from hens fed the control diet had the highest content of total omega 6 fatty acid ($p < 0.001$), while eggs produced from hens fed the OA diet had an intermediate level of total omega 6 fatty acid, and eggs produced from hens fed the HOPN diet had the lowest omega 6 content in comparison to the other treatment groups (Table 6). At week 5, 6, and 8, eggs produced from hens fed the control diet had significantly higher levels of total omega 6 content in comparison to eggs produced from hens fed the HOPN

diet. Nonetheless, there were no significant differences in n3 docosahexaenoic (C22:6 n3) acid content in eggs produced from hens fed the three treatment groups at any of the experimental time points (Table 6). Additionally, eggs were analyzed for the following fatty acid acids: butyric, caproic, caprylic, undecanoic, lauric, tridecanoic, myristic, myristoleic, pentadecylic, pentadecenoic, margaric, margaroleic, arachidic, gadoleic, eicosadienoic, homo-gamma-linolenic, eicosatrienoic, arachiconic, n3 timnodonic, heneicosanic, behenic, erucic, brassic, lignoceric, and nervonic acid, of which no levels were detected (data not shown).

Numerous feeding trials have demonstrated that modification of the fatty acid profile in the diets of food production animals significantly alters the lipid content and fatty acid profile of the meat [32–34] and/or eggs produced [10,35–39]. Similarly, this study demonstrates that eggs produced from older production hens (57 weeks of lay) fed a HOPN or OA diet had significantly reduced saturated fatty acid and trans-fat content with enhanced monounsaturated oleic fatty acid content as compared to conventional eggs.

3.4. Sensory Evaluation

Of the 109-consumer panelists, 60% were female and 34% were male (data not shown). All consumers were under the age of 65, with 50% of the consumer population between the age of 18 to 25 (data not shown). A total of 37% of the consumer population reported the consumption of scrambled eggs multiple times per week, and 34% reported consuming scrambled eggs at least once per week (data not shown). Recruited consumer panelists scored scrambled eggs similarly in appearance liking, aroma liking, color liking, overall liking, flavor liking, texture Just About Right (JAR), and purchase intent between the three treatment groups ($p < 0.05$, Table 7) using a 9-point hedonic scale: extremely dislike = 1 and extremely like = 9.

Table 7. Consumer [1] acceptance scores for scrambled egg samples produced from laying hens fed experimental diets [2].

		Control	OA	HO PN
Appearance Liking [3]		6.6 [a]	6.2 [b]	6.2 [b]
Aroma Liking [3]		6.6 [a]	6.5 [a]	6.4 [a]
Color Liking [3]		6.6 [a]	6.3 [a]	6.5 [a]
Overall Liking [3]		7.0 [a]	6.6 [a]	6.6 [a]
Flavor Liking [3]		6.9 [a]	6.6 [a]	6.6 [a]
Overall Flavor JAR [4]	Not Enough Flavor	20.2% [a]	18.3% [a]	23.9% [a]
	JAR	66.1% [a]	65.1% [a]	63.3% [a]
	Too Much Flavor	13.8% [a]	16.5% [a]	12.8% [a]
Aftertaste JAR [5]	Not Strong Enough	10.0% [a]	6.0% [a]	4.9% [a]
	JAR	60.0% [a]	50.0% [a]	53.7% [a]
	Too Strong	30.0% [a]	44.0% [a]	41.5% [a]
Texture JAR [4]	Much Too Soft	12.8% [a]	4.6% [a]	12.8% [a]
	JAR	73.4 [a]	65.1% [a]	67.9% [a]
Purchase Intent [6]		3.7 [a]	3.5 [a]	3.4 [a]

[1] In total, 109 consumer panelists scored scrambled shell egg samples for comparative sensory attributes. [2] Experimental diets: Control = soybean meal + corn, OA = control diet spiked with 2.64% oleic fatty acid oil, HOPN = high-oleic peanut + corn. [3] Liking attributes scores: 1 = extremely dislike and 9 = extremely like. [4] Just About Right (JAR) scores: 1 or 2 = too little, 3 = just about right, and 4 or 5 = too much. The reported percentage of consumers that selected each option used Chi-square for statistical analysis. [5] Aftertaste liking and JAR scores were from consumers who detected an aftertaste in the product. [6] Purchase intent scores: 1 or 2 = would not buy, 3 = unsure, 4 or 5 = would buy. [a,b] Means within the same row lacking a common superscript differ significantly ($p < 0.05$).

Food flavor is consistently rated as one of the most important elements determining consumer product consumption, timeframe on the food market, purchase intent, and repeat purchase [40,41]. Other studies have reported that eggs produced from hens fed diets supplemented with unsaturated fatty acids from marine oils or linseed oils have undesirable off-flavors in the eggs [42] and/or meat [42] produced. In contrast, this study demonstrated that consumer panelists equally scored and preferred scrambled eggs produced from hens fed diets containing unsaturated fatty acids from high-oleic peanuts or

oleic acid, with no reports of conceived off-flavors. Thus, in this study, we aimed to not only examine the effects of feeding production hens HOPN on the fatty acid profile of the shell eggs produced, but also to determine the effect on sensory attributes and consumer acceptance of the eggs produced. Hence, feeding a high-oleic peanut diet and/or OA supplemented diet did not adversely affect the sensory attributes or consumer acceptance of the eggs produced.

4. Conclusions

Lastly, this study helps to substantiate the utilization of whole unblanched high-oleic peanuts as a valued alternative feed ingredient for poultry to enhance the eggs produced with β-carotene and reduced saturated fatty acid content. Furthermore, this study also confirms the use of high-oleic peanuts with the skin intact as an energy- and protein-rich alternative feed ingredient for older production hens to prevent the production of oversized eggs, while naturally enhancing yolk pigments and carotenoid content.

Author Contributions: All authors actively contributed to the care and husbandry of all research animals, while co-authors O.T.T., R.M., and K.E.A. were active participants in the data analysis, data interpretation, and preparation of the manuscript. Co-authors T.C.V., and E.S. collected and tabulated all body weights, feed intake, egg weights and egg quality data. Co-author A.K.R. additionally assisted with formatting and editing the final manuscript for publication. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The procedures used in these studies were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #17-001-A).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. The sensory protocol was reviewed and deemed exempt by the NC State University Institutional Review Board (IRB) for human subjects.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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The Effect of Feeding Hens a Peanut Skin-Containing Diet on Hen Performance, and Shell Egg Quality and Lipid Chemistry

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Abstract: Peanut skins are a considerable waste product with little current economic value or use. We aimed to determine the dietary effects of peanut skins on layer production performance and egg quality and chemistry of the eggs produced. Two hundred commercial hens were randomly assigned to four treatments (five replicates) and fed ad libitum for 8 weeks: conventional control diet, diet containing 24% high-oleic peanut (HOPN), diet containing 3% peanut skin (PN Skin), and a diet with 2.5% oleic acid (OA). Hens fed the HOPN diet had significantly reduced body weights relative to the control and PN Skin treatments, producing fewer total eggs over the 8-week experimental period. Eggs weights were similar between the control and PN Skin treatments at weeks 2 and 4, while eggs from the PN Skin treatment group were heavier than other treatments at weeks 6 and 8 of the experiment. Eggs produced from the HOPN treatment had reduced saturated fatty acid (FA) content in comparison to the other treatment groups, while similar between PN Skin and control eggs at week 8 of the experiment. This study suggests that PN skins may be a suitable alternative layer feed ingredient.

Keywords: alternative layer feed ingredient; peanut skins; high-oleic peanuts; shell eggs; layers; poultry feeding trial

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1. Introduction

Feed ingredients used to make dietary rations for food production animals account for approximately 60–70% of the total production cost annually [1]. Peanut skins, which are an abundant low-value waste by-product of the peanut industry, contain residual nutrients that may serve as an energy-rich, antioxidant-rich, affordable feed additive or ingredient for production animals. Peanut skins contain 19% fat, 12% fiber, and 14% to 15% polyphenolic compounds [2]. Nevertheless, approximately 70 million pounds of peanut skins are discarded annually with no identified uses and little to no economic value [3].

Dairy and beef cattle feeding trials have shown that peanut skin dietary inclusion rates greater than 8–16% inhibits protein digestion and absorption due to the high content of tannin and procyanidin [4,5]. Interestingly, reduction in the tannin and procyanidin content in peanut skins by ammoniation did not improve protein digestibility, nitrogen retention, or production performance in steers [6]. In contrast, a small goat feeding trial using whole peanuts and/or peanut skins in the diets of goats demonstrated that whole peanuts and/or peanut skins had similar rates of rumen digestibility as conventional forages such as alfalfa hay cubes, while peanut skins providing a high level of dietary antioxidants [7]. While there are several published reports on the use of peanut skins as a feed additive in ruminant diets, there are no published peanut skin feeding trials to date in monogastric production animals. Hence, in this study, we aimed to determine the effect of peanut skins as a feed ingredient on the production performance of layers.

Secondly, we aimed to determine the effect of peanut skins on the chemical composition and quality of the eggs produced from layers fed a peanut skin-containing diet. Poultry feeding trials using carotenoid-rich feed ingredients such as tomato powder, alfalfa concentrate, and marigold extract demonstrate significant enrichment of egg yolk color intensity and carotenoid content in eggs produced from quail [8] and layers [9] versus conventionally fed hens. However, commercial use of these feeding programs is costly and often not viable due to the high cost of inclusion in the diets. Interestingly, studies have shown that hens fed a diet containing peanuts with the skin intact produced eggs enriched in yolk color (2-fold) and in β-carotene content conventional eggs [10]. For this reason, we aimed to determine the effect of feeding peanut skins or oleic acid on egg yolk color and/or chemistry in the eggs produced from hens fed a peanut skin or oleic acid-supplemented diet.

2. Materials and Methods

All animal research procedures used in these feeding trials were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #17-001-A).

2.1. Experimental Design, Animal Husbandry, Dietary Treatments, and Hen Performance

Two hundred 40-week-of-lay Hy-Line W36 hens were randomly assigned to one of 4 isonitrogenous (18% crude protein) and isocaloric (3080 kcal/kg) treatments, with 5 replicates per treatment, to meet and/or exceed the NRC requirements for layers. Hens were individually housed and fed *ad libitum* for 8 weeks one of the following dietary treatments: control conventional soybean meal + corn, 24% unblanched high-oleic peanut (HOPN), 3% peanut skin (PN Skin), or 2.5% food-grade oleic acid (OA)-supplemented diet. High-oleic peanuts were crushed using a roller mill into crumbles prior to inclusion in the finished HOPN diet. The OA diet was prepared by supplementing the control diet with 2.5% food-grade OA (Millipore Sigma, Burlington, MA, USA). Peanut skins were collected after the blanching process and were ground finely using a blender into a powder prior to inclusion in the diet. There were five replicates per treatment with hens individually housed in battery cages (each cage 12 inches wide × 18 inches deep × 18 inches height) in one room at the Chicken Education Unit, NC State University (Raleigh, NC, USA). Hens were provided feed and water *ad libitum* and 14 L:D 8 weeks. Finished feed samples were analyzed for aflatoxin and microbiological contaminants by the NC Department of Agriculture and Consumer Services, Food and Drug Protection Division Laboratory (Raleigh, NC, USA).

Body weights were recorded for each individual hen at week 1 and week 8, with feed weights recorded weekly. Shell eggs were collected, enumerated, and weighed daily. The total number of eggs produced per treatment was calculated for each experimental week and for the total 8-week feeding trial. The average feed conversion ratio (FCR) was calculated as total feed consumed over the 8-week feeding (kg)/dozens of eggs produced for each treatment group over the 8-week feeding trial.

2.2. Egg Quality and Grading

Bi-weekly DSM egg yolk color, Haugh unit (HU), albumen height, vitelline membrane strength, and USDA grade were determined bi-weekly with 15 eggs per treatment by randomly selecting 3 eggs from each replicate. Fresh shell eggs were collected on the day of quality assessment and USDA grading. Haugh unit values were determined using methods described by Haugh [11] and were recorded with the Technical Services and Supplies (TSS) QCD system (Dunnington, York, UK). The QCD system was calibrated to the DSM Color Fan consisting of a series of 15 colored plastic tabs with a range of yolk colors from light yellow to orange-red (color index 1 to 15) defined by Vuillemier [12]. In general, a texture analyzer (TA.XTplus, Stable Micro Systems Ltd., Surrey, UK) was used to measure the shell strength and vitelline membrane strength by the breaking strength using

a 5 kg load cell per the manufacturer's instructions (Stable Micro Systems Ltd., Surrey, UK) with measurements in grams of force. Vitelline membrane strength was determined using methods described by Jones et al. 2005 with a 2 mm/s test speed and 0.0001 kg trigger force [13]. Modified methods of Jones et al. 2002 were used to measure shell strength with a 2 mm/second test speed and a 0.001 kg trigger force [14].

2.3. β-Carotene, Lipid Content, and Fatty Acid Analysis

All experimental diets and eggs were analyzed for total cholesterol, crude fat, fatty acid, and β-carotene content in triplicate by an AOAC-certified lab, ATC Scientific (Little Rock, AK, USA), using AOAC-approved standard chemistry methods. Each egg sample was mixed for homogeneity in a whirl-pak® (Millipore Sigma, St. Louis, MO, USA) bag for 3 min using a Smasher™ Lab Blender (Weber Scientific, Hamilton, NJ, USA). Subsequently, all egg samples were frozen at −20 °C and stored frozen until chemical analysis within two weeks of collection. Frozen homogenous egg samples were shipped on dry ice overnight to the vendor for analysis within 2 weeks of collection. Lipid (total cholesterol, crude fat) and fatty acid analysis of homogenous egg samples and feed samples were analyzed using direct methylation methods as described by Toomer et al. [10]. Total cholesterol was measured as mg cholesterol/100 g sample weight (feed or egg), while crude fat was measured as percentage of gram crude fat/gram sample weight (feed or egg). Fatty acid content was measured as percentage of gram of fatty acid/gram total lipid content of the sample (feed or egg). Methods used to determine β-carotene content in eggs are detailed in the AOAC 958.05 [15] color of egg yolk method. Egg fat hydrolysis methods were determined using the AOAC method 954.02 [16]. Gross energy analysis of feed samples was performed by ATC Scientific using an adiabatic oxygen bomb calorimeter with standard methods.

2.4. Statistical Analysis

Each hen served as the experimental unit for all performance data. All performance data were evaluated for significance by one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$ using SAS statistical software (version 9.4). If ANOVA results were significant ($p < 0.05$), Tukey's multiple comparisons t-test was conducted to compare the mean of each treatment group with the mean of every other treatment at $p < 0.05$ significance level. Comparisons were made between body weights (50 birds/treatment), feed intake (50 birds/treatment), feed conversion ratio (50 birds/treatment), and egg weights (total # of eggs collected over the 8-week feeding trial).

Fifteen eggs per treatment (3 eggs per replicate randomly selected) were statistically analyzed by one-way ANOVA ($p < 0.05$) using SAS. Means were separated by least-squares means with Tukey–Kramer adjustment for multiple comparisons ($p < 0.05$) for treatment differences in egg quality parameters (Haugh unit, vitelline membrane strength, shell strength, yolk color score), egg β-carotene content, and egg lipid content (crude fat, total cholesterol, fatty acid content) with 60 total egg samples at each time point (0 week, 2 week, 4 week, 6 week, 8 week). Means were separated by least-squares means with Tukey–Kramer adjustment for multiple comparisons ($p < 0.05$) for treatment differences.

3. Results

3.1. Feed Analysis

Four experimental diets were formulated (Table 1) to be isocaloric (3080 kcal/kg) and isonitrogenous (18% crude protein). In addition, chemical analysis was performed to determine the crude protein, crude fat, gross energy, and fatty acid profile of the experimental diets (Table 2). As expected, the HOPN dietary treatment had the highest level of oleic fatty acid content relative to the other treatment groups, while the control, PN Skin, and OA dietary treatment groups had the highest levels of linoleic fatty acid content relative to the HOPN dietary treatment (Table 2). Interestingly, the PN Skin dietary

treatment had the greatest percent of omega 3 fatty acid content (Table 2) relative to the other treatment groups.

Table 1. Feed formulation of experimental laying hen diets.

Treatments [1]				
	Control	**HOPN**	**PN Skin**	**OA**
Ingredients	% (by weight)			
Soybean Meal	20.4	0	12.0	10.0
Corn	47.5	36.9	56.9	57.0
High-Oleic Peanut [2]	0	24.0	0	0.0
Soybean Oil	7.8	0	4.4	0.0
Wheat Bran	6.0	20.0	5.0	8.7
Soy Protein Isolate	5.0	5.5	7.5	7.8
Peanut Skin	0	0	3.0	0
Oleic Acid Oil	0.0	0.0	0.0	2.5
Calcium Carbonate	10.8	10.8	9.1	11.3
Dicalcium Phosphorus	1.5	1.2	1.6	1.5
Sodium Chloride	0.3	0.3	0.3	0.3
L-Lysine	0	0.5	0.1	0.2
DL-Methionine	0.2	0.3	0.3	0.2
L-Tryptophan	0	0	0	0
L-Threonine	0	0.1	0	0
Choline Chloride	0.2	0.2	0.2	0.2
[3] Santoquin®	0.1	0.1	0.1	0.1
Mineral Premix [4]	0.2	0.2	0.2	0.1
Vitamin Premix [5]	0.1	0.1	0.1	0.1
Selenium Premix [6]	0.1	0.1	0.1	0.1
Metabolizable Energy [7]	3080	3080	3080	3080

[1] Four isocaloric, isonitrogenous (18% protein) diets were fed to Hy-Line W36 hens for 8 weeks. [2] Treatments: control = conventional soybean meal and corn mash diet, HOPN = (24%) unblanched high-oleic peanut crumbles and corn mash diet, PN Skin = control diet supplemented with 3.0% ground peanut skins, OA = control diet supplemented with 2.5% food-grade oleic fatty acid oil. [3] High-oleic peanuts = unblanched raw whole high-oleic peanut crumbles. [3] Santoquin® = Feed antioxidant and preservative to prevent fat oxidation in stored feed (Novus International, St. Charles, MO, USA). [4] Mineral premix manufactured by NCSU FeedMill, supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt. [5] Vitamin premix manufactured by NCSU FeedMill supplied the following per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid. [6] Selenium premix manufactured by NCSU FeedMill = 1 mg selenium premix provided 0.2 mg Se (as Na_2SeO_3) per kg of diet. [7] Metabolizable energy = kcal/kg feed.

Table 2. Chemical analysis of experimental laying hen diets.

Treatments [1]				
	Control	**HOPN**	**PNSkin**	**OA**
Component	% (by weight)			
Crude Fat [2]	8.4	13.9	8.7	5.1
Crude Protein	19.4	18.5	20.2	19.0
Fiber	2.3	3.2	1.9	2.4
* Palmitic	10.8	6.7	10.2	10.8
* Steric	3.8	3.2	3.6	2.7
* Oleic	22.6	74.3	27.8	25.9
* Elaidic	1.3	0.7	1.2	1.0
* Linoleic	52.5	7.1	48.4	45.8
* Omega 3	6.618	0.1	58.5	3.2
* Omega 6	53.2	1.4	49.4	47.8
Gross Energy [3]	3506	3757	3308	3085

[1] Treatments: control = conventional soybean meal and corn mash diet, HOPN = unblanched high-oleic peanut crumbles (24%) and corn mash diet, PN Skin = control diet supplemented with 3.0% ground peanut skins, OA = control diet supplemented with 2.5% food-grade oleic fatty acid oil. Lipid (crude fat, total cholesterol, fatty acid) and beta-carotene analysis was performed by an AOAC-certified lab, ATC Scientific (Little Rock, AR, USA), using AOAC-approved standard methods. [2] Crude fat content = g crude fat/g total sample weight * 100, * fatty acid content = g of fatty acid/g total lipid * 100. Each value represents the mean ± the standard error for each triplicate sample. [3] Gross energy = kcal/kg feed.

3.2. Hen Performance and Egg Weights

Hens fed the OA diet had body weights that were significantly less than the body weights of hens fed the control and PN Skin diets ($p < 0.05$), while body weights were similar between the HOPN and OA dietary treatments at week 1 (Table 3). At week 8, hens fed the HOPN diet had significantly smaller body weights relative to the body weights of hens fed the control and PN Skin ($p < 0.05$) diets, while body weights were similar between hens fed the HOPN and OA diets. In addition, hens fed the HOPN experimental diet had significantly reduced feed intake ($p < 0.001$) and fewer dozens of eggs produced ($p < 0.05$) in comparison to the other treatment groups (Table 3). Nevertheless, there were no significant treatment differences in feed conversion ratio over the 8-week feeding trial.

The weekly average egg weights (Table 4) were the smallest in eggs produced from hens fed the HOPN diet relative to the other treatment groups at week 1 of the feeding trial ($p < 0.05$). At week 2, week 4, week 6, and week 8, egg weights from hens fed the HOPN and OA experiment diet were significantly smaller than eggs produced from hens fed the control and PN Skin experimental diets, while egg weights produced from hens fed the OA experimental diet were significantly greater than eggs produced from hens fed the HOPN diet (week 1, week 2, week 4, and week 8; $p < 0.0001$). Egg weights were similar between the control and PN Skin treatment groups at week 1, week 2, and week 4, while egg weights were significantly higher in the PN Skin treatment group at week 6 and week 8 relative to the controls (Table 4).

Table 3. Performance of hens fed an unblanched high-oleic peanut or peanut skin diet and housed in battery cages.

	Body Weights (kg)	FCR [2]	Feed Intake	Dozen Eggs (kg)	Produced
Treatment [1]	Week 1	Week 8	(kg feed/)	Total for 8 weeks	Dozen eggs
Control	1.6 ± 0.03 [a]	1.6 ± 0.4 [a]	2.3 ± 0.05	9.5 ± 0.2 [a]	21.1 ± 1.1 [a]
HOPN	1.5 ± 0.03 [ab]	1.5 ± 0.4 [b]	2.3 ± 0.05	8.5 ± 0.2 [b]	17.5 ± 1.1 [b]
PN Skin	1.5 ± 0.03 [a]	1.6 ± 0.4 [a]	2.3 ± 0.05	9.3 ± 0.2 [a]	20.2 ± 1.1 [a]
OA	1.5 ± 0.03 [b]	1.5 ± 0.4 [b]	2.3 ± 0.05	9.5 ± 0.2 [a]	21.6 ± 1.1 [a]
p-value *	0.04	0.03	0.80	0.0002	0.01

Two hundred Hy-Line W36 hens (40 week of lay) were assigned to one of 4 isonitrogenous (18% crude protein) and isocaloric (3080 kcal/kg) treatments (5 replicates per treatment) and fed 8 weeks *ad libitum*. Body weights were collected at week 1 and week 8 of the study. [1] Treatments: control = conventional soybean meal and corn mash diet, HOPN = 24% unblanched high-oleic peanut crumbles and corn mash diet, PN Skin = control diet supplemented with 3.0% ground peanut skins, OA = control diet supplemented with 2.5% food-grade oleic fatty acid oil. [2] Feed conversion ratio (FCR) = kg total feed intake over the 8-week/total dozen eggs produced over 8 weeks for each treatment group. Each value (body weights and feed intake) represents the mean ± the standard error. [a,b] Means within the same column lacking a common superscript differ significantly ($p < 0.05$). * *p*-value = differences determined by ANOVA $p < 0.05$.

Table 4. Egg weights from hens fed an unblanched high-oleic peanut or peanut skin diet and housed in battery cages.

	Weekly Egg Weights [1] (g)				
Treatment [2]	**Week 1**	**Week 2**	**Week 4**	**Week 6**	**Week 8**
Control	59.5 ± 0.5 [a]	60.5 ± 0.4 [a]	60.3 ± 0.4 [a]	60.9 ± 0.4 [b]	60.4 ± 0.4 [b]
HO PN	58.3 ± 0.5 [b]	58.4 ± 0.4 [c]	58.2 ± 0.4 [c]	59.3 ± 0.4 [c]	58.3 ± 0.4 [d]
PN Skin	60.1 ± 0.5 [a]	60.8 ± 0.4 [a]	60.7 ± 0.4 [a]	61.8 ± 0.4 [a]	61.9 ± 0.4 [a]
OA	59.6 ± 0.5 [a]	59.6 ± 0.4 [b]	59.5 ± 0.4 [b]	59.7 ± 0.4 [c]	59.2 ± 0.4 [c]
p-value *	0.02	<0.0001	<0.0001	<0.0001	<0.0001

Two hundred 40-week of lay Hy-Line W36 hens were assigned to one of 4 isonitrogenous (18% crude protein) and isocaloric (3080 kcal/kg) treatments (5 replicates per treatment) and fed 8 weeks *ad libitum*. Body weights were collected at week 1 and week 8 of the study. [1] Weights (g) of eggs were determined daily and weekly for each treatment group. Data represent the weekly (1, 2, 4, 6 and 8 weeks) averages ± standard error for each time point for each treatment group. [2] Treatments: control = conventional soybean meal and corn mash diet, HOPN = 24% unblanched high-oleic peanut crumbles and corn mash diet, PN Skin = control diet supplemented with 3.0% ground peanut skins, OA = control diet supplemented with 2.5% food-grade oleic fatty acid oil. Each value represents the mean ± the standard error. [a,b,c,d] Means within the same column lacking a common superscript differ significantly ($p < 0.05$). * *p*-value = differences determined by ANOVA $p < 0.05$.

3.3. Egg Grading and Quality

All eggs produced in this 8-week feeding trial were graded as USDA Grade AA of superior quality, with thick, firm egg whites and defect-free egg yolks. Moreover, all shells were clean and without defects. There were a minimal number of blood spots or number of meat spots and no statistical difference at the 95% confidence interval between eggs produced from the treatment groups (data not shown). There were no significant differences in 8-week average shell strength or vitelline membrane strength between shell eggs produced from hens fed the four different treatments (Table 5). However, the HU used as a measurement of egg quality was similar between shell eggs produced from hens fed the control, HOPN, and PN Skin dietary treatments, while the 8-week average HU of eggs produced from hens fed the OA diet was significantly lower than shell eggs from the HOPN and PN Skin treatment groups ($p < 0.05$). Of most interest, the 8-week average yolk color was significantly less in eggs produced from hens fed the HOPN experimental diet in comparison to the other treatment groups ($p < 0.0001$).

Table 5. Egg quality of eggs produced from hens fed an unblanched high-oleic peanut or peanut skin diet and housed in battery cages.

.	Shell Strength	Vitelline Membrane	Haugh Unit	Yolk Color
	(g force)	Strength (g force)	(HU)	Roche (1–15) [1]
Treatment [2]	Weekly Average (8-Week Study)			
Control	3742 ± 209	0.2 ± 0.007	83.3 ± 1.7 [ab]	3.0 ± 0.2 [a]
HO PN	3828 ± 209	0.2 ± 0.007	86.1 ± 1.7 [a]	1.8 ± 0.2 [b]
PN Skin	3770 ± 209	0.2 ± 0.007	85.4 ± 1.7 [a]	2.9 ± 0.2 [a]
OA	3979 ± 209	0.2 ± 0.007	81.7 ± 1.7 [b]	2.9 ± 0.2 [a]
p-value *	0.68	0.31	0.04	<0.0001

Two hundred Hy-Line W36 hens (40-week of lay) were assigned to one of 4 isonitrogenous (18% crude protein) and isocaloric (3080 kcal/kg) treatments (5 replicates per treatment) and fed 8 weeks *ad libitum*. Eggs were collected on the day of quality assessment with 15 eggs per treatment, with 3 eggs randomly selected per replicate. Each value represents the average values over the 8-week period ± SEM. [1] Yolk color = Roche Color Fan color index 1–15 (lightest to darkest color intensity). [2] Treatments: control = conventional soybean meal and corn mash diet, HOPN = 24% unblanched high-oleic peanut crumbles and corn mash diet, PN Skin = control diet supplemented with 3.0% ground peanut skins, OA = control diet supplemented with 2.5% food-grade oleic fatty acid oil. * *p*-value = differences determined by ANOVA $p < 0.05$. [a,b] is described as items within a column sharing the same superscript are similar, so that means that Control and OA are similar statistically, while HOPN, PN Skin and Control are statistically similar, but OA and HOPN and PN Skin are statistically different.

3.4. Egg Chemistry

There were no significant treatment differences in total cholesterol and crude fat (CF) levels in eggs produced from hens fed the four dietary treatment groups at any of the time points measured (Table 6). Eggs produced from hens fed the HOPN diet had the lowest content of saturated fatty acid levels of palmitic and stearic acid, in comparison to eggs produced from the other dietary treatment groups at week 2, week 4, week 6, and week 8 ($p < 0.0001$, Table 6). In contrast, eggs produced from hens fed the OA dietary treatment had the highest content of palmitic saturated fatty acid levels in comparison to eggs produced from the other dietary treatment groups at week 2, week 4, and week 6 ($p < 0.0001$). Palmitic fatty acid content was similar between eggs produced from hens fed the control diet and PN Skin diet at week 4, week 6, and week 8 of the experimental timeframe. Moreover, eggs produced from hens fed the OA diet had significantly reduced levels of stearic saturated fatty acid levels in comparison to eggs produced from hens fed the control diet and PN Skin diet at week 2, week 4, week 6, and week 8 ($p < 0.0001$). Stearic saturated fatty acid levels were similar between eggs produced from hens fed the control diet and PN Skin diet at week 2, week 4, and week 8 ($p < 0.0001$).

Eggs produced from hens fed the HOPN dietary treatment had the lowest level of trans-fat elaidic acid in comparison to eggs produced from hens fed the other dietary treatments at week 4, week 6, and week 8 ($p < 0.0001$, Table 6). However, eggs produced from hens fed the control diet and the PN Skin diet had similar levels of elaidic fatty acid content at week 4, week 6, and week 8. Ironically, at week 2, eggs produced from hens fed the HOPN treatment had the highest content of elaidic acid content compared to eggs produced from the other treatment groups. Oleic fatty acid content was highest in eggs produced from hens fed the HOPN experimental diet, followed by eggs produced from hens fed the OA and PN Skin experimental diets at week 2, week 4, week 6, and week 8 ($p < 0.0001$, Table 6). Eggs produced from hens fed the control diet had the lowest levels of oleic acid content relative to eggs produced from hens fed the other dietary treatments at all time points measured.

Table 6. Lipid and fatty acid content of eggs produced from hens fed unblanched high-oleic peanut or peanut skins and housed in battery cages.

Week	Trmt [1]	Cholesterol	CF	Palmitic	Stearic	Elaidic	Oleic
2	Control	N/A	4.7 ± 0.9	23.1 ± 0.3 [b]	8.8 ± 0.1 [a]	30.7 ± 0.4 [d]	30.7 ± 0.4 [d]
	HO PN	N/A	5.5 ± 0.9	17.8 ± 0.3 [c]	6.0 ± 0.1 [c]	62.3 ± 0.4 [a]	62.3 ± 0.4 [a]
	PN Skin	N/A	5.7 ± 0.9	24.2 ± 0.3 [a]	8.9 ± 0.1 [a]	35.6 ± 0.4 [c]	35.5 ± 0.4 [c]
	OA	N/A	4.7 ± 0.9	24.9 ± 0.3 [a]	7.5 ± 0.1 [b]	44.9 ± 0.4 [b]	44.9 ± 0.4 [b]
	p-value *	N/A	0.63	0.001	<0.0001	<0.0001	<0.0001
4	Control	255 ± 41	6.4 ± 0.8	24.1 ± 0.2 [b]	9.2 ± 0.2 [a]	1.4 ± 0.1 [b]	32.8 ± 0.3 [d]
	HO PN	240 ± 41	5.6 ± 0.8	17.9 ± 0.2 [c]	5.9 ± 0.2 [c]	0.9 ± 0.1 [c]	63.9 ± 0.3 [a]
	PN Skin	211 ± 41	4.9 ± 0.8	24.0 ± 0.2 [b]	8.8 ± 0.2 [a]	1.3 ± 0.1 [b]	36.6 ± 0.3 [c]
	OA	229 ±41	6.2 ± 0.8	25.4 ± 0.2 [a]	7.5 ± 0.2 [b]	1.6 ± 0.1 [a]	46.3 ± 0.3 [b]
	p-value *	0.75	0.30	<0.0001	<0.0001	0.0002	<0.0001
6	Control	261 ± 35	5.7 ± 0.8	22.9 ± 0.4 [b]	29.9 ± 0.3 [a]	1.3 ± 0.05 [b]	30.0 ± 0.8 [d]
	HO PN	296 ± 35	6.5 ± 0.8	17.1 ± 0.4 [c]	5.6 ± 0.3 [d]	0.9 ± 0.05 [c]	58.3 ± 0.8 [a]
	PN Skin	248 ± 35	5.7 ± 0.8	23.5 ± 0.4 [b]	8.4 ± 0.3 [b]	1.3 ± 0.05 [b]	34.5 ± 0.8 [c]
	OA	291 ± 35	6.8 ± 0.8	24.4 ± 0.4 [a]	7.4 ± 0.3 [c]	1.6 ± 0.05 [a]	43.4 ± 0.8 [b]
	p-value *	0.50	0.40	<0.0001	<0.0001	<0.0001	<0.0001
8	Control	312 ± 22	9.6 ± 0.2	23.3 ± 0.4 [a]	8.9 ± 0.2 [a]	1.2 ± 0.05 [b]	30.7 ± 0.8 [c]
	HO PN	298 ± 22	9.8 ± 0.2	18.1 ± 0.4 [b]	6.2 ± 0.2 [c]	0.9 ± 0.05 [c]	58.6 ± 0.8 [a]
	PN Skin	281 ± 22	9.3 ± 0.2	22.8 ± 0.4 [a]	8.5 ± 0.2 [a]	1.2 ± 0.05 [b]	42.7 ± 0.8 [b]
	OA	279 ± 22	9.6 ± 0.2	23.3 ± 0.4 [a]	7.3 ± 0.2 [b]	1.4 ± 0.05 [a]	41.2 ± 0.8 [b]
	p-value *	0.40	0.08	<0.0001	<0.0001	<0.0001	<0.0001

Two hundred Hy-Line W36 hens (40-week of lay) were assigned to one of 4 isonitrogenous (18% crude protein) and isocaloric (3080 kcal/kg) treatments (5 replicates per treatment) and fed 8 weeks *ad libitum*. Eggs were collected weekly, and 15 eggs/treatment (3 eggs randomly selected/replicate) N = 60 were chemically analyzed bi-weekly at an AOAC-certified commercial lab, ATC Scientific (Little Rock, AR, USA), using standard AOAC-approved methods. N/A = total cholesterol could not be analyzed at week 2 of the study due to lack of sample volume. Each value represents the average values over the bi-weekly period ± SEM. [1] Treatments: control = conventional soybean meal and corn mash diet, HOPN = unblanched high-oleic peanut crumbles (24%) and corn mash diet, PN Skin = control diet supplemented with 3.0% ground peanut skins, OA = control diet supplemented with 2.5% food-grade oleic fatty acid oil. * *p*-value = differences determined by ANOVA. [a,b,c,d] Means within the same column lacking a common superscript differ significantly ($p < 0.05$).

Omega 3, omega 6, linoleic and linolenic fatty acid content was the lowest in eggs produced from hens fed the HOPN experimental diet, followed by eggs produced from hens fed the OA experimental diet, relative to the other dietary treatment groups at week 2, week 4, week 6, and week 8, with the exception of omega 3 content at week 6 ($p < 0.0001$, Table 7). Eggs produced from hens fed the control diet had the highest levels of omega 3, omega 6, linoleic and linolenic fatty acid content, followed by eggs produced by hens fed the PN Skin dietary treatment relative to eggs produced from hens fed the other dietary treatments at week 2, week 4, week 6 and week 8. There were no significant treatment differences in β-Carotene content in eggs produced from hens fed the four dietary treatments at any time point measured.

Table 7. β-Carotene and fatty acid content of eggs produced from hens fed unblanched high-oleic peanut or peanut skins and housed in battery cages.

	Week	Omega 3	Omega 6	Linoleic	Linolenic	β-Carotene
2	Control	1.8 ± 0.02 ^{a}	30.5 ± 0.5 ^{a}	24.9 ± 0.2 ^{a}	1.6 ± 0.02 ^{a}	3.9 ± 0.6
	HO PN	0.3 ± 0.02 ^{d}	9.57 ± 0.5 ^{d}	7.20 ± 0.2 ^{d}	0.2 ± 0.02 ^{d}	3.2 ± 0.6
	PN Skin	1.4 ± 0.02	24.6 ± 0.5 ^{b}	21.9 ± 0.2 ^{b}	1.2 ± 0.02 ^{b}	4.6 ± 0.6
	OA	0.5 ± 0.02 ^{c}	15.9 ± 0.5 ^{c}	13.0 ± 0.2 ^{c}	0.3 ± 0.02 ^{c}	4.0 ± 0.6
	p-value *	<0.0001	<0.0001	<0.0001	<0.0001	0.19
4	Control	1.7 ± 0.05 ^{a}	27.0 ± 0.3 ^{a}	25.2 ± 0.3 ^{a}	1.5 ± 0.04 ^{a}	4.2 ± 0.9
	HO PN	0.3 ± 0.05 ^{d}	8.17 ± 0.3	6.69 ± 0.3 ^{d}	0.1 ± 0.04 ^{d}	3.4 ± 0.9
	PN Skin	1.4 ± 0.05 ^{b}	24.0 ± 0.3 ^{b}	21.7 ± 0.3	1.2 ± 0.04 ^{b}	3.4 ± 0.9
	OA	0.5 ± 0.05	14.5 ± 0.3 ^{c}	12.9 ± 0.3 ^{c}	0.3 ± 0.04 ^{c}	3.2 ± 0.9
	p-value *	<0.0001	<0.0001	<0.0001	<0.0001	0.68
6	Control	1.5 ± 0.03 ^{a}	28.8 ± 0.4	23.2 ± 0.4 ^{a}	1.4 ± 0.03 ^{a}	2.1 ± 0.4
	HO PN	0.1 ± 0.03 ^{c}	8.6 ± 0.4 ^{d}	6.4 ± 0.4 ^{d}	0.1 ± 0.03 ^{d}	2.2 ± 0.4
	PN Skin	1.2 ± 0.03 ^{b}	23.8 ± 0.4 ^{b}	21.2 ± 0.4 ^{b}	1.2 ± 0.03 ^{b}	2.2 ± 0.4
	OA	0.03 ± 0.03 ^{d}	13.7 ± 0.4 ^{c}	11.5 ± 0.4 ^{c}	0.2 ± 0.03 ^{c}	2.5 ± 0.4
	p-value *	<0.000	<0.0001	<0.0001	<0.0001	0.73
8	Control	1.6 ± 0.05 ^{a}	27.7 ± 0.4 ^{a}	25.1 ± 0.4 ^{a}	1.5 ± 0.04 ^{a}	7.1 ± 1.0
	HO PN	0.2 ± 0.05 ^{d}	9.1 ± 0.4 ^{d}	6.8 ± 0.4 ^{d}	0.1 ± 0.04 ^{d}	4.6 ± 1.0
	PN Skin	1.1 ± 0.05 ^{b}	22.5 ± 0.4 ^{b}	19.9 ± 0.4 ^{b}	1.0 ± 0.04 ^{b}	5.9 ± 1.0
	OA	0.3 ± 0.05	14.3 ± 0.4 ^{c}	11.9 ± 0.4 ^{c}	0.3 ± 0.04 ^{c}	5.4 ± 1.0
	p-value *	<0.0001	<0.0001	<0.0001	<0.0001	0.16

Two hundred Hy-Line W36 hens (40-week of lay) were assigned to one of 4 isonitrogenous (18% crude protein) and isocaloric (3080 kcal/kg) treatments (5 replicates per treatment) and fed 8 weeks *ad libitum*. Eggs were collected weekly, and 15 eggs/treatment (3 eggs randomly selected/replicate) N = 60 were chemically analyzed bi-weekly at an AOAC-certified commercial lab, ATC Scientific (Little Rock, AR, USA), using standard AOAC-approved methods. Each value represents the average values over the bi-weekly period ± SEM. Treatments: control = conventional soybean meal and corn mash diet, HOPN = 24% unblanched high-oleic peanut crumbles and corn mash diet, PN Skin = control diet supplemented with 3.0% ground peanut skins, OA = control diet supplemented with 2.5% food-grade oleic fatty acid oil. * *p*-value = differences determined by ANOVA. ^{a,b,c,d} Means within the same column lacking a common superscript differ significantly ($p < 0.05$).

4. Discussion

Numerous feeding trials have demonstrated that the feedstock rations rich in carotenoids (tomato powder, alfalfa, marigold extract) and/or unsaturated fatty acids are transferred to the eggs [10,17]. Studies have also demonstrated improved bioavailability of lutein from enriched eggs in comparison to lutein found in spinach or dietary supplements [18] with enhanced intestinal absorption of lutein when consumed with dietary lipids, suggesting that eggs may be a superior delivery system for some carotenoids. However, the inclusion of specialty feed ingredients (alfalfa meal, marigold, fish meal, linseed meal) is not cost-effective or commercially viable for animal food production. On the contrary, few studies have examined the enrichment of consumable food products (eggs or meat) using agricultural waste by-products rich in polyphenolic compounds such as peanut skins as poultry feedstock ration. Value-addition of agricultural waste by-products, such as peanut skins to poultry feedstock rations, could promote agricultural sustainability and provide creative solutions for agricultural waste by-products with considerable residual nutritional value.

Overall, this study demonstrates that peanut skins can be effectively used in the diets of egg-producing hens at inclusion levels of 3% of the conventional diet, without

adversely affecting hen performance (feed intake, FCR, dozens of eggs produced), egg quality (HU, shell strength, albumen height, vitelline membrane strength, yolk color) or the fatty acid profile of the eggs produced. Additionally, this study parallels previous poultry feeding trials demonstrating that unblanched high-oleic peanuts enrich the eggs and meat produced with unsaturated fatty acids with reduced saturated and trans fats, with reduced egg mass compared to the controls [10] and broiler chickens [19].

While hens fed the HOPN diet had reduced feed intake, 8-week average body weights, and total dozens of eggs produced relative to the other treatments, there were no significant differences in the FCR between the treatment groups. In previous experiments, we demonstrated that hens fed the HOPN dietary treatment had reduced feed intake due to increased ileal fat digestibility and apparent metabolizable energy compared to the other treatment groups [20]. Hence, HOPN fed birds consumed less of a more energetically dense diet to meet the metabolic needs in comparison to the other dietary treatment groups.

In general, egg weights were increased in the PN Skin treatment group relative to the other treatments in the last 4 weeks of the study, while egg weights from the HOPN and OA treatment groups were smaller than the other treatment groups. Similarly, other studies have demonstrated that feeding hens diets rich in unsaturated fatty acids, such as conjugated linoleic acid (CLA), reduce egg weights and body weights [21,22], suggesting that dietary supplementation with CLA causes a reduction in hen body weights, similar to weight loss in humans consuming dietary CLA, which correlates with reduced egg weights and/or size. Egg size has been shown to be greatly influenced by body weight [23]. With every 45 g of body weight increase, there is approximately a 0.5 g increase in egg size from 18 weeks of age in laying hens [24].

In contrast, our previous studies demonstrated that yolk color in eggs produced from hens fed a diet containing unblanched high-oleic peanuts had an approximately 2-fold increase in yolk color in comparison to conventional eggs [10], while in this study, egg yolk color was significantly less in eggs produced from hens fed the HOPN diet relative to the other treatment groups. Eggs produced from hens fed the PN Skin and control diets had similar levels of palmitic and elaidic fatty acid for most of the study, while eggs produced from the HOPN and OA treatment groups had reduced saturated and trans fatty acid levels comparatively.

Chemical analysis of the four experimental diets revealed increased levels of omega 3 fatty acid levels in the PN Skin diet relative to the other dietary treatments. Nonetheless, omega 3 fatty acid level in eggs produced from hens fed the PN Skin experimental diet was similar between all treatment groups. The soybean oil, whole peanuts and/or peanut skins, and yellow corn (very low levels) are the predominate feed ingredients containing omega 3 fatty acids [25–27], which may have correlated to elevated omega 3 fatty acid content found in the PN Skin experimental diets that contained modest amounts of each of these feed ingredients relative to the other dietary treatments.

In contrast to our previously published reports [10], β-carotene content in this study was not significantly different between eggs produced from hens fed the four different dietary treatment groups at any of the experimental time points measured in this study. Studies conducted by Pattee and Purcell (1967) revealed that peanut oil extracted from young peanuts contained 60 μg of β-carotene and 138 μg of lutein per liter, while peanut oil extracted from more mature peanuts had lower concentrations of these carotenoids [28]. However, the determination of peanut maturity has been correlated with the increasing color of the mesocarp from white to yellow, orange, brown, and black [29]. Peanuts have an indeterminate growth pattern, in which at harvest, the combine collects peanut pods ranging in different maturity levels present on the plant [30]. Therefore, a given peanut harvest may contain a higher percentage of young/immature pods that contain elevated levels of carotenoids in the seed and oil [28], suggesting that a potentially higher percentage of young/immature peanuts may have been harvested for use in our earlier layer hen feeding trials with unblanched high-oleic peanuts in which enriched the eggs produced with unsaturated fatty acids and β-carotene [10].

β-carotene is a lipid-soluble carotenoid found abundantly in plants and responsible for the rich yellow and deep orange colors in plants [31]. Conventional commercial eggs are rich in lutein and zeaxanthin [32], which are carotenoids that are most likely transferred from yellow corn in the diet to the egg yolks. Layer feeding trials have demonstrated the transfer of carotenoids and their pigments from the diet to the yolks of eggs produced [9]. While our previous layer feeding trials demonstrated that eggs produced from hens fed a HOPN diet had significantly increased β-carotene levels and yolk color relative to conventional eggs, in which the rich yellow/deep orange pigment of β-carotene was transferred to the eggs. However, in this study, β-carotene content was not elevated, and hence the yellow/orange pigments were not available to transfer from the diet to the yolks of eggs produced by hens fed the HOPN or PN Skin diets. Moreover, eggs produced from hens fed the HOPN diet had less available dietary carotenoids from yellow corn (lutein and zeaxanthin) in the diet relative to the other treatment groups (Table 1 content of yellow corn: control 47.5%, 36.9%, PN Skin 56.9%, OA 57.0%), which may have also correlated to reduced yolk color scores.

Most importantly, this study reports similar body weights, feed intake, FCR, and egg chemistry between the PN Skin and control treatment groups, implying the effective use of PN skins as an alternative layer feed ingredient. These results support the value-added use of peanut skins as a poultry feed ingredient, an abundant agricultural waste by-product of the peanut industry. While this study has positive implications for the use of peanut skins as an alternative poultry feed ingredient, this study fails to parallel commercial egg production commonly using floor pens or alternative housing systems. Moreover, we aim to repeat this study with larger sample sizes to more closely parallel industry. In addition, we aim to conduct additional feeding trials with hens housed in floor pens and fed a peanut and/or peanut skin-containing diet for comparative analysis of the production performance to hens housed in battery cages and fed a peanut and/or peanut skin-containing diet.

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Institutional Review Board Statement: The procedures used in these studies were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #17-001-A).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. The sensory protocol was reviewed and deemed exempt by the NC State University Institutional Review Board (IRB) for human subjects.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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Productive Performance, Carcass Traits, and Meat Quality in Finishing Lambs Supplemented with a Polyherbal Mixture

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Abstract: The objective of this study was to evaluate the effects of dietary supplementation of a polyherbal mixture (HM) containing saponins, flavonoids, and polysaccharides on productive performance, carcass characteristics and meat quality of lambs during the final fattening period. Thirty-six Dorper × Katahdin lambs (23.27 ± 1.23 kg body weight (BW)) were housed in individual pens and were assigned to four treatments ($n = 9$) with different doses of HM: 0 (CON), 1 (HM1), 2 (HM2) and 3 (HM3) g of HM kg^{-1} of DM for 56 days. Data were analysed as a completely randomized design using the MIXED and GLM procedures of statistical analysis system (SAS), and linear and quadratic effects were tested to evaluate the effects of the HM level. DM digestibility decreased in lambs fed HM3 ($p < 0.05$). There was no effect of HM on daily weight gain, dry matter intake, final BW, feed conversion, carcass characteristics, colour (L* and a*) and meat chemical composition. Meat pH, cooking loss and drip loss increased linearly ($p < 0.05$) when the HM dose was increased. The Warner-Bratzler shear force (WBSF) of meat was lower ($p < 0.05$) in lambs fed HM3. In conclusion, dietary inclusion of 3 g HM kg^1 of DM improves meat tenderness. However, high doses of HM in the diet may decrease the digestibility of DM and increase the cooking loss and drip loss of lamb meat during the final fattening period.

Keywords: fattening lamb; saponins; bioactive compounds; mutton tenderness

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1. Introduction

Antibiotics have been commonly used as growth promoters in animals. However, the emergence of bacteria resistant to these drugs has led to the search for alternative products with similar effects to antibiotics, but of natural origin [1]. Dietary supplementation with herbal products seems to be a promising strategy to improve the productive performance, carcass characteristics and meat quality of small ruminants [2]. Some polyherbal mixtures (HM) prepared with medicinal plants have shown positive effects on productive performance, meat and carcass quality characteristics of steers and lambs during the final fattening period [3–5]. On the other hand, in calves, it has been reported that the use of HM can improve growth and health status during the pre-ruminant period until weaning by modifying gene expression [6]. However, the effects of bioactive compounds (for example, saponins and flavonoids) of HM in biological systems, may depend on the efficiency of their absorption and extensive metabolic transformation [7].

Previous studies [8,9] have shown that some plants containing saponins, flavonoids and polysaccharides can improve antioxidant status, ruminal fermentation, immune response and productive performance in sheep. Likewise, some HM containing saponins,

flavonoids and tannins have been shown to have a positive impact on nutrient utilization efficiency in goats [10]. Other products containing saponins have shown positive impact on energy metabolism and on the duodenal flux of amino acids [11], ruminal fermentation rate [12,13], rumen microbial populations [14], and production of volatile fatty acids [12–14]. Similarly, flavonoids can modulate the ruminal microbiome, improve rumen fermentation and metabolic status to improve the productive performance and health of ruminants [15]. Some HM containing flavonoids have shown positive impact on antioxidant status [7], and ruminal microbial populations of lambs [16]. In addition, flavonoid supplementation modifies the expression of genes in the rumen epithelium that could be related to inflammation and animal behaviour modulation [17].

Some plant parts containing saponins have also been used to improve the meat quality of adult goats and kids [18,19]. However, there is limited information on the effects of plants or HM containing saponins, flavonoids, and polysaccharides on the productivity, carcass characteristics, and meat quality of lambs. The botanical origin, the dose, and the composition of the diet used can influence the biological response that saponins have on ruminants [20]. Although, the effects of using saponins in ruminant feed have been investigated in animals fed diets containing a high proportion of forage [11,13]; information on the effects of these bioactive metabolites in ruminants fed high concentrate diet is limited and inconsistent [19,21]. Some saponin extracts improve ruminal fermentation and increase the efficiency of energy use in the animals, which could result in better productive performance [11]. However, the effects of saponins on ruminal fermentation may differ depending on the ruminal pH [22], which varies according to the dietary level of concentrate. Due to the beneficial effects of herbal products and their secondary metabolites, it has been hypothesized that supplementation with HM as a source of saponins, flavonoids and polysaccharides can contribute to improving the productivity of the lambs during the final fattening period, without affecting the quality of the meat or the characteristics of the carcass. The objective of this study was to evaluate the effects of increasing doses of an HM containing saponins, flavonoids, and polysaccharides on the productive performance, carcass characteristics, and meat quality of lambs fed high-concentrate diets.

2. Materials and Methods

2.1. Location

The experiment was conducted at the Teaching and Research Unit of Small Ruminants located at the Experimental Farm of the Universidad Autónoma Chapingo, Mexico, which is located at 19°22′ north latitude and 98°35′ west longitude, with an altitude of 2250 m. The climate is temperate subhumid, with rain during the summer and dry during the winter, with average annual precipitation and temperatures of 665 mm and 15.2 °C, respectively [23]. The study was conducted during the summer, under hot and rainy conditions. The care and handling procedures for the lambs were carried out following the guidelines of the Official Mexican Standard (NOM-062-ZOO-1995).

2.2. Polyherbal Mixture Characteristics

The HM used was Peptasan® (Nuproxa S. de RL. de CV. Querétaro, México), which is a commercial polyherbal formula labelled to contain 150 g kg^{-1} of saponins. In addition, Peptasan® is composed of parts from the *Saccharum officinarum*, *Balanites roxburghi* and *Acacia concinna* plants. *S. officinarum* contains polysaccharides with immunostimulating effects [24]; *B. roxburghii* contains saponins and flavonoids with antioxidant, anti-inflammatory, antimicrobial and antiviral properties [25]; and *A. concinna* contains saponins with immunomodulatory properties [26].

2.3. Diet Composition

HM was fed to the lambs through diets formulated to have weight gains of 300 g d^{-1} [27]. HM (1, 2 or 3 g kg^{-1} of diet DM basis) was premixed with minor ingredients (vitamin and mineral supplement, limestone and salt) before incorporation into complete mixed

diets. The lambs were fed a finishing diet (total mixed ration) comprised 30.3% ground corn, 24.1% ground sorghum, 8.1% soybean meal, 7.1% wheat bran, 7.4% corn gluten, 2.3% bypass fat, 19.4% oat straw, 0.5% vitamin and mineral supplement, 0.5% salt, and 0.3% limestone (DM basis). Oat straw was ground in a hammer mill (Azteca 20, Molinos Azteca, Guadalajara, México) with a 3.8 cm screen before incorporation into total mixed ration. The nutrient composition of the basal diet was 15.53% crude protein, 2.58% ether extract, 13.57% acid detergent fiber, 26.14% neutral detergent fiber, 5.47% ash and 2.8 Mcal of metabolizable energy according to NRC [27] DM basis.

2.4. Animals and Experimental Design

Thirty-six male Dorper × Katahdin lambs (23.27 ± 1.23 kg BW, 4–5 months old) were randomly distributed in four treatments: (1) basal diet without HM (CON); (2) HM1, CON + 1 g of HM kg^{-1} dry matter (DM); (3) HM2, CON + 2 g of HM kg^{-1} DM; and (4) HM3, CON + 3 g of HM kg^{-1} DM. The lambs were placed in individual pens (2.6 m × 0.8 m) equipped with automatic drinkers and individual feeders. Prior to the start of the experimental phase, lambs were vaccinated against *Clostridium* and *Pasteurella* (2.5 mL $lamb^{-1}$, Bobact® 8 MSD-Merck, Kenilworth, NJ, USA), and dewormed through an oral administration of Koptisin ovine® (10 mg kg^{-1} BW, Chinoin, Labs, Mexico City, Mexico). Additionally, 1 mL $lamb^{-1}$ of vitamins containing 500,000 IU of vitamin A, 75,500 IU of vitamin D and 50 mg of vitamin E (Vigantol® Bayer, Mexico City, Mexico) was provided on day 1 of the adaptation period. The lambs had an adaptation period to the basal diet of 14 days, and the experimental phase lasted 56 days. During the adaptation period, the lambs received oat straw as a ruminal pH buffer, and the experimental diets were administered at increasing levels (20, 40, 60, 80 and 100% of the total ration) for 14 days (3 days per level, except for 100%), until the oat straw was reduced to 0%. The feed was provided at 09:00 and 17:00 h, and the drinking water was supplied ad libitum. Individual BW was recorded before the morning feeding on days 1, 14, 28, 42 and 56, of the experimental phase. The amount of diet offered and refused was recorded daily to estimate dry matter intake (DMI, kg d^{-1}). The amount of feed offered was always 10% higher than the previous intake to ensure ad libitum intake. Daily weight gain (DWG, kg d^{-1}) was calculated between feeding period intervals. The feed conversion ratio (FCR) was expressed as feed consumption per unit of body weight gain. Figure 1 shows the experimental procedure.

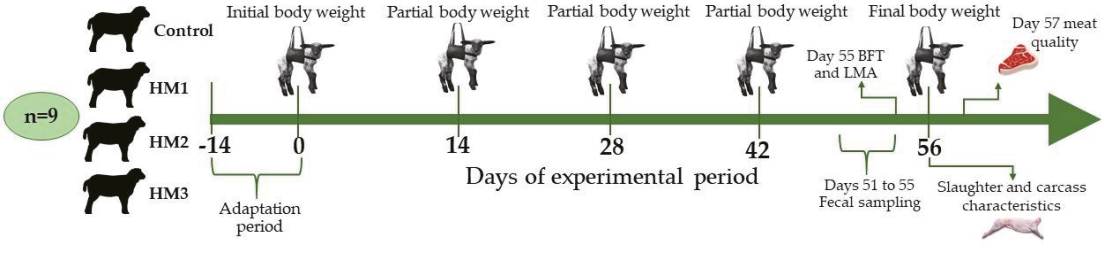

Figure 1. Completely randomized design and sampling times of lambs supplemented with a polyherbal mixture (HM) during the final fattening period; *n* = 9—indicate the number of animals sampled in each treatment; Control—basal diet without HM; HM1—basal diet + 1 g of HM kg^{-1} of dry matter (DM); HM2—basal diet + 2 g of HM kg^{-1} of DM; HM3—basal diet + 3 g of HM kg^{-1} of DM; BFT—backfat thickness; LMA—*longissimus* muscle area.

2.5. Sampling and Analyses of Feeds

Samples of feed provided and rejected were collected daily to determine the chemical composition. Prior to the analysis, the food samples were dried at 55 °C in a forced air oven and then ground in a Wiley mill (model 4, Arthur Thomas Co. Philadelphia, PA, USA). The variables determined were dry matter, crude protein, ether extract and ash [28]. Acid

detergent fibre and neutral detergent fibre was determined using the procedures described by Van Soest et al. [29].

2.6. Apparent Dry Matter Digestibility

Faecal samples were collected from each animal during five consecutive days (in the morning at 08:00 a.m. and in the afternoon at 16:00 p.m. before feed delivery) starting on day 51, directly from the rectum [5]. Feed and orts were collected daily during the same period. Acid-insoluble ash was employed as a marker of internal tract digestibility to analyse the apparent total tract DM digestibility [30].

2.7. Carcass Characteristics

The *longissimus* muscle area (LMA) and the backfat thickness (BFT) located between the 12th and 13th ribs of the lamb were measured on day 55 of the experiment using a Sonovet 600 (Medison, Inc., Cypress, CA, USA) with a 7.5 Mhz transducer [31]. After the last weighing (day 56 of the experiment) the lambs were fasted for 18 h before being slaughtered. All lambs were slaughtered on the same day. The slaughter process was conducted in a commercial slaughterhouse in accordance with standard procedures of the Official Mexican Standard (NOM-033-SAG/ZOO-2014). Lambs were stunned (captive bolt), exsanguinated and skinned. Immediately after the slaughter, the hot carcass weight was registered (HCW). The hot carcass yield (HCY) was determined through HCY = (HCW/FBW) $*$ 100, as it was described by Zimerman et al. [32]. In addition, the skin, head, legs, testicles, rumen (empty), liver, spleen, kidneys, heart, lungs, small intestine (empty), and large intestine (empty) were each weighed separately.

2.8. Meat Quality

After 1 h post-mortem, the right *Longissimus thoracis* (LT) muscle between the 7th and 11th ribs was removed from the carcass with a scalpel and used for pH, colour, Warner-Bratzler shear force (WBSF), chemical composition, drip loss and cooking loss analysis. Samples of *LT* muscle (approximately 600 g) were collected from the carcass and then frozen at −20 °C for a subsequent meat quality analysis.

Prior to the analysis of cooking losses (CL) and Warner-Bratzler shear force (WBSF), the samples were thawed for 24 h at 4 °C in a cooler protected from drafts and the meat samples were analysed in triplicate. CL was determined according by Vazquez-Mendoza et al. [33]; for this purpose, fillets with 2.5 cm thick were roasted on a grill (Toastmaster cool-edge-grill, Macon, MO, USA) until they reached an internal temperature of 70 °C, which was monitored with a thermometer (Brannan & Sons, Cleator Moor, Cumbria, UK). When the temperature reached 70 °C, the fillets were removed from the grill and allowed to cool to room temperature (20–25 °C). To calculate the percentage of CL, each fillet was weighed before and after the procedure (weight of raw meat−weight of cooked meat)/weight of raw meat ×100), as it was described by Vazquez-Mendoza et al. [33]. In order to measure the WBSF, 2.5 cm thick meat fillets (three per lamb) were cooked at 70 °C using the CL method, as sited above. WBSF was measuring using an Instron® universal testing machine (model 1132, Instron, Canton, MA, USA) with a Warner-Bratzler accessory [34]. Meat colour was determined on cuts of the *longissimus dorsi* muscle 24 h after slaughter using a Minolta CM-2006d spectrophotometer (Konica model, Minolta Holdings Inc., Osaka, Japan). Lightness (L*), redness (a*) and yellowness (b*) as meat quality attributes were evaluated using the procedure described by Miltenburg et al. [35]. With the values of a* and b*, the Chroma (C*) and Hue (H*) indices were calculated using the equations: Chroma = $(a^{*}\,2 + b^{*}\,2)^{0.5}$ and Hue = $\tan^{-1}$ (b*/a*) × 57.29 both expressed in degrees [36]. Colour coordinate values were obtained using the average of three measurements of colour for each sample. Meat pH was measured following the procedure described by Negrete et al. [37]. This was measured in triplicate on 3 g of *longissimus dorsi* muscle homogenized in 20 mL of deionized water using a blender Waring 51BL32 (model 700, Torrington, CT, USA), and using a Hanna® pH meter (Model HI 98127, Waterproof Tester, Woonsocket, RI, USA). Drip loss value was calculated

as weight loss of the fresh meat sample (90 g) placed in a plastic bag after storage for 24 h at 4 °C. Drip loss was determined in triplicate as percentage of water released from fresh muscle [38].

Prior to the proximate analysis of meat, the samples were thawed for 24 h at 4 °C. The subcutaneous fat and connective tissue were separated from the muscle using a scalpel, and the meat was ground and homogenized for 5 min with a mixer. Meat samples were analysed in triplicate to determine the moisture, lipid, protein and ash content as a percentage of the muscle sample following AOAC procedures [28].

2.9. Statistical Analysis

All statistical analyses were performed using the SAS statistical program [39]. First, it was performed the normality test on all variables using the UNIVARIATE procedure. BW, DMI, DWG and FCR data were analysed for each period with a completely randomized design with repeated measures over time, using the MIXED procedure. Initially, initial BW was included as a covariate to adjust the variables DWG, DMI and final BW. However, this covariate was removed from the model because it was not significant ($p > 0.05$). Different variance–covariance structures were verified to fit the statistical model, and the compound symmetry structure showed the best fit according to the criteria of the lowest values of BIC and AIC [40]. The full statistical model used was:

$$Y_{ijk} = \mu + T_i + P_j + (T \times P)_{ij} + A_k + e_{ijk} \quad (1)$$

where Y_{ijk} represents the value measured at period j and treatment i for the lamb k, μ represents the overall mean, T_i represents the fixed effect of HM treatments (i = 1, 2, 3, 4), P_j represents the fixed effect of the period within four feeding periods (j = period 1: 1–14, period 2: 15–28, period 3: 29–42 and period 4: 43–56 d), $(T \times P)_{ij}$ represents the fixed effect of interaction between treatment and period, A_k represents the random effect of lambs provided different diets (k = 1, 2, 3, ... 36), and e_{ijk} represents the random residual error.

On the other hand, data on carcass characteristics, animal organs and meat quality were analysed using the GLM procedure. Each lamb was considered an experimental unit. Initially, final BW was included as a covariate to adjust all variables (carcass characteristics, organs and meat quality). However, this covariate was removed from the model because it was not significant ($p > 0.05$). The statistical model used was: $Y_{ijk} = \mu + T_i + e_{ij}$, in which μ is the mean value, T_i is the treatment effect (fixed), and e_{ij} is the error term.

Linear and quadratic orthogonal polynomials were used to evaluate the effects of HM level on all variables evaluated. Means of treatments were compared using the Tukey test, and significant differences were considered when $p \leq 0.05$. In addition, a trend was considered when $p > 0.05$ and ≤ 0.10.

3. Results

3.1. Productive Performance and Digestibility

Final body weight (FBW) was not affected by treatments (Table 1). For dry matter intake (DMI), no significant differences were found among the treatments during the experimental period. On the other hand, DWG showed a tendency of linear decrease ($p = 0.06$), and the lambs that were supplemented with HM3 performed lower than the lambs fed with the other diets. However, the feed conversion ratio was not affected by the level of HM added to the diet. On the other hand, the dry matter digestibility (DMD) decreased linearly ($p = 0.03$) as the dose of HM in the diet increased. The lowest digestibility of DM was observed in lambs fed HM3 diet (Table 1).

Table 1. Productive performance of lambs supplemented with a polyherbal mixture [1] during the final fattening period.

Parameter	Treatment					*p*-Value	
	CON	HM1	HM2	HM3	EEM	Linear	Quadratic
Initial body weight (IBW) kg	23.15	23.45	22.93	23.55	1.233	0.90	0.90
Final body weight (FBW) kg	41.93	39.88	40.13	38.80	1.608	0.21	0.82
Dry matter intake (DMI) kg d^{-1}	1.161	1.083	1.059	1.034	0.056	0.12	0.64
Daily weight gain (DWG) kg d^{-1}	0.335 *	0.293	0.307	0.272 *	0.020	0.06	0.85
Feed conversion ratio (FCR) DMI/DWG	3.49	3.74	3.54	3.91	0.196	0.23	0.76
Dry matter Digestibility (DMD) %	75.71 ^{a}	74.72 ^{ab}	72.31 ^{ab}	70.39 ^{b}	1.528	0.03	0.76

[1] Peptasan® based on *Saccharum officinarum*, *Balanites roxburghi and*, *Acacia concinna*. CON—basal diet without polyherbal mixture (HM); HM1—basal diet + 1 g of HM kg^{-1} of DM; HM2—basal diet + 2 g of HM kg^{-1} of DM; HM3—basal diet + 3 g of HM kg^{-1} of DM; EEM—standard error of the treatment means; ^{a,b}—means within a row with different subscripts differ when $p \leq 0.05$; *—indicates a tendency.

3.2. Carcass Traits

No differences were observed in hot carcass weight, hot carcass yield, backfat thickness, *longissimus dorsi* muscle area, weight of internal organs (empty rumen, small intestine, large intestine, lungs and trachea, heart, liver, kidneys, spleen), nor in the weight of testicles, skin, feet and head by the effect of supplementation with the HM (Table 2).

Table 2. Carcass traits and organ weights of lambs supplemented with a polyherbal mixture [1] during the final fattening period.

Parameter	Treatment					*p*-Value	
	CON	HM1	HM2	HM3	EEM	Linear	Quadratic
Hot carcass weight kg	20.73	19.43	19.38	18.88	0.757	0.11	0.22
Hot carcass yield %	49.47	48.72	48.28	48.95	0.767	0.57	0.54
Backfat thickness mm	3.00	3.11	3.00	3.11	0.114	0.67	0.99
Muscle area *longissimus dorsi* cm^2	11.24	10.90	10.92	10.66	0.312	0.22	0.90
Rumen (empty) kg	1.188	1.152	1.134	1.139	0.047	0.43	0.47
Small intestine (empty) kg	0.882	0.839	0.896	0.913	0.046	0.47	0.34
Large intestine (empty) kg	1.046	1.042	1.024	1.045	0.053	0.93	0.86
Lungs and Trachea kg	0.699	0.686	0.679	0.638	0.040	0.30	0.41
Heart kg	0.198	0.172	0.176	0.192	0.009	0.74	0.92
Liver, kg	0.823	0.842	0.839	0.800	0.034	0.64	0.71
Kidneys kg	0.337	0.352	0.328	0.316	0.019	0.31	0.24
Spleen kg	0.076	0.079	0.083	0.078	0.006	0.70	0.60
Testicles kg	0.690	0.717	0.718	0.634	0.055	0.50	0.62
Skin kg	2.914	2.718	2.834	2.527	0.159	0.15	0.40
Feet kg	0.882	0.824	0.833	0.807	0.041	0.25	0.44
Head kg	1.967	2.025	1.986	1.937	0.072	0.69	0.64

[1] Peptasan® based on *Saccharum officinarum*, *Balanites roxburghi* and, *Acacia concinna*. CON—basal diet without polyherbal mixture (HM); HM1—basal diet + 1 g of HM kg^{-1} of DM; HM2—basal diet + 2 g of HM kg^{-1} of DM; HM3—basal diet + 3 g of HM kg^{-1} of DM; EEM—standard error of the treatment means.

3.3. Meat Quality

Meat pH, cooking loss and drip loss increased linearly ($p < 0.05$) as the dose of HM in the diet increased (Table 3). The WBSF of meat decreased linearly as the level of HM in the diet increased ($p = 0.02$). On the other hand, no significant changes were observed in meat colour variables, with the exception of yellowness (b*), which decreased as dietary HM dose increased ($p = 0.04$). The chemical composition (moisture, protein, fat and ash) of the meat was not affected by the dose of HM in the diet.

Table 3. Meat characteristics of lambs supplemented with a polyherbal mixture [1] during the final fattening period.

Parameter	Treatment					*p*-Value	
	CON	HM1	HM2	HM3	EEM	Linear	Quadratic
Meat pH (24 h)	5.50 ^{ab}	5.36 ^{b}	5.69 ^{a}	5.84 ^{a}	0.14	0.04	0.32
WBSF kg cm^{-2}	6.47 ^{a}	6.29 ^{a}	5.53 ^{ab}	4.73 ^{b}	0.57	0.02	0.58
Cooking loss (%)	16.89	18.72	19.28	20.13	1.09	0.04	0.65
Dripp loss (%)	3.55 ^{b}	4.07 ^{ab}	4.84 ^{a}	4.81 ^{a}	0.38	0.01	0.48
Lightness (*L**)	36.22	36.20	33.45	34.77	1.27	0.22	0.60
Redness (*a**)	9.23	8.45	9.05	9.23	0.44	0.75	0.28
Yellowness (*b**)	10.28 ^{a}	9.11 ^{b}	9.45 ^{ab}	8.73 ^{b}	0.45	0.04	0.62
Chroma	13.87	12.46	13.12	12.74	0.51	0.25	0.33
Hue $^{\circ}$	47.81 ^{a}	47.12 ^{ab}	46.48 ^{ab}	43.40 ^{b}	1.65	0.07	0.47
Moisture, g 100 g^{-1}	73.70	73.69	73.69	73.58	0.48	0.97	0.99
Crude protein, g 100 g^{-1}	20.38	20.47	20.59	20.48	0.38	0.94	0.88
Fat, g 100 g^{-1}	2.45	2.46	2.45	2.49	0.07	0.99	0.98
Ash, g 100 g^{-1}	1.34	1.33	1.33	1.32	0.03	0.82	0.98

[1] Peptasan® based on *Saccharum officinarum, Balanites roxburghi* and, *Acacia concinna*. WBSF—Warner-Bratzler shear force; CON—basal diet without polyherbal mixture (HM); HM1—basal diet + 1 g of HM kg^{-1} of DM; HM2—basal diet + 2 g of HM kg^{-1} of DM; HM3—basal diet + 3 g of HM kg^{-1} of DM; EEM—standard error of the treatment means; ^{a,b}—means within a row with different subscripts differ when $p \leq 0.05$.

4. Discussion

Some plants containing saponins, polysaccharides and flavonoids have shown positive effects on antioxidant capacity and immune status in ruminants [8,41]. In addition, saponins have been reported to improve the energy utilization efficiency and increase the duodenal flux of amino acids and microbial protein [11]. Consequently, lambs supplemented with herbal products containing saponins, polysaccharides, and flavonoids would be expected to have higher growth rates. However, although in our study FBW and DWG were not affected by HM, a linear reduction trend was observed in DWG of lambs fed the HM3 diet, which could be a consequence of the lower dry matter digestibility observed with HM3. This suggests that high doses of HM in the diet could affect the growth rate of lambs when it is used for prolonged periods. Similar results were previously reported by Liu et al. [42] in lambs supplemented with *Medicago sativa* saponin extracts (0, 0.5, 1, 2 and 4 g kg^{-1} DM for 90 days); and by Nasri et al. [43] who examined the effects of increasing doses of *Quillaja saponaria* saponin extracts (0, 30, 60 and 90 mg kg^{-1} DM for 57 days) in lambs fed high concentrate diets. In the latter investigation, BW and DWG was similar among treatments, regardless of the dose of saponins used. In another study, Wang et al. [9] investigated the effects of supplementing lambs with *Astragalus membranaceus* roots (0, 20, 50 and 80 g kg^{-1} DM for 56 days) containing saponins, polysaccharides and flavonoids. In that study, BW was not affected, but DWG was higher in the treatments supplemented with *Astragalus membranaceus*, perhaps as a consequence of the beneficial effects that the saponins, flavonoids and polysaccharides of the plant had on the antioxidant and immune status, and on the serum concentration of growth hormone in the animals.

Some plants containing saponins, polysaccharides and flavonoids increase the relative abundance of fibre-degrading bacteria in the rumen [41]. This could result in higher fibre and feed digestibility and could also increase ruminal passage rate and dry matter intake. However, in our study, DMI was similar among lambs of all treatments during the experimental period. Although HM could increase the rate of passage, saponins are natural surfactant glycosides, which may have a bitter and astringent taste for animals [44]. This can cause low palatability of the diet, which would partially explain the absence of changes observed in DMI. In a similar study, Liu et al. [42] investigated the effects of extracts of *Medicago sativa* saponins (0, 0.5, 1, 2 and 4 g kg^{-1} DM for 90 days) on the productive performance of lambs. In that study, DMI increased linearly as the dose of saponins in the diet increased. This suggests that the lambs are able to adapt to consume saponins, but this adaptation could require long periods of supplementation.

Some plant-extracted saponins have shown promising effects on improving feed utilization efficiency because they can suppress enteric methane emissions through direct effects on ruminal microorganisms [12,14]. In the present study, FCR was similar among treatments, suggesting that HM did not affect feed utilization efficiency. The absence of significant changes in FCR could be explained by the fact that DMI and DWG were also not affected by the treatments. Similar results were previously reported by Mandal et al. [18] in goats supplemented with 5 g d^{-1} of *Acacia concinna* pods for 90 days, and by Liu et al. [42] in lambs supplemented with alfalfa saponin extracts (0.5, 1, 2 and 4 g kg^{-1} DM for 90 days). In their study, they observed that FCR was similar among treatments, even though feed digestibility was higher in lambs supplemented with saponins.

Previous studies have reported that digestion and utilization of nutrients in the diet of ruminants could be improved by dietary supplementation of saponins [12,45], and plants containing saponins, polysaccharides and flavonoids [41]. However, in our study, a negative effect of HM on DM digestibility was observed. Similar results were previously reported by Nasri et al. [43] in lambs supplemented with saponin extracts from *Quillaja saponaria* at dietary concentrations of 30, 60 and 90 mg kg^{-1} DM; and by Nasehi et al. [21] in lambs supplemented with increasing doses (0, 6.1, 8.7 and 11.3 g kg^{-1} DM) of saponins from the green tea plant (*Camellia sinensis)*. Their results showed that saponins reduced the digestibility of DM of the lambs but did not affect their productive performance.

Regarding carcass characteristics, HCW and HCY were not affected by dietary supplementation of HM. No information is available on the effects of HM containing saponins, polysaccharides and flavonoids on sheep or goat carcass characteristics. However, results that are congruent with our findings were previously reported by Nasri et al. [43] on lambs supplemented with increasing doses of saponin extracts from *Quillaja saponaria* (0, 30, 60 and 90 mg kg^{-1} DM for 57 days); and by Abdallah et al. [46] on sheep supplemented with 10 and 15% dried *Astragalus membranaceus* roots containing saponins, flavonoids and polysaccharides. Their results showed that HCW and HCY were not affected by dietary supplementation of saponins, and neither were they affected by the mixture of saponins, polysaccharides and flavonoids from *Astragalus membranaceus*. The limited information on the effects of HM on ruminant carcass characteristics makes it difficult to explain the results observed in this and other studies. However, the similarity of BFT in the carcass of lambs from all treatments may partially explain the absence of changes in HCY in the present study.

BFT and LMA were also not affected by the HM dietary supplementation. The mechanism of action of herbal products and their bioactive compounds on lipogenesis has not been studied in lambs [4]. However, Liang et al. [47] observed that, in beef cattle fed with high-grain rations, supplementation of flavonoid extracts in the diet increased BFT through changes in the differential expression of genes involved in lipid metabolism. In the present study BFT was not affected by the inclusion of HM in the diet, even though it contains parts of the plant *Balanites roxburghii*, which contains flavonoids [25]. This suggests that the effects of flavonoids on BFT are dependent on botanical origin. Given that fat deposition, physical and chemical carcass characteristics of lambs are influenced by breed, sex, age and weight [48,49], the homogeneity of these characteristics in the lambs used in the present study partially explains the absence of changes in LMA and BFT.

Regarding the internal and external organs of lambs, similar results were previously reported by Hundal et al. [19] in goats supplemented with 2% of *Macrotyloma uniflorum* seeds containing saponins; and by Abdallah et al. [46] in sheep supplemented with 10 and 15% of *Astragalus membranaceus* roots containing saponins, flavonoids and polysaccharides. They observed that the weight of the kidneys on sheep supplemented with the highest dose of saponins, flavonoids and polysaccharides from *A. membranaceus* was higher than that on sheep from the other treatments, but there was no effect on the other internal organs. Information on the effects of herbal products or their bioactive compounds on the size and weight of internal organs in ruminants is still limited, which makes it difficult to explain the results observed in this study. However, differences in the internal organs of sheep are

influenced by the breed, sex and age of the animals [50], and by the feeding regime [51]. In the present study all these factors were controlled, which would partially explain the absence of significant changes.

The lowest pH of the meat was observed in the lambs with the HM1 treatment, while in the animals of the other treatments the pH was similar, within the normal range of 5.5 to 5.8 suggested by Sañudo et al. [52]. Abdallah et al. [46] did not observe pH changes in the meat of lambs supplemented with 10 and 15% *Astragalus membranaceus* roots containing saponins, flavonoids and polysaccharides. In another study, Nasri et al. [43] also did not observe pH changes in the meat of lambs supplemented with saponin extracts from *Quillaja saponaria* at concentrations of 30, 60 and 90 mg kg^{-1} DM. However, it was observed that the pH of the meat in lambs of all treatments was below the normal range, similar to what was observed in our study with the HM1 treatment. Therefore, the effects of HM on the pH of the meat observed in the present study could be related to the presence of bioactive compounds. The pH is important for preserving meat during storage. A low pH has a bacteriostatic effect, while a pH above the normal range favours the growth of proteolytic microorganisms [53,54]. This suggests that supplementation of low doses of HM in the diet could promote favourable bacteriostatic effects in lamb meat, and thus increase its shelf life.

Ponnampalam et al. [55] mentioned that an ultimate pH > 5.8 is associated with alterations in drip loss and WBSF. In addition, in sheep meat, Watanabe et al. [56] reported a curvilinear association between ultimate pH and WBSF values, with a toughness peak at pH around 6.0 and improvements in tenderness at pH below and above 6.0. In our study, WBSF decreased as the dose of HM increased; however, this result must be carefully interpreted considering the low number of replicates used and the high coefficient of variation observed (30.74%, data not shown). Similar results were previously reported by Qin et al. [57] in lambs fed pomace (7.8 and 16% for 80 days) obtained from *Hippophae rhamnoides* fruits, which contained 0.69 and 1.02% flavonoids, respectively. In that experiment, WBSF decreased when the flavonoid dose increased. In another study, Abdalla et al. [46] observed no significant changes in WBSF of meat from lambs supplemented with saponins, flavonoids and polysaccharides from *Astragalus membranaceus* roots. WBSF is a well-known method for estimating the meat tenderness [57], consequently, the lower WBSF observed in the present study suggests that dietary supplementation of HM could improve the lamb meat tenderness. Although the exact mechanism is unknown, the changes in WBSF observed in this and other studies suggest that bioactive compounds contained in some plants facilitate the activation of some peptidases such as calpains and cathepsins, which help prevent and delay post-mortem muscle fibre stiffening [58]. It is also possible that these bioactive compounds act by reducing calpastatin activity, allowing a higher rate of myofibril protein degradation [59]. This hypothesis is supported by the observed linear increase in drip loss as WBSF decreased, because drip water losses may increase when calpastatin activity decreases [60]. Furthermore, Webb and Agbeniga [61] reported a linear relationship between WBSF and drip loss, in which higher drip loss was associated with rapid tenderisation and lower WBSF of the meat.

Drip loss is associated with the capacity to retain water in the muscle, with the juiciness and the tenderness of the meat [46,49]. In the present study, the drip loss of meat increased when the dose of HM increased, indicating that high doses of HM could affect the water retention capacity, tenderness and juiciness of meat. Abdallah et al. [46] investigated the effects of dietary supplementation with dried *Astragalus membranaceus* roots containing saponins, flavonoids, and polysaccharides, and observed that meat drip loss decreased in response to *A. membranaceus* supplementation. However, WBSF was similar in the meat of lambs from all treatments. Although the exact mechanism involved is unknown, the higher drip loss observed in the meat analysed in the present study could be related to the observed changes in WBSF, as previously discussed.

Colour is an important attribute of meat quality because it is the first aspect that attracts consumers when choosing fresh meat [62]. A variety of secondary compounds

from plants can improve oxidative stability and prevent discolouration of meat of small ruminants [2]. In the present study, HM did not affect the values of L*, a*, Chroma and Hue °. However, b* decreased in response to supplementation of HM in the diet. This result could be positive because consumers generally do not expect to find high b* in fresh meat [63]. Similarly, previous studies [46,64] reported that supplementation with medicinal plants containing saponins, polysaccharides, and flavonoids also did not affect the colouration of meat from lambs and goats.

There is little information on the use of HM containing saponins, polysaccharides and flavonoids as a colour preservative in ruminant meat. The pigment content of meat can modify its colouration [35]. Likewise, the inclusion of some medicinal plants containing flavonoids increases the hypertrophy of muscle fibres in lambs [57], which could dilute the content of muscle pigments and consequently alter meat colour [65,66]. These findings suggest that the HM used could increase muscle hypertrophy, which would partially explain the observed reduction in b*. On the other hand, Luo et al. [64] reported that dietary supplementation of medicinal plants containing saponins, polysaccharides and flavonoids altered the pigment content on the meat of small ruminants. Similar effects of consumption of these metabolites would partially explain the b* changes in the meat of lambs supplemented with HM in the present study.

In the present study, the chemical composition of lamb meat was similar in all treatments, perhaps as a consequence of the low impact of HM supplementation on the nutritional composition of the diet. In a similar study, Abdallah et al. [46] investigated the effects of *Astragalus membranaceus* roots (0, 10 and 15% for 47 days) containing saponins, polysaccharides and flavonoids on sheep meat quality. In that research, the moisture, protein and ash content of the meat was similar among treatments. However, they observed that fat content decreased in sheep that ate *A. membranaceus* roots. Furthermore, in our study, HM supplementation had little impact on the final BW of the lambs, all being of the same breed and age, which partially explains the absence of significant changes in the chemical composition of the meat [48,49,54].

5. Conclusions

The results of this study indicate that dietary supplementation with HM reduces dry matter digestibility (linear effect). However, the inclusion of up to 3 g HM kg^{-1} DM does not affect productive performance, carcass characteristics, chemical composition, and meat colouration (lightness and redness) of lambs fed high concentrate diets during the final fattening period. Meat yellowness decreases (linear effect) in response to HM supplementation in the diet, which could be positive because consumers, in general, do not expect to find high yellowness in fresh meat. On the other hand, meat pH, cooking loss and drip loss increase linearly as the dose of HM in the diet increases (linear effect). In addition, Warner-Bratzler shear force decreases as the dose of HM increases (linear effect). Thus, Peptasan® HM could be used to improve meat tenderness of lambs fed high concentrate diets. However, this result must be carefully interpreted considering the low number of replicates used. In addition, the increased drip loss in response to HM supplementation could be a risk of microbial spoilage during meat storage. Therefore, it is convenient to carry out meat quality analyses at the muscle level to evaluate the impact of other doses of this HM in rations with different proportion of concentrate for lambs in different experimental periods and physiological stages.

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Effect of *Enterococcus faecium* AL41 (CCM8558) and Its Enterocin M on the Physicochemical Properties and Mineral Content of Rabbit Meat

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Abstract: Improving rabbit meat quality using natural substances has become an area of research activity in rabbit nutrition due to stabilization of husbandry health and economy. The present study evaluates the effect of bacteriocin-producing, beneficial strain *Enterococcus faecium* AL41/CCM8558 and its enterocin M (EntM) on the quality and mineral content of rabbit meat. Seventy-two Hycole rabbits (aged 35 days) were divided into EG1 (CCM8558 strain; 1.0×10^9 CFU/mL; 500 µL/animal/d), EG2 (EntM; 50 µL/animal/d), and control group (CG). The additives were administrated in drinking water for 21 days. Significant increase in meat phosphorus (EG1: $p < 0.05$; EG2: $p < 0.0001$) and iron (EG1, EG2: $p < 0.001$) contents was noted; sodium and zinc levels were only slightly higher in experimental groups compared with control data. The calcium (EG1, EG2: $p < 0.001$), potassium, and copper (EG1: $p < 0.01$) concentrations were reduced. The treatment did not have a negative influence on physicochemical traits of rabbit meat. Based on these results, we conclude that diet supplementation with beneficial strain *E. faecium* CCM8558 and its EntM could enhance the quality and mineral content of rabbit meat, with the focus on its iron and phosphorus contents.

Keywords: enterocin; beneficial strain; meat quality; minerals; rabbit meat

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1. Introduction

In the last decade, the number of well-educated consumers focusing on a healthy lifestyle, diet and food sources is increasing. Rabbit meat is tender white meat, suitable for preparing delicious, nutritious, and mainly, healthy food, and greatly valued for its high protein level (20–21%) with essential amino acids of high digestibility, low fat content with a favorable proportion among saturated, monounsaturated, and polyunsaturated fatty acids, and it is almost free of cholesterol. Rabbit meat also provides a moderate amount of energy and low sodium content, but it is rich in potassium, magnesium, phosphorus, selenium, and B vitamins (as the richest source of vitamin B_{12} [1–3]). Due to these dietary properties, its frequent consumption is highly recommended, e.g., for pregnant women, children, elderly people, and patients with cardiovascular disorders. Moreover, the fortification of the rabbit diet with functional compounds has an increasing tendency and, in this way, rabbit meat can represent a functional food [2]. The majority of studies have been focused on the qualitative parameters and sensory properties of rabbit meat [1,4–6]. Other works present the fatty acid profile, oxidative stability and lipid metabolism of rabbit meat, and their influence using natural additives [7–9]. Although the mineral content of rabbit meat has been investigated by several researchers [2,10–13], in vivo studies about the influence of natural feed additives on rabbit meat minerals are scarce [14,15].

In recent years, many alternatives—probiotics, prebiotics, enzymes, bacteriocins, organic acids, herbs, and their extracts have been tested in rabbits as feed additives to

enhance their productivity and health [2]. It is well known that probiotics—beneficial bacteria can improve gut microbial balance, positively influence metabolism and nutrient digestibility as well as mucosal immunity, and maintain the health, growth, and productivity of animals. The majority of studies suggested a positive effect of dietary natural feed additives' inclusion on meat quality and that these compounds could be useful to improve nutritional properties—minerals, fatty, and amino acids of rabbit meat. While most of the works present the environmental, feeding, genetics, biological factors, and technological (pear-slaughter, transportation, processing) effects on rabbit carcass and meat quality [1], only a few of them have presented the effect of probiotics and/or bacteriocins on the rabbit meat mineral composition [14,15]; information about probiotic influence on meat of other monogastric animals, such as chickens and pigs is also rare [16,17]. Therefore, this study aimed to investigate the impact of feed administration by bacteriocin-producing and beneficial strain *Enterococcus faecium* AL41 (deponed to Czech Culture Collection of Microorganisms in Brno, Czech Republic, CCM8558) and its enterocin M (EntM) on the mineral content and quality of rabbit meat. Moreover, EntM is a new, not commercial bacteriocin, which can help to extend existing knowledge about bacteriocins' application in animal husbandry with a focus on the meat's nutritional quality—this is the novelty of this study.

2. Materials and Methods

2.1. Experiment Schedule, Diet, Slaughtering, and Sampling

The experiment was performed in co-operation with our colleagues at the National Agricultural and Food Centre (NAFC, Nitra, Slovakia). All applicable international, national and/or institutional guidelines for the care and use of animals were followed appropriately, and all experimental procedures were approved by the Slovak State Veterinary and Food Administration and Ethics Committees of both (permission code: SK CH 17016 and SK U 18016).

Seventy-two Hycole rabbits (weaned at age of 35 days, both sexes, equal male to female ratio per treatment) were used in this experiment. Rabbits were divided into 2 experimental groups (EG1, EG2) and 1 control group (CG), with 24 animals in each group. The average live weight of rabbits at the start of the experiment were 830.0 g $\pm$ 165.2 in EG1, 833.0 g $\pm$ 116.9 in EG2, and in CG it was 729.0 g $\pm$ 152.5. The rabbits were kept in standard cages (type D-KV-72, 61 $\times$ 34 $\times$ 33 cm, supplied by Kovobel company, Domažlice, Czech Republic) with two animals per cage. A cycle of 16 h of light and 8 h of dark was used through the experiment. Temperature (20 $\pm$ 4 °C) and humidity (70 $\pm$ 5%) were maintained throughout the experiment by heating and ventilation systems, and data were recorded continuously with a digital thermograph positioned at the same level as the cages. Animals were fed a commercial pelleted diet for growing rabbits (ANPRO-FEED, VKZII Bučany, Slovakia; Table 1) during the whole experiment with access to water ad libitum. Rabbits in group EG1 received bacteriocin-producing *E. faecium* CCM8558 strain possessing probiotic properties (1.0×10^9 CFU/mL) in their drinking water at a dose of 500 µL/animal/day for 21 days. It was marked by rifampicin to differ it from the total enterococci and prepared as described previously by Strompfová et al. [18]. The animals in group EG2 were administered EntM (prepared according to Mareková et al. [19]), a dose of 50 µL/animal/day, with activity 12,800 AU/mL for 21 days. Activity of EntM was tested by the agar spot test according to De Vuyst et al. [20] against the principal indicator strain *E. avium* EA5 (isolate from feces of piglet, our laboratory). The doses of additives and their manner of application were decided based on our previous in vitro studies testing the inhibitory activity of EntM against target bacteria and an experiment with rabbit-derived bacteriocin-producing strain *E. faecium* CCM7420 [21]. Based on our previous experiments, that these additives can be dissolved in distilled water and/or phosphate buffer [19], the additives were applied firstly to 100 mL of drinking water in all cages, and after consuming this volume, the rabbits had access to water ad libitum. Control rabbits (group CG) had the same conditions, but without additives being applied to their drinking water, and they

were fed a commercial diet. Drinking water was provided through nipple drinkers. The experiment lasted for 42 days.

Table 1. Ingredients, chemical composition, and nutritive value of diets.

Ingredients	(%)		(g.kg^{-1})
Extracted clover (grass) meal	27.00	Mineral and vitamin premix [1]	3.00
Extracted sugar beet	10.00	Crude protein	189.38
Oats	13.00	Crude fiber	191.88
Wheat bran	6.00	Fat	39.00
Soybean meal	7.50	Ash	80.00
Sunflower meal	14.00	Organic compounds	921.00
Monocalcium phosphate	0.60	Starch	178.00
Dicalcium carbonate	0.90	Lysin	7.50
Salt	0.30	Methionine + cysteine	6.50
Carob	2.50	Choline chloride	0.80
DL-Methionine + wheat meal	0.10 + 0.10	ME	11.92 MJ

[1] Premix provided per kg diet: vitamin A, 10,000 IU; vitamin D_3, 2000 IU; vitamin E acetate, 30 mg; vitamin B_2, 5 mg; vitamin B_6, 2 mg; vitamin B_{12}, 8 mg; Ca, 9.25 g; P, 6.2 g; Na, 1.6 g; Mg, 1.0 g; k, 10.8 g; Fe, 327.5 mg; Mn, 80 mg; Zn, 0.7 mg.

At days 21 and 42, eight animals from each group were randomly selected for slaughter; they were stunned with electronarcosis (50 Hz, 0.3 A/rabbit/4 s) in an experimental slaughterhouse, immediately hung by the hind legs on the processing line, and quickly bled by cutting the jugular veins and the carotid arteries. After the bleeding, the *Longissimus thoracis* and *lumborum* (LTL) muscles were separated by removing the skin, fat and connective tissue, chilled, and stored 24 h at 4 °C until physicochemical analysis started.

2.2. Physico-Chemical, Mineral, and Statistical Analysis

The ultimate pH was determined 48 h postmortem (p.m.) with a Radelkis OP-109 (Jenway, Essex, UK) with a combined electrode penetrating 3 mm into the LTL. Color measurements were taken on MLD surface of the carcass at 24 h after bleeding. Color characteristics were expressed using the CIE L*a*b system (lightness-L*, 0: black and 100: white), (redness and greenness-a*; yellowness and blueness-b*) using a Lab. Miniscan (HunterLab, Reston, VA, USA). Lightness measurements at room temperature were also taken. Total water, protein and fat contents were estimated using an INFRATEC 1265 spectroscope (FOSS, Tecator AB, Höganäs, Sweden) and expressed in g/100 g. The Near Infrared Transmission (NIT) principle is based on the fact that the measured sample absorbs the Near Infrared light at different wavelengths according to different characteristics such as fat or protein content [22]. From these values, the energy content was calculated [EC (kJ/100 g) = 16.75 × Protein content (g/100 g) + 37.68 × Fat content (g/100 g)]; [23]. Water holding capacity (WHC) was determined by compress method at constant pressure [24]. The analyzed samples (0.3 g in weight) were placed on filter papers (Schleicher and Shuell No. 2040B, Dassel, Germany) with tweezers previously weighed. Together with the papers, samples were sandwiched between Plexiglass plates and then subjected to a pressure of 5 kg for 5 min. The results were calculated from the difference in weight between the slips with aspirating spot and the pure filter paper. The ash content was determined by burning in Muffle furnace at 530 ± 20 °C according to STN 570185.

For macro and micro element analysis, samples were ashed at 550 °C, the ash was dissolved in 10 mL of HCl (1:3), and minerals were determined by AAS iCE 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Phosphorus content was determined by molybdovanadate reagent on Camspec M501 (Spectronic Campes Ltd., Leeds, UK).

Treatment effects on tested parameters were analyzed using one-way analysis of variance (ANOVA) with Tukey post hoc test. All statistical analyses were performed using GraphPad Prism statistical software (GraphPad Prism version 6.0, GraphPad Software, San Diego, CA, USA). Differences between the mean values of the different dietary treatments were considered statistically significant at $p < 0.05$. Data are expressed as means and standard deviations of the mean (SD).

3. Results

Mineral Profile of Rabbit Meat

The effect of dietary supplementation with *E. faecium* CCM8558 and its EntM on mineral profile of rabbit meat is presented in Table 2. Feeding of CCM8558 strain and EntM significantly increased the phosphorus (EG1: $p < 0.05$; EG2: $p < 0.0001$) and iron concentrations (EG1, EG2: $p < 0.001$), while sodium and zinc levels were only slightly higher in experimental groups, compared with control. On the other hand, calcium (EG1, EG2: $p < 0.001$) and copper (EG1: $p < 0.01$) values significantly decreased during additives' application. The lowest calcium and the highest phosphorus level were measured in EG1.

Table 2. Mineral levels (mg/100 g fresh matter/muscle, means ± SD) in *Longissimus thoracis* and *lumborum* of rabbits.

	EG1	EG2	CG	*p*-Value
Day 21 of the Experiment (at 56 Days of Age)				
Calcium (mg/100 g)	6.73 ± 0.01 [a]	12.07 ± 0.54 [b]	17.83 ± 1.50 [c]	<0.0001
Phosphorus (mg/100 g)	259.50 ± 22.06 [a]	215.13 ± 36.94 [b]	193.33 ± 5.20 [b]	0.0012
Magnesium (mg/100 g)	23.30 ± 0.02	23.53 ± 0.03	24.10 ± 1.40	0.2430
Natrium (mg/100 g)	31.10 ± 0.06	30.70 ± 0.06	29.53 ± 4.10	0.5063
Potassium (mg/100 g)	382.87 ± 8.54	372.80 ± 10.47	409.10 ± 39.89	0.0542
Iron (mg/100 g)	0.487 ± 0.013 [a]	0.495 ± 0.067 [a]	0.390 ± 0.040 [b]	0.0019
Manganese (mg/100 g)	0.061 ± 0.022 [a]	0.065 ± 0.003 [b]	0.062 ± 0.058 [b]	0.9800
Zinc (mg/100 g)	1.203 ± 0.415	1.216 ± 0.149	1.158 ± 0.259	0.9385
Copper (mg/100 g)	0.188 ± 0.039 [a]	0.215 ± 0.021	0.251 ± 0.030 [b]	0.0104

[a,b,c] Means with a different superscript in the same row are significantly different ($p < 0.05$). EG1: experimental group 1 (*E. faecium* CCM8558 strain); EG2: experimental group 2 (enterocin M); CG: control group.

Within microminerals, the iron content significantly increased in EG1 and EG2 ($p < 0.01$), while reduced copper level was noted in both experimental groups.

Higher pH values ($p < 0.01$, Table 3) were noted through EntM application (EG2), compared with CG. The color measurements differed in all tested parameters: increase of lightness (EG1: $p < 0.01$, EG2: $p < 0.05$) and yellowness was noted, whereas the redness was decreased. No significant differences were found in protein, lipid, energy, ash, and water contents during additives' administration.

Table 3. Physicochemical properties of *Longissimus thoracis* and *lumborum* of rabbits (means ± SD).

	EG1	EG2	CG	*p*-Value
Day 21 of the Experiment (at 56 Days of Age)				
Average live weight (g)	1575.5 g ± 189.5 [a]	1729.7 g ± 252.5 [b]	1451.8 g ± 202.5 [a]	<0.0001
pH 48 h *postmortem*	5.37 ± 0.02 [a]	5.48 ± 0.09 [b]	5.36 ± 0.02 [a]	0.0034
*L** (lightness)	55.53 ± 3.66 [a]	52.79 ± 1.80 [a]	47.87 ± 3.56 [b]	0.0024
*a** (redness)	0.55 ± 0.24	0.57 ± 0.08	0.62 ± 0.11	0.7398
*b** (yellowness)	8.30 ± 0.69	8.08 ± 0.59	7.37 ± 0.78	0.0820
Water content (g/100 g)	75.90 ± 0.17 [a]	76.27 ± 0.15 [a]	75.87 ± 0.23 [b]	0.0033
Protein content (g/100 g)	21.37 ± 0.21	21.03 ± 0.23	21.40 ± 0.66	0.2707
Fat content (g/100 g)	1.73 ± 0.23	1.70 ± 0.10	1.73 ± 0.49	0.9824
Energy value (kJ/100 g)	426.53 ± 4.21 [a]	416.35 ± 1.98 [b]	423.76 ± 8.65 [a,b]	0.0196
Water holding capacity (g/100 g)	25.99 ± 2.18	30.35 ± 1.46	28.68 ± 5.28	0.1151
Ash (g/100 g)	1.033 ± 0.003	1.033 ± 0.003	1.033 ± 0.003	1.0000
Day 42 of the Experiment (at 77 Days of Age)				
Average live weight (g)	2321.0 g ± 317.8 [a]	2626.4 g ± 192.9 [b]	2174.0 g ± 243.0 [a]	0.0005
pH 48 h post mortem	5.54 ± 0.03 [a]	5.63 ± 0.09 [b]	5.65 ± 0.04 [b]	0.0134
*L** (lightness)	51.78 ± 4.14	50.04 ± 1.71	51.79 ± 1.19	0.4468
*a** (redness)	0.68 ± 0.29 [a,b]	1.22 ± 1.21 [a]	0.08 ± 0.05 [b]	0.0470
*b** (yellowness)	7.34 ± 0.74	7.56 ± 0.61	7.89 ± 0.68	0.3918
Water content (g/100 g)	75.33 ± 0.38	75.70 ± 0.46	75.50 ± 0.44	0.3511
Protein content (g/100 g)	21.93 ± 0.15	21.83 ± 0.38	22.00 ± 0.26	0.5825
Fat content (g/100 g)	1.73 ± 0.23	1.47 ± 0.25	1.50 ± 0.20	0.1301
Energy value (kJ/100 g)	432.69 ± 11.14	420.97 ± 11.49	425.02 ± 11.27	0.2224
Water holding capacity (g/100 g)	31.09 ± 5.16 [a,b]	25.75 ± 2.82 [a]	33.55 ± 5.18 [b]	0.0267
Ash (g/100 g)	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.0000

[a,b] Means with a different superscript in the same row are significantly different ($p < 0.05$). EG1: experimental group 1 (*E. faecium* CCM8558 strain); EG2: experimental group 2 (enterocin M); CG: control group.

4. Discussion

There is a great variety in macrominerals and trace elements content of rabbit meat among different studies. Whereas the potassium content framed within those reported in the literature [1,2,10], phosphorus, sodium and manganese level were below, and calcium was over the range presented by formerly mentioned authors. Moreover, Hermida et al. [10] presented higher iron and manganese, and lower zinc and copper concentrations in rabbit meat, compared with our findings.

The lowest level of calcium in EG1 was similar to the values presented by Dalle Zotte [1]. On the other hand, Nistor et al. [13] reported higher calcium content of rabbit meat (21.4 mg/100 g). Opposite to calcium, the phosphorus level in EG1 (259.50 mg/100 g) was the highest. The indigestible carbohydrates compounds like oligofructose, galactooligosaccharides, and inulin have been found to cause improved mineral retention/absorption by the host organism because of their ability to bind and sequester the minerals, and these carbohydrate-mineral complexes pass unabsorbed through the small intestine onto the colon when the minerals are released from the complex and absorbed. The application of probiotics acts on the nondigestible carbohydrates, causing the short chain fatty acids (SCFAs) rise which can affect an increased absorption of minerals like magnesium and calcium. Moreover, the probiotic enhanced SCFAs can stimulate Vitamin D receptor expression on the eukaryotic cells, which regulate the absorption of calcium from diet, and its metabolism in mammals [25]. The probiotics can also increase the calcium transporters like TRPV6 and calcireticulin Sp100 in the intestine [26]. Phosphorus is needed for the growth of muscle tissue for being involved in the energy metabolism. Its content was higher compared to our previous experiment with autochthonous *E. faecium* CCM7420 rabbit-derived strain [14] as well as to data reported by the other authors [1,2,10] but still lower than it was reported by Nistor et al. [13]. Wang et al. [27] also demonstrated improved phosphorus absorption and utilization in broilers after *E. faecium* CGMCC 2516 microcapsules' application. Rabbit meat contains more potassium than other types of meat and is recommended for hypertension diet. Although the potassium concentrations decreased in both experimental groups compared to CG, the data were comparable to values reported by Dalle Zotte and Szendrő [2].

Increased iron content was noted similarly to our previous results achieved during beneficial E. faecium CCM7420 strain application [14]. Other authors presented higher iron values in rabbit meat: 1.1–1.3 mg/100 g [1] and 0.66–0.99 mg/100 g [11]. It is well known that iron is better absorbed in solution in an acidic environment. In the intestine, the pancreas pours a very alkaline fluid into the upper small intestine and makes the whole contents alkaline; this creates a problem for mineral and trace elements, e.g., iron, zinc absorption. In this case, probiotics could improve the intestinal microbial balance due to lactic acid bacteria, create a beneficial acidic environment in the gastrointestinal tract, and increase the minerals' absorption and their subsequent transferring/inclusion into the meat. The acidic environment can enhance the ionization of minerals that in turn results in passive diffusion [28]. Some studies have been considered showing better iron, copper, and zinc absorption due to improvement of the microbial balance by dietary inclusion of microorganisms—yeasts—in several animals [29–31]. We also hypothesized enhanced enzymatic activity due to improved intestinal microbiota during CCM8558 strain and its EntM application. Another possible way of better mineral absorption could be the enlargement of the absorption surface by proliferation of enterocytes. This fact is also confirmed by improved morphometry parameters, recorded during our previous experiments when beneficial *E. faecium* CCM7420 strain and the EntM were applied to rabbits [32,33]. Copper is an essential trace mineral which performs important biochemical functions, which is usually deficient in the typical human diet. Regarding the copper content, the results vary widely [10,11,34]; our findings agree with those reported by Hermida et al. (0.03–0.21 mg/100 g; [10]). Despite the reduced (but still in the range characteristic for rabbit meat) copper level in both experimental groups compared with

control data, the dietetic value of rabbit meat was not negatively influenced during the beneficial CCM8558 strain and its EntM application.

The majority of reports present higher pH_{24} (24 h postmortem) values than 5.70. Lower pH_{48} (48 h postmortem) values (in the range 5.37–5.65) obtained in this experiment could be explained by the depletion of glycogen reserve in muscles during refrigeration and by longer storage time. Similar pH_{48} values were detected also during the beneficial *E. faecium* CCM7420 strain (5.34–5.65) and phyto-additives' (5.61–5.71) application in rabbits [14,35]. Lower values of lipids were determined, but the protein content of rabbit meat was not influenced using the CCM8558 strain and its EntM. Redness is influenced with the degree of iron oxidation in the heme pigment in myoglobin. At high pH levels, oxymyoglobin is rapidly turned into dark red color reduced myoglobin, showing a positive relationship between these parameters [36]; our findings also confirm this relationship. Higher values of yellowness could be related to free radicals, produced by lipid oxidation during storage and/or manipulation, which can oxidize heme pigments, causing discoloration of meat and meat products [37]. Lower lipid value than the average [1] was determined by us, similarly to Lauková et al. [38] after gallidermin application in rabbits, but contradictory to results achieved during probiotic BioPlus 2B® preparation or phyto-additives' supplementation in rabbits [35,39]. Positive correlation between lipids and energy was found in rabbits of EG1 group, mainly at day 42 (after probiotic strain cessation). The protein content of rabbit meat was not influenced using CCM8558 strain and its EntM, similarly, to results noted through gallidermin administration in rabbits [38]. Improved energy values were previously described also during phyto-additives' application in rabbits [35].

5. Conclusions

This study contributes to updating data in the literature concerning the effect of bacteriocin-producing and beneficial strain *E. faecium* CCM8558, and its enterocin M on the quality and mineral content of rabbit meat. Higher phosphorus and iron levels were recorded during both CCM8558 strain and EntM administration in rabbits. During EntM application, improved pH48, lightness, and water content was noted. In general, physicochemical parameters and nutritional value of rabbit meat were not negatively influenced during bioactive compounds' administration. This study has impact for basic research primarily due to spreading knowledge regarding the rabbit meat's mineral composition and secondarily due to the opportunity to compare the beneficial effect of different natural additives—probiotics and enterocins in rabbits under in vivo conditions. Nevertheless, further investigations are needed to assess the efficacy of CCM8558 and EntM in rabbit husbandries.

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Data Availability Statement: Data are available upon reasonable request to the corresponding author.

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Bioavailability of Dietary Zinc Sources and Their Effect on Mineral and Antioxidant Status in Lambs

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Abstract: This study investigated the relative bioavailability (RBV) of zinc from different sources used as feed additives in ruminant nutrition based on Zn concentration and the activity of Zn-dependent enzymes in lamb tissues. Thirty-two male lambs of Improved Valachian breed (three months old) were randomly assigned to four dietary treatments. For 120 days, the lambs were fed either the total mix ration (TMR) providing 29.6 mg Zn/kg or the TMR supplemented with either zinc sulphate ($ZnSO_4$), zinc chelate of glycine hydrate (ZnGly), or zinc chelate of protein hydrolysate (ZnProt). The supplemented diets contained a total of 80 mg Zn/kg. Supplementation with $ZnSO_4$ increased Zn concentration in the liver, while the highest Zn uptake was in the kidneys of lambs fed the ZnProt diet. The ZnGly supplemented diet elevated the activity of the Cu/Zn-dependent enzyme superoxide dismutase (Cu/Zn SOD) in the liver. Regardless of Zn source, Zn supplementation resulted in increased total antioxidant status (TAS) in the pancreas. The estimated RBV of Zn based on linear regression slope ratios did not differ among the Zn sources. Our results indicate similar availability of Zn from organic dietary sources as from commonly used zinc sulphate; however, their effects on mineral and antioxidant status may differ slightly in growing lambs.

Keywords: feed additives; trace minerals; bioavailability; lambs; antioxidant enzymes

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1. Introduction

Definitions for mineral bioavailability vary because of the complexity of the issue. From a nutritional point of view, the bioavailability of minerals can be defined as the proportion of ingested trace elements utilized for their specific physiological and biochemical functions at the site of action [1]. Mineral bioavailability includes gastrointestinal digestion, absorption, metabolism, tissue distribution, and bioactivity, but it depends on many factors such as feed composition, co-ingested compounds, mineral dosage and chemical form, and the mineral status of animals [2]. Moreover, due to the substantial impacts of homeostatic mechanisms on the plasma and tissue concentrations of minerals, it is difficult to correctly estimate mineral bioavailability based on the above definition.

Zinc as an essential integral part of many important enzyme systems is involved in keeping the health and performance of ruminants at their maximum [3]. Dietary zinc deficiency could lead to impaired growth, reproduction, and immune dysfunction with increased susceptibility to infections in growing animals [3–5]. Although Zn requirements for growing lambs and calves are recommended at 33 mg Zn/kg of dry matter (DM) in a complete diet, young ruminants require additional Zn to support accretion of body protein during periods of rapid growth, and improvement in growth performance, health and reproduction, mineral and antioxidant status, and immunity in ruminants has been reported after Zn supplementation [3,6–9]. In addition, Zn supplementation with Zn nanoparticles can offer an effective approach to maintaining the high production and health of ruminants due to the improvement in animal reproductive efficiency, immunomodulatory properties, and enhancing the microbial biomass production, while it can also help to reduce methane emission in livestock [10].

The methods of assessment of Zn bioavailability include Zn absorption and retention, plasma or tissue Zn concentrations, animal growth, the biologically active form of Zn, and prevention of signs of Zn deficiency [11]. Intake of Zn from organic sources has been found to increase nutrient digestibility and Zn absorption and retention in lambs compared with inorganic Zn supplementation [7,12,13], but the results of other studies focusing on zinc bioavailability in ruminants are contradictory [14–18]. Interaction of Zn with other metal ions in the diet is a dietary factor that can modify Zn bioavailability. Complex interactions among minerals within the digestive tract can affect the absorption of zinc as well as the feed's effectiveness in promoting the health and productivity of ruminants, but organic Zn chelates might permit Zn and other minerals in the diet to circumvent factors that inhibit absorption of their inorganic forms [17,19]. Therefore, in this study, we decided to estimate the relative bioavailability of different zinc sources used as feed additives in ruminant nutrition based on Zn concentrations as well as the activity of Zn-dependent enzymes in lamb tissues and to investigate the effect of organic Zn sources on the mineral and antioxidant status in lambs.

2. Materials and Methods

All experimental protocols involving animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and Animal Research: Reporting In Vivo Experiments (ARRIVE guidelines). All methods and procedures reported herein were carried out in line with European Union Directive 2010/63/EU for animal experiments, and the experimental protocol was approved by the Ethical Committee of the Institute of Animal Physiology of the Slovak Academy of Sciences and by the State Veterinary and Food Office (Ro-4160/13-221).

2.1. Animals, Diets, and Experimental Design

Thirty-two castrated male lambs (Improved Valachian breed) aged three months were selected from the flock of a commercial farm (Olšavica–Brutovce, Slovakia) with an initial mean body weight 19.7 ± 3.2 kg. The lambs were housed at the Slovak Academy of Sciences Institute of Animal Physiology Research Centre. They were allocated to one of four dietary treatments (n = 8) in a completely randomized design and fed an unsupplemented total mix ration (TMR) consisting of grass hay and feed concentrate (600 and 300 g/day, respectively) in the adaptation period lasting four weeks. During the experimental period, lasting 120 days, at four months of age (mean BW 24.7 ± 2.97 kg), the lambs were fed the TMR consisting of grass hay and feed concentrate (800 and 350 g/day, respectively) providing a total of 29.6 mg Zn/kg for the control lambs (C, control). The concentrates for the experimental dietary treatments were supplemented either with zinc sulphate as an inorganic Zn source ($ZnSO_4$; $ZnSO_4 \cdot H_2O$, Sigma–Aldrich, St. Louis, MO, USA), or organic sources of Zn, zinc chelate of glycine hydrate (ZnGly; Glycinoplex-Zn 26%, Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) and zinc chelate of protein hydrolysate (ZnProt; Bioplex®-Zn 15%, Alltech Inc., Nicholasville, KY, USA). Commercially produced Zn organic chelates (ZnGly, ZnProt) are more stable complexes produced by reacting Zn with amino acid hydrate or hydrolyzed protein protecting Zn in the gut from the adverse effects of other feed compounds and minerals and make it more absorbable and available to lambs. The TMRs for all dietary treatments were formulated to meet National Research Council (NRC, 2007) requirements [20], and all supplemented diets (TMRs for the supplemented dietary treatments) were prepared so as to contain a total of 80 mg Zn/kg of a complete diet (as-fed basis). The TMR composition is shown in Table 1.

Table 1. Ingredients and chemical composition of the total mix ration (TMR) [1] on a dry matter (DM) basis.

Ingredient	g/kg of DM
Grass hay	709
Barley ground	165
Wheat bran	81
Soybean meal	41
Limestone	4
Chemical composition (analyzed)	
Dry matter (g/kg)	891
Crude protein	113
Acid detergent fiber	230
Neutral detergent fiber	357
Ash	66
Mn, mg/kg of DM	34
Zn, mg/kg of DM	33
Cu, mg/kg of DM	8.5

[1] Common TMR fed to all lambs during the trial. The control received only the TMR, other dietary treatments received the TMR consisting of grass hay and concentrate supplemented with different Zn sources.

During the adaptation period, the lambs were housed in floor pens with bedding (n = 2 lambs/pen, 1.64 × 1.25 m) equipped with an automatic water supplier and plastic feeders. At the beginning of the experimental feeding period, each lamb was housed individually in a pen and fed the TMR consisting of grass hay and concentrate (800 and 350 g/day, respectively). The feed concentrate contained ground barley, wheat bran, and soybean meal as the main ingredients. The addition of the Zn sources to ground concentrate was analytically confirmed (n = 8). The analyzed total Zn concentrations in the TMRs of the supplemented experimental treatments were 78.11 mg Zn/kg for $ZnSO_4$, 79.79 mg Zn/kg for the ZnGly, and 77.49 mg Zn/kg for ZnProt treatments. Throughout the experiment, the animals were fed twice a day and had free access to fresh potable water. Once a week each lamb was offered a trace mineral lick without Zn composed of Ca 16.2, Na 316, Mg 32, Cu 0.5, Co 0.06, I 0.02, and Se 0.01 (g/kg). Feed intake from the daily ration was evaluated separately for each lamb, and the body weights were recorded monthly.

2.2. Sample Collection and Analysis

The total amount of feed refused by each lamb was collected and recorded daily at 8:00 a.m. All refused feed samples were dried and ground to pass through a 1 mm screen and stored for subsequent analysis.

Jugular blood samples were collected into acid-washed heparinized tubes from each lamb once a month during the experimental period. Blood samples were centrifuged at 1180× g for 10 min at 4 °C, and plasma was removed for mineral analysis. Fresh blood samples were immediately used for the determination of superoxide dismutase activity in erythrocytes. At the end of the experiment, six lambs from each treatment group were slaughtered, and tissue samples were collected. Tissue samples were collected from the left lobe of the liver, the left kidney cortex, apex of the heart and the pancreas as well as from the skeletal muscles *m. longissimus dorsi* and *m. psoas major*. Sampling was conducted from identical locations of each relevant tissue using a ceramic knife. All tissues and plasma samples were stored in acid-washed plastic vials or tubes at −80 °C until further analysis.

Dry matter from feed and tissue samples was determined according to the Association of Official Analytical Chemists (AOAC) method [21] by drying samples to a constant weight at 105 °C. After drying the samples of diet ingredients at 60 °C for 48 h, the samples were analyzed for neutral detergent fiber (NDF) and acid detergent fiber (ADF) using the method proposed by Van Soest et al. [22]. NDF was assayed without heat-stable amylase and expressed inclusive of residual ash. ADF was also expressed inclusive of residual ash. ADF and NDF concentrations were determined using the ANKOM2000 Fibre Analyzer

(Ankom Technology, Macedon, NY, USA). Standard methods [21] were used to analyze ash (No. 942.05), nitrogen (No. 968.06), and crude protein (No. 990.03) using the Kjeldahl method after acid digestion.

2.3. Mineral Analysis

Trace mineral concentrations in the feed components, plasma, and tissue samples were analyzed using a double-beam atomic absorption spectrophotometer (AA-7000 Series, Shimadzu Co., Kyoto, Japan) with a graphite furnace (GFA-7000, Shimadzu Co., Kyoto, Japan). The microwave-assisted digestion method using closed pressure vessels (MWS 4 Speedwave, Berghof Co., Eningen, Germany) was used for decomposition of all samples in two replicates. All samples, except plasma, were wet acid-digested with a mixture of concentrated nitric acid and hydrogen peroxide. Mineral concentration of Mn in muscles was determined using a graphite furnace atomic absorption spectrophotometer with deuterium background correction and pyrolytic-coated graphite tubes [23]. The certificate reference materials of bovine liver BCR-185, bovine muscle ERM-BB184, and pig kidney ERM-BB186 from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and ClinCheck control of lyophilized human blood (Recipe, Munich, Germany) were included in each analysis to verify instrument accuracy. Mineral concentrations in all samples except plasma were expressed as mg/kg of DM and in plasma as mg/L.

2.4. Enzyme Analysis

All antioxidant assays of plasma and animal tissues are described in more detail in the study by Čobanova [14]. The activity of superoxide dismutase (SOD, EC 1.15.1.1) in the erythrocytes was measured using the RANSOD kit (Randox, Crumlin, UK). The activity of SOD was expressed in U/g Hb for the specific activity. Total SOD and Cu/Zn superoxide dismutase (Cu/Zn SOD) activity in the liver, pancreas, and kidney cortex of each lamb was measured by means of spectrophotometric assay according to the procedure proposed by Marklund and Marklund [24], whereby one unit of enzyme activity was defined as the amount of enzyme required to inhibit pyrogallol autoxidation by 50%.

The activity of glutathione peroxidase (GPx, EC 1.11.1.9) in blood and hemoglobin (Hb) content were analyzed using commercial kits from Randox, Crumlin, UK, and GPx activity in lamb tissues was assayed by measuring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) according to Paglia and Valentine [25]. The assays were carried out at 25 °C, and hydrogen peroxide was used as the substrate. The enzyme activities of SOD and GPx are expressed as units of enzyme per milligram and per gram of tissue protein, respectively.

The total antioxidant status (TAS) of the blood plasma and lamb tissues was determined by means of the ferric-reducing antioxidant power (FRAP) assay using the modified method of Benzie and Strain [26]. TAS is expressed as μmol Fe^{2+} per gram of tissue protein or mmol Fe^{2+} per liter of plasma.

Lipid oxidation in the serum and tissues was estimated using the modified fluorometric TBARS method according to Jo and Ahn [27]. Lipid peroxidation was expressed as μmol of malondialdehyde (MDA) formed per L of plasma or nmol MDA per gram of tissue protein. Total protein in the tissue homogenates was assayed using the Bradford method [28].

The total concentration of thiol groups (TSH) was found spectrophotometrically based on the reaction of 5,5′-dithio-bis (2-nitrobenzoic acid) with protein thiol groups [29]. The concentrations of the TSH groups are expressed in mmol per liter of plasma or μmol per g of tissue.

Metallothionein 1 (MT1) plasma concentration was determined with the colorimetric method using commercial ELISA kits (Sheep Zn-MT ELISA kit, NeoScientific, London, UK) following the manufacturer's instructions. The optical density of the samples was measured using a microplate reader (Apollo 11 LB913, Berthold Technologies GmHB & Co., KG, Bad Wildbad, Germany).

2.5. Statistical Analysis

All statistical analyses were performed using the GraphPad Prism software (GraphPad Prism version 9.0, GraphPad Software, San Diego, CA, USA), and the differences in plasma mineral concentrations between the dietary treatments after 30, 60, and 90 days of Zn supplementation were evaluated using two-way repeated measures ANOVA with the post hoc Tukey's multiple comparisons test (Supplementary Materials, Table S1). Other parameters were analyzed using one-way ANOVA with the post hoc Tukey's multiple comparison test using dietary treatment as the source of variation and lamb as the experimental unit. Differences between the mean values of the different dietary treatments were considered statistically significant at $p < 0.05$. The values in the tables are the means and the pooled standard errors of the mean (SEM).

Relative bioavailability was estimated based on a single dietary Zn concentration from Zn sources, using zinc sulphate as the standard source, by means of slope ratio comparisons from linear regression [30]. Regressions were calculated based on supplemental Zn level as the independent variable, and differences between Zn sources were determined based on the differences in their respective regression coefficients.

3. Results

3.1. Growth Performance

During the whole experimental period, no form of feed supplementation with Zn from any source influenced either feed intake or body weight/gain compared to unsupplemented control lambs (Table 2).

Table 2. Growth performance of lambs for the 120 day feeding period differing in Zn source (80 mg Zn/kg). ADFI: average daily feed intake; ADG: average daily gain.

Parameters	Dietary Treatments [1]				SEM	*p*-Value [2]
	C	$ZnSO_4$	ZnGly	ZnProt		
Initial body weight, kg	24.89	24.76	24.39	24.78	0.526	0.9981
Final body weight, kg	31.46	30.96	31.29	31.11	0.521	0.9892
ADFI, g/d	1059	1059	1081	1059	12.49	0.9102
ADG, g/d	54.82	51.64	57.60	53.74	2.484	0.8700
Feed/gain	20.32	20.83	20.47	20.97	0.831	0.9931

[1] C: basal diet, $ZnSO_4$: zinc sulphate, ZnGly: zinc chelate of glycine hydrate, and ZnProt: zinc chelate of protein hydrolysate. [2] Values ($n = 8$/treatment) are presented as the least squares means.

3.2. Mineral Status

The 120 day period of feed supplementation with organic Zn proteinate (ZnProt) increased the plasma Zn concentration compared to the control treatment ($p = 0.028$, Table 3), while the MT concentration in plasma was reduced during the ZnProt treatment ($p < 0.05$). Plasma Cu concentration increased with $ZnSO_4$ treatment compared to the control and ZnProt treatments ($p < 0.01$) and, therefore, the highest Zn/Cu ratio was observed in the plasma of lambs fed the diet enriched with ZnProt ($p < 0.01$). Plasma concentrations of zinc did not differ between the dietary treatments after 30, 60, and 90 days of Zn supplementation; however, the effects of time and treatment were detected (Supplementary Materials, Table S1). Concentrations of Cu and Fe in plasma were not affected by dietary treatments in all collection periods (i.e., 30, 60, and 90 days of Zn supplementation), but the time effect was detected in both minerals (Table S1).

Feed supplementation with Zn sulphate increased Zn concentration in liver tissue ($p < 0.05$) compared to the control treatment (Table 4). The diet with ZnProt significantly increased Zn concentrations in the kidney cortex compared to other treatments ($p < 0.01$). Zn concentrations in other tissues (muscles, spleen, pancreas, heart, rib bone) were not affected by the dietary treatment.

Table 3. Activity of antioxidant enzymes (i.e., SOD and GPx) in blood, plasma mineral concentration, and other plasma parameters of lambs fed diets (80 mg Zn/kg) differing in Zn source after the 120 day feeding period.

Parameters	Dietary Treatments [1]				SEM	*p*-Value [2]
	C	$ZnSO_4$	ZnGly	ZnProt		
SOD, U/g Hb	2221 [ab]	2620 [a]	1561 [ab]	1244 [b]	329	0.0402
GPx, U/g Hb	224 [ab]	281 [a]	263 [ab]	175 [b]	14.5	0.0391
Zn, mg/L	0.917 [a]	0.947 [ab]	0.994 [ab]	1.15 [b]	0.029	0.0359
Cu, mg/L	0.535 [a]	0.773 [b]	0.703 [ab]	0.517 [a]	0.032	0.0019
Zn/Cu ratio	1.09 [ab]	0.79 [b]	0.97 [bc]	1.28 [c]	0.05	0.0024
Fe, mg/L	2.45	2.53	2.35	2.65	0.06	0.4136
MT, µg/L	2.28 [a]	2.20 [ab]	2.06 [ab]	1.95 [b]	0.03	0.0392
ALB, g/L	34.3	33.2	34.9	35.1	0.33	0.1474
TSH, mmol/L	0.389	0.412	0.393	0.447	0.01	0.1919
TAS, µmol/L	0.396	0.353	0.362	0.375	0.01	0.0948
MDA, µmol/L	0.160	0.146	0.175	0.163	0.01	0.4099

SOD: superoxide dismutase, GPx: glutathione peroxidase, MT: metallothionein, ALB: albumin, TSH: total thiol groups, TAS: total antioxidant status, and MDA: malondialdehyde. [1] C: basal diet, $ZnSO_4$: zinc sulphate, ZnGly: zinc chelate of glycine hydrate, and ZnProt: zinc chelate of protein hydrolysate. [2] Values (n = 6/treatment) are presented as the least squares means. Means within lines with different superscript letters are significantly different ($p < 0.05$).

Table 4. Tissue Zn concentrations in lambs fed diets differing in Zn source (80 mg Mn/kg) for the 120 day feeding period.

Tissue, mg/kg of DM	Dietary Treatments [1]				SEM	*p*-Value [2]
	C	$ZnSO_4$	ZnGly	ZnProt		
Liver	118.4 [a]	134.7 [b]	130.3 [ab]	126.4 [ab]	1.99	0.0354
Kidney	109.9 [a]	110.6 [a]	107.8 [a]	120.2 [b]	1.41	0.0038
Muscle						
Longissimus dorsi	109.8	108.8	103.8	107.7	2.04	0.7508
Psoas major	101.3	105.9	106.6	109.3	2.36	0.6981
Spleen	97.64	97.83	101.3	101.6	1.12	0.5772
Pancreas	68.9	66.9	68.5	70.1	0.62	0.5322
Heart	68.11	66.57	68.96	66.86	0.76	0.7805
Rib bone, mg/kg ash	140.9	132.1	131.3	146.2	2.44	0.1764

[1] C: basal diet, $ZnSO_4$: zinc sulphate, ZnGly: zinc chelate of glycine hydrate, and ZnProt: zinc chelate of protein hydrolysate. [2] Values (n = 6/treatment) are presented as the least squares means. Means within lines with different superscript letters are significantly different ($p < 0.05$).

The tissue concentrations of Mn, Fe, and Cu in liver, kidney, muscles, spleen, pancreas, heart and rib bone are presented in Table 5. Increased Cu concentrations in the pancreas and decreased Mn concentrations in the heart muscle were observed in the lambs fed diets supplemented with both organic Zn sources ($p < 0.01$ and $p < 0.05$, respectively). The ZnProt-enriched diet decreased liver Cu concentrations compared to the control lambs ($p < 0.05$). Cu concentrations increased in the kidney cortex of lambs given $ZnSO_4$ and ZnGly treatments compared to the control animals ($p < 0.001$). Feed supplementation with ZnGly decreased Fe concentrations in kidney and muscles (*m. psoas major*) compared to ZnProt treatment ($p < 0.05$) and decreased muscle Mn (*m. longissimus dorsi*) compared with $ZnSO_4$ but increased Mn concentrations in the pancreas in comparison with the control and ZnProt treatments. Intake of the diets supplemented with Zn from both organic sources decreased Fe concentrations in the heart compared to the control and $ZnSO_4$ treatment ($p < 0.01$).

Table 5. Tissue concentrations of Cu, Fe, and Mn in lambs fed diets differing in Zn source (80 mg Mn/kg) for the 120 day feeding period.

Tissue, mg/kg of DM	Dietary Treatments [1]				SEM	*p*-Value [2]
	C	$ZnSO_4$	ZnGly	ZnProt		
Copper						
Liver	302.2 [a]	240.0 [ab]	252.7 [ab]	221.1 [b]	10.4	0.0353
Kidney	20.13 [a]	21.32 [b]	20.52 [ab]	21.40 [b]	0.17	0.0011
Pancreas	4.384 [a]	4.701 [a]	5.232 [b]	5.378 [b]	0.12	0.0081
Heart	14.56	14.01	13.81	12.47	0.29	0.1384
Spleen	4.233	4.142	4.207	4.147	0.12	0.9953
Muscle						
Longissimus dorsi	2.853	2.611	2.899	2.605	0.21	0.9493
Psoas major	1.278	1.187	1.260	1.281	0.07	0.9705
Iron						
Liver	230.5	188.0	232.8	230.0	13.4	0.6152
Kidney	204.2 [a]	199.1 [ab]	172.5 [b]	207.8 [a]	5.00	0.0164
Pancreas	73.47	74.62	75.00	87.15	2.15	0.2443
Heart	150.4 [a]	151.7 [a]	135.2 [b]	135.2 [b]	2.13	0.0042
Spleen	1364	975.4	1037	1531	114.6	0.4184
Muscle						
Longissimus dorsi	63.09	63.12	56.06	69.54	2.13	0.2997
Psoas major	56.07 [ab]	60.31 [ab]	51.84 [a]	62.02 [b]	1.23	0.0300
Manganese						
Liver	8.733	9.124	9.579	9.990	0.29	0.5105
Kidney	5.087	5.496	5.320	5.077	0.11	0.4678
Pancreas	7.134 [a]	8.021 [ab]	8.440 [b]	8.121 [a]	0.16	0.0126
Heart	1.340 [ab]	1.408 [a]	1.300 [b]	1.133 [b]	0.04	0.0623
Spleen	1.214	1.195	1.439	1.393	0.04	0.1432
Muscle						
Longissimus dorsi	0.321 [ab]	0.348 [a]	0.237 [b]	0.327 [ab]	0.02	0.0450
Psoas major	0.334	0.368	0.275	0.278	0.02	0.1004

[1] C: basal diet, $ZnSO_4$: zinc sulphate, ZnGly: zinc chelate of glycine hydrate, and ZnProt: zinc chelate of protein hydrolysate. [2] Values (n = 6/treatment) are presented as the least squares means. Means within lines with different superscript letters are significantly different ($p < 0.05$).

3.3. Antioxidant Status

Activity of both antioxidant enzymes SOD and GPx decreased in the erythrocytes of lambs on the diet supplemented with ZnProt compared to $ZnSO_4$ treatment only (both $p < 0.05$). Other parameters of plasma antioxidant status (ALB, TAS, TSH, and MDA) were not affected by dietary treatment (Table 3).

The highest activity of total SOD as well as of Cu/Zn SOD was observed in liver tissue of lambs fed the ZnGly-enriched diet compared to all other lambs ($p < 0.0001$, Table 6). SOD activity in other tissues was not affected by the dietary treatment. Regardless of the source, Zn supplementation increased total antioxidant status (TAS) in the pancreas of all treated lambs ($p < 0.0001$). Other antioxidant parameters, such as GPx activity or concentrations of MDA, TSH, and NPSH in liver, kidney and pancreas tissues, were not affected by Zn feed supplementation.

Table 6. Antioxidant parameters in tissues of lambs fed diets (80 mg Zn/kg) differing in Zn source [1].

Enzyme Activity	Dietary Treatments [1]				SEM	*p*-Value [2]
	C	$ZnSO_4$	ZnGly	ZnProt		
Liver						
SOD, U/mg protein	90.8 [a]	123 [b]	161 [c]	108 [ab]	6.60	0.0001
Cu/Zn SOD, U/mg protein	78.7 [a]	107 [ab]	130 [b]	95.4 [a]	5.45	0.0021
GPx, U/g protein	19.6	21.6	19.7	19.6	0.55	0.5282
MDA, nmol/g protein	157	165.5	142.1	161.8	6.53	0.6374
TAS, µmol/g protein	38.7	37.37	37.58	39.96	1.20	0.8805
TSH, µmol/g tissue	15.5	16.0	15.3	15.3	0.24	0.8028
NPSH, µmol/g tissue	5.20	4.75	4.50	4.73	0.10	0.1481
Kidney cortex						
SOD, U/mg protein	55.9	47.4	50.0	46.9	2.17	0.4627
Cu/Zn SOD, U/mg protein	44.8	35.6	37.8	37.8	2.03	0.4218
GPx, U/g protein	21.5	21.9	21.5	21.3	0.62	0.9907
MDA, nmol/g protein	74.2	75.2	69.6	80.4	1.95	0.3056
TAS, µmol/g protein	21.5	21.4	21.5	21.9	0.51	0.9896
TSH, µmol/g tissue	8.31	8.54	8.31	9.32	0.16	0.1426
NPSH, µmol/g tissue	2.44	2.70	2.43	2.71	0.05	0.1242
Pancreas						
SOD, U/mg protein	5.38	5.29	5.78	4.20	0.36	0.4847
Cu/Zn SOD, U/mg protein	5.12	4.93	5.51	4.04	0.35	0.5142
GPx, U/g protein	17.9	14.7	19.8	17.3	1.10	0.4590
MDA, nmol/g protein	69.6	72.9	69.6	71.7	3.16	0.9801
TAS, µmol/g protein	3.31 [a]	6.68 [b]	9.08 [b]	7.40 [b]	0.55	0.0001
TSH, µmol/g tissue	8.70	9.05	10.11	8.93	0.26	0.3787
NPSH, µmol/g tissue	1.92	1.73	1.98	1.71	0.06	0.3463

[1] C: basal diet, $ZnSO_4$: zinc sulphate, ZnGly: zinc chelate of glycine hydrate, and ZnPro: zinc chelate of protein hydrolysate. [2] Values ($n = 6$/treatment) are presented as the least squares means. Means within lines with different superscript letters are significantly different ($p < 0.05$).

3.4. Bioavailability

Relative bioavailability (RVB) of Zn sources was estimated based on the linear regression slope ratios of Zn concentrations and the activity of the Zn-dependent enzyme Cu/Zn SOD in lamb tissues regressed on the basis of Zn supplemental levels (Table 7). $ZnSO_4$ used as the Zn standard was assigned a value of 100%. The slopes and estimated RBV values based on Zn concentrations in plasma, liver, and kidney as well as liver activity of Cu/Zn SOD did not differ among the Zn feed additives ($p > 0.05$). The best parameter for the assessment of RVB was Cu/Zn SOD activity in the liver ($R^2 = 0.44$), and based on this parameter, RBV tended to be higher in lambs fed ZnGly ($p < 0.085$).

Table 7. Estimated relative bioavailability (RBV) of Zn sources based on linear regression slope ratios of Zn concentrations in tissues and liver activity of Cu/Zn SOD on Zn supplementation level (mg).

Dependent Variable	Zinc Source	Regression Coefficient		RBV, %	*p*-Value
		Slope	SE		
Plasma Zn, mg/L [a]	Zn sulphate	0.000405	0.000576	100	0.6154
	Zn glycinate	0.001357	0.000839	335	
	Zn proteinate	0.001029	0.000641	254	
Liver Zn, mg/kg DM [b]	Zn sulphate	0.2335	0.0841	100	0.7136
	Zn glycinate	0.1903	0.1028	81.5	
	Zn proteinate	0.1309	0.0679	56.1	
Kidney Zn, mg/kg DM [c]	Zn sulphate	0.1220	0.0726	100	0.8926
	Zn glycinate	0.1046	0.0711	85.7	
	Zn proteinate	0.1538	0.0776	126	
Liver Cu/Zn SOD, U/mg protein [d]	Zn sulphate	0.4089	0.1450	100	0.085
	Zn glycinate	0.7338	0.1840	179	
	Zn proteinate	0.2378	0.1272	58.2	

[a] Intercept = 0.57, $R^2 = 0.16$, and SD = 0.086; [b] Intercept = 118, $R^2 = 0.33$, and SD = 11.9; [c] Intercept = 106, $R^2 = 0.23$, and SD = 9.72; [d] Intercept = 78.7, $R^2 = 0.44$, and SD = 24.6.

4. Discussion

Twelve-week feed supplementation with 80 mg of Zn/kg did not affect plasma Zn concentrations measured once a month compared to control. However, after the 120 day feeding period with differing Zn dietary sources, the plasma Zn concentration increased in lambs fed the diet supplemented with ZnProt. In many studies, long-term supplementation of the basal diet with a low Zn concentration range (10–30 mg of Zn/kg) as well as Zn dietary source affected neither plasma nor tissue Zn concentrations in ruminants [6,12,31–33]. We decided to supplement the TMR with close to the proposed total maximum contents of Zn for ruminants in the EU [34] to contain a total of 80 mg Zn/kg of complete diet from different Zn sources. Zn plasma levels reflected dietary Zn concentrations, when sheep were fed with Zn-marginal or -deficient diets containing only 14–22 mg Zn/kg DM [17,35,36]. Our results indicate that the unsupplemented TMR containing 30 mg of Zn/kg was sufficient to meet the physiological requirements for growing lambs. Because the offered TMR was not Zn-deficient and plasma Zn concentrations were maintained within a relatively narrow value span because of efficient homeostatic regulation [37], differences in plasma Zn levels between the dietary treatments were not significant. Although we found a time effect on Zn plasma concentrations, a long period of Zn supplementation caused changes in Zn absorption and excretion to maintain homeostasis; therefore, no differences in Zn bioavailability were detected.

Although Zn plasma seems to not be a reliable marker for assessment of Zn bioavailability [3,12], and we found positive correlation between Zn concentrations in plasma and pancreas, liver, and kidney. Zn concentrations in liver, kidney, pancreas, and bone are considered as suitable markers for assessment of Zn bioavailability in ruminants [11,31,38]. However, increased Zn concentrations were observed mainly in these tissues in ruminants fed diets supplemented with high Zn concentrations (300–1400 mg Zn/kg), with the greatest Zn deposition in plasma and/or tissues in animals fed diets enriched with organic Zn sources [33,39,40]. Differences between Zn feed additives have not been found in tissue mineral deposition using low or normal Zn supplementation levels [6,31–33,41]. Despite the fact that Zn concentrations in tissues are controlled by homeostatic changes in Zn absorption and/or endogenous excretion [4,37], and we supplemented our lambs' diet with up to 80 mg of Zn/kg, increased Zn concentrations were found in the kidneys of lambs fed the ZnProt diet compared to other supplemented or unsupplemented lambs, and Zn levels were elevated in the liver of lambs receiving $ZnSO_4$ treatment compared to controls but with no differences between Zn sources. Calves receiving $ZnSO_4$ at 20 mg of Zn/kg for 56 or 98 days had higher Zn content in their liver than animals fed ZnProt, but after increasing supplemental Zn levels to 500 mg Zn/kg, greater absorption and retention of Zn from ZnProt were observed, resulting in increased Zn deposition in liver, kidney, and plasma [31]. Our results suggest that feed supplementation with Zn from different sources at 80 mg of Zn/kg of diet resulted in Zn being absorbed and utilized at similar rates, and higher Zn levels in the kidneys of lambs fed the ZnProt diet may indicate some differences in the post-absorptive metabolism of Zn from an organic source [9,16,31].

Zinc as an activator of the enzyme SOD as well as other antioxidant proteins and molecules contributes to the proper functioning of the antioxidant defense system [5,42,43]. Regardless of Zn source, Zn supplementation improves the antioxidant status of ruminants due to the increased antioxidant activity of SOD and glutathione peroxidase (GPx) [7,14,44], while in our study, the total antioxidant status improved in the pancreas and the SOD activity in the liver of all supplemented lambs. Intake of supplemented diets with zinc sulphate and Zn glycinate significantly increased liver activity of total SOD, and the highest Cu/Zn SOD activity was determined in the liver of lambs given the ZnGly diet only. It seems that ZnGly could increase the antioxidant activity of Cu/Zn SOD in the liver, which is supported by the hypothesis of higher Zn availability from ZnGly maintaining the normal enzyme activity in the body; however, ZnGly treatment affected Cu/Zn SOD and/or ALP activity in the liver and serum of monogastric animals [45–47]. Zn supplementation from organic Zn sources increased Cu/Zn SOD activity as well as Zn concentrations in the liver

of ruminants compared to $ZnSO_4$ [31,33,48,49]. Surprisingly, we found remarkably reduced activity in both antioxidant enzymes, SOD and GPx, in the liver and/or blood as well as plasma metallothionein concentration in lambs supplemented with ZnProt; however, the lambs receiving ZnProt treatment had the highest Zn concentration in kidney and plasma after the 120 d feeding period. This could indicate that Zn from ZnProt was not associated with SOD enzyme synthesis and may be metabolized differently from other Zn sources [31].

The most appropriate criteria for estimating the relative bioavailability (RVB) of zinc in mature ruminants is Zn concentration in the liver, kidney, and pancreas or liver metallothionein concentration [3,31,39,50], when the RVB of zinc sources was expressed relative to zinc sulphate as a standard source. We decided to use Zn concentrations in liver, kidney, and pancreas tissue to estimate the RVB of Zn sources; however, the tissue Zn deposition in particular did not differ significantly from the other supplemented treatments. Since the bioavailability of minerals is defined as the proportion of ingested element from feed which is utilized for specific physiological and biochemical functions at the site of action [1,51], we also used the liver activity of Cu/Zn SOD to estimate RVB, as that was where the significant differences between the individual treatments were found. Our results show that the RBV of Zn did not differ among the Zn sources, because there were no significant differences between their linear regression slopes. The best parameter for bioavailability assessment appears to be the activity of Cu/Zn SOD in the liver, as the highest coefficient of determination for this parameter was found there. This is in accord with other studies reporting no differences in Zn utilization and RVB among Zn sources in ruminants [33,39,41,52]. Potentially better bioavailability of organic Zn chelates or complexes in ruminants has been reported in a few studies based on improved gut absorption, tissue retention of Zn, and/or higher activity of Cu/Zn SOD in liver [17,48]. Cao et al. [39] reported that Zn proteinate was a more available source of Zn for lambs than $ZnSO_4$ and other organic Zn sources due to the higher Zn uptake by kidney and pancreas tissues. Although we also recorded higher Zn concentrations in kidney and plasma of lambs fed the ZnProt diet and liver activity of SOD in ZnGly treatment, we assumed similar relative bioavailability of all Zn sources used based on our linear regression slope ratio comparisons.

Potentially greater absorption of Zn from organic sources, slower release of Zn from Zn chelates or complexes and differing post-absorptive metabolism may affect mineral tissue deposition and mineral status in ruminants. Zn, Cu, Mn, and Fe are chemically similar, so interaction of these elements appears to be tissue-specific due to the competition among the minerals sharing the same transporter systems at the level of membrane transport [53,54]. It has been suggested that Zn supplementation stimulates the production of metallothioneins (MTs) in the intestine and other tissues [55]. In our study Zn supplementation affected mineral status in lambs due to the different mineral tissue deposition. Intake of the diets supplemented with Zn from both organic sources resulted in increased Cu deposition in the pancreas and reduced Mn uptake by the heart muscle. ZnProt supplementation reduced plasma and hepatic Cu concentrations, which could indicate sequestration of dietary Cu in intestinal MT induced by feeding with the ZnProt diet. Low intracellular available Cu bound to MT could affect Fe and Mn transport through the Cu-dependent protein hephaestin and a common transporter, ferroportin [56,57]. Unfortunately, we did not measure MT concentrations in the intestine mucosa, so our results cannot clearly indicate binding of Cu to MT. Further investigation is needed to elucidate whether Zn intake from ZnProt can induce a high metallothionein level in the intestinal mucosa and post-absorptive metabolism of ZnProt as well.

5. Conclusions

We can conclude that organic Zn sources, zinc chelate of glycine hydrate, and zinc chelate of protein hydrolysate, given adequate levels of dietary Zn, were absorbed and utilized in a similar way as inorganic Zn feed additive zinc sulphate in growing lambs,

and Zn supplementation increased antioxidant status in the pancreas regardless of Zn source. Relative bioavailability of zinc did not differ among the Zn feed additives; however, the effect of Zn from organic sources on mineral deposition and antioxidant status may differ slightly in lambs. Further research is needed in order to elucidate the differences in post-absorptive metabolism of Zn from organic sources.

Similarities in bioavailability of Zn sources indicate that the lambs' Zn requirements were met (33 mg Zn/kg of DM), regardless of Zn supplemental source. However, feed supplementation with Zn from the inorganic and organic sources at 80 mg of Zn/kg of complete feedstuffs might improve the antioxidant status of growing ruminants without any effect on the growth performance.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11111093/s1, Table S1. Trace mineral concentrations in plasma after 30, 60 and 90 days of Zn supplementation (80 mg Zn/kg).

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The Effect of Dried Grape Pomace Feeding on Nutrients Digestibility and Serum Biochemical Profile of Wethers

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Abstract: The aim of this study was to find the effect of dried grape pomace (GP) feeding on the nutrients digestibility coefficients and biochemical parameters of sheep blood serum. The experiment was divided into three feeding periods—C (control), GP1 (1% grape pomace concentration), and GP2 (2% grape pomace concentration). Wethers in three groups in balance cages were housed for right feces collection. The C feed diet consisted of hay, ground wheat, soybean meal, mineral and vitamin lick. An experimental diet with 1% and 2% addition of GP from the daily dry matter intake was fed. After that, digestibility coefficients (in %) were calculated by the difference between nutrient intake and excretion. Furthermore, in the wethers' blood, biochemical parameters (mineral, energetic, nitrogen, and enzymatic profile) were analyzed. After the GP2 feeding, statistically significant higher digestibility of CP (crude protein), NFC (nonfiber carbohydrates), NDF (neutral detergent fiber), and OM (organic matter) was found. However, the addition of dried GP increased significantly the content of Cl^- and decreased the value of glucose, nevertheless, their concentrations were within the reference interval. Parameters of the wethers' blood serum nitrogen and enzymatic profile were not affected by GP feeding. Dried grape pomace in an amount of 2% diet dry matter can be considered a suitable source of nutrients in sheep feeding, which in addition should improve the digestibility of diet crude protein.

Keywords: biochemical parameters; grape by-products; nutrition; sheep; utilization

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1. Introduction

The wine industry produces annually millions of tons of grape by-products, which are valuable resources of biologically active substances that have many potential uses, also in animal nutrition [1]. Grape pomace (GP) is a by-product from the wine industry and represents about 15–20% of the weight of the grape bunch [2]. The GP is a suitable feed additive for animal nutrition [3–8]. The product can be fed fresh, dried, or ensiled [9]. The nutritive value of grape pomace is variable depending on the grape-growing region, cultivar, technology of winemaking, and the proportion of seeds and pulp [10–12]. The GP is a source of health benefits: flavonoids with antioxidant and anti-inflammatory activity [13–15] that can improve rumen fermentation [16] and delay gas production [17]. Digestibility of crude protein, organic matter, and NDF (neutral detergent fiber) was increased in sheep receiving GP [18,19]. Many studies have focused on the biochemical profile of small ruminant's

blood with impact on the effect of breed, age, gender, location, and season [20–24]. The effect of different dosages of GP on biochemical parameters of ruminants' blood in different experiments was realized in dairy cows [25], in calves [26], or in sheep [27]. Our previous studies have analyzed the effects of various natural substances obtained as by-products of agricultural production on animal nutrient digestibility, health status, or reproductive efficiency [28–34]. These studies indicate the great potential of these products for use in animal nutrition, however, the GP addition in animal feeding has to be further examined. The hypothesis is that GP addition to the ruminants' daily diet will increase the nutrients digestibility without the negative effect on the animals' health. Based on the above, the aim of this study was to describe the effect of dried GP feeding on the nutrients digestibility coefficients and blood serum biochemical parameters of wethers.

2. Materials and Methods

2.1. The Materials Animals and Housing

Experiments were conducted at the Experimental Center of Livestock at the Department of Animal Husbandry (Slovak University of Agriculture in Nitra). The wethers were of Ile de France breed, obtained from the University farm in Kolinany (Slovak University of Agriculture in Nitra) with an average weight of 34.05 ± 1.97 kg and age of 4 months. The study consisted of 3 groups: control—C, 1% grape pomace—GP1, and 2% grape pomace—GP2 (Table 1). During the preparatory time period, wethers were free housed in group without bedding in pens. Then, the wethers were housed in balance cages individually to monitor proper individual daily diet intake and feces collection in the balance period. The experiment complied with animal health care standards. The animals were under veterinary control and cared for by experienced animal caretakers during the whole experiment. The routine manipulation with animals during the experiment did not cause disproportionate and excessive stress. The conditions of animal care, manipulations, and use corresponded with the instructions of the Ethics Committee of the Slovak University of Agriculture in Nitra, Protocol No. 48/2013.

Table 1. Experiment scheme.

Control		**Grape Pomace Addition**	
C (*n* = 8)		**GP1 (*n* = 8) GP2 (*n* = 8)**	
Preparatory period 14 days	Balance period 5 days	Preparatory period 7 days	Balance period 5 days

C—control group, GP 1—grape pomace 1% from daily dry matter intake, GP 2—grape pomace 2% from daily dry matter intake.

2.2. Feeding and Experimental Design

The composition of experimental and control daily diets are listed in Table 2. Grape pomace of the Pinot Gris variety (*Vitis vinifera* L.) was obtained from the academic vinery (Slovak University of Agriculture in Nitra). The nutrient content of feed components is shown in Table 3. During the whole experiment, animals were fed two times per day. Half of the daily diet was fed during the morning and another 50% was fed during the afternoon. Water, mineral and vitamin lick was accessible ad libitum. The concentration of biologically active substances (total polyphenols: 27.38 ± 1.38 mg GAE/g—equivalent of gallic acid) was determined in a previous study [35]. The control (C) daily diet from meadow hay, ground wheat, soybean meal, and mineral and vitamin lick was formed. The preparatory period before C diet feeding was 14 days (Table 1). Following this, the experimental balance period lasted 5 days. Daily diet GP1 and GP2 consisted of meadow hay, ground wheat, soybean meal, mineral and vitamin lick, and dried GP (1 and 2% of daily dry matter intake, respectively). The preparatory period before experimental variant GP1 and GP2 lasted 7 days and the balance period 5 days. The difference between the experimental variants was only in the concentrations of dried GP in the diet.

Table 2. Feed rations used in the digestibility experiment.

Feeds (g)	Feeding Groups		
	C	GP1	GP2
Meadow hay	700.0	700.0	700.0
Ground wheat	118.6	118.6	118.6
Soybean meal	238.6	238.6	238.6
Grape pomace (dried)	-	10.3 *	20.6 **
Mineral and vitamin lick	ad libitum	ad libitum	ad libitum

* 1% from daily dry matter intake, ** 2% from daily dry matter intake, mineral and vitamin lick (Jan Valasek, Ludrova, Slovakia) content was as follows: MnO (as Mn) 3100 mg, ZnO (as Zn) 4800 mg, $Ca(IO_3)_2$ (as I) 125 mg, Se 31 mg, $CoSO_4.7H_2O$ (as Co) 42 mg, vit. A 300,000 i.u., vit. D3 125,000 i.u., vit. E 100 mg, ash 95%, Ca 9.9%, P 5.0%, Na 13.7%, Mg 5.1% in 1 kg of dry matter.

Table 3. Chemical composition of feed components.

	Meadow Hay	Wheat	Soybean Meal	Grape Pomace
DM *	873.85	909.75	898.95	942.25
CP	69.12	125.86	484.85	98.70
EE	10.41	17.29	15.52	84.19
CF	388.29	31.55	52.04	183.98
ADF	459.15	43.56	103.9	380.87
NDF	697.17	116.77	117.03	459.67
NFE	478.45	805.79	377.89	593.42
NFC	169.56	720.57	312.89	317.72
OM	946.26	980.49	930.28	960.28
Ash	53.74	19.51	69.72	39.72
Ca	4.58	0.40	3.39	4.46
P	2.28	4.29	7.70	3.21
Mg	1.52	1.45	3.65	1.20
Na	0.30	0.20	0.30	0.26
K	12.82	5.07	24.86	12.89

DM: dry matter, CP: crude protein, EE: ether extract, CF: crude fiber, ADF: acid detergent fiber, NDF: neutral detergent fiber, NFE: nitrogen free extract, NFC: nonfiber carbohydrates, OM: organic matter, * in g/kg of original matter, other nutrients in g/kg of dry matter.

2.3. Blood Sampling and Analyses

Blood samples were collected from *vena jugularis externa* on the morning of the last day of the nutrition balance experiment in each variant. Sampling and analysis of blood were realized. For biochemical analysis of blood serum blood samples were centrifuged at 1006× *g* for 30 min. Potassium (K), sodium (Na), and chloride (Cl) ions were analyzed by an EasyLite analyzer (Medica, Bedford, MA, USA) with an ion-selective electrode [36,37]. Blood serum concentrations of calcium (Ca), magnesium (Mg), phosphorus (P), triglycerides (TG), cholesterol (CHOL), glucose (GLU), total protein (TP), urea, albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), were determined using DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) kits on the Randox RX Monza analyzer (Randox Laboratories, Crumlin, UK) [37,38]. Globulin (GLB) was calculated mathematically by subtracting the serum levels of albumins from serum total proteins [32].

2.4. Feed and Feces Collection, Analysis and Determination of Digestibility

During the balance period once daily in the morning the rests and samples of feeds, daily diets, and feces were collected. The content of organic and inorganic nutrients was analyzed in the rests and samples of feeds and in pooled samples of feces for each animal for 5 days. Dry matter content (DM) was analyzed by gravimetric method at 103 °C, crude protein (CP) by Kjeldahl method, ether extract (EE) by gravimetric method according to the Soxhlet principle, crude fiber (CF) by gravimetric method as a residue insoluble in

acid and alkaline media after deduction of ash (Fibertec System, Tecator), acid detergent fiber (ADF) by gravimetric method as a residue after hydrolysis in acid detergent solution (Fibertec System, Tecator), neutral detergent fiber (NDF) by gravimetric method as a residue after hydrolysis in neutral detergent solution (Fibertec System, Tecator) and ash (A) by gravimetric method at 550 °C (muffle furnace) were determined. The content of organic matter (OM), nitrogen free extract (NFE), and nonfiber carbohydrates (NFC) were calculated according to formulas:

$$\text{OM} = \text{DM} - \text{A (g/kg)} \quad (1)$$

$$\text{NFE} = \text{DM} - (\text{CP} + \text{EE} + \text{CF} + \text{A}) \text{ (g/kg)} \quad (2)$$

$$\text{NFC} = \text{DM} - (\text{CP} + \text{EE} + \text{NDF} + \text{A}) \text{ (g/kg)} \quad (3)$$

The content of Ca, Mg, Na, K was determined by High Resolution Continuum Source Atomic Absorption Spectrometer contrAA 700 (ANALYTIC JENA, Jena, Germany) and content of P by 6400 Spectrophotometer (JENWAY, Montreal, QC, Canada). In vivo apparent digestibility coefficients of CP, EE, CF, NFE, NFC, OM, ADF, and NDF in the diets (in %) were calculated as:

$$\text{In vivo digestibility coefficient} = [(\text{nutrient intake} - \text{nutrient excreted})/\text{nutrient intake}] \times 100 \text{ (\%)} \quad (4)$$

2.5. Statistical Analysis

Statistical evaluation of results by IBM SPSS v26.0 was realized. For calculation of basic statistical characteristics (mean and standard deviation), determination of the significance of differences and comparison of the results between the control and experimental diets within the variables (Tukey Test). One-way ANOVA was performed at the level $p < 0.05$.

3. Results and Discussion

3.1. Nutrient Digestibility

The apparent digestibility of crude protein was affected by dried grape pomace addition (Table 4). In the control group (C) a significantly lower digestibility coefficient of crude protein ($p < 0.05$), compared to the GP1 and GP2 was observed. This result corresponds with findings that were reported by some authors [18,19]. According to Guerra-Rivas et al. [11] the diet fed to the sheep (control vs. grape pomace) had minor effects on ruminal degradation parameters of crude protein. Ishida et al. [39] found lower digestibility of crude protein of grape pomace in comparison to the control diet (65.69 vs. 75.14%). It can be assumed that this was due to a higher proportion of grape pomace from dry matter intake (24% from dry matter intake of wethers). This trend was also confirmed by Abarghuei et al. [40] and Jayanegara et al. [41]. Differences in the digestibility of other nutrients between the control and experimental group GP1 were not significant. However, the apparent digestibility of nonfiber carbohydrates (NFC), organic matter (OM), and neutral detergent fiber (NDF) of the diets significantly ($p < 0.05$) increased by higher dose of dried grape pomace (C vs. GP2; NFC $p = 0.018$; OM $p = 0.022$; NDF $p = 0.015$). This trend of increasing the digestibility of organic matter and NDF, with an increase in their intake, was also confirmed by Bahrami et al. [42] and Foiklang et al. [16]. On the contrary, Baumgartel et al. [43] observed decreasing nutrient digestibility between basal and test diet including grape pomace. After the addition of GP to the ruminants' diets, higher OM digestibility was found [44].

Table 4. Digestibility coefficients (%) of the different feeding groups.

	Feeding Groups		
	C	**GP1**	**GP2**
CP	70.22 ^{a} ± 1.19	72.17 ^{b} ± 1.46	73.49 ^{b} ± 0.98
EE	58.10 ± 3.45	63.03 ± 0.58	60.76 ± 2.69
CF	47.25 ± 4.04	50.25 ± 1.94	51.30 ± 1.14
NFE	67.31 ± 2.30	66.91 ± 1.44	69.85 ± 0.51
NFC	77.52 ^{a} ± 1.07	77.78 ^{a} ± 1.58	79.93 ^{b} ± 0.16
OM	62.32 ^{a} ± 2.83	62.93 ^{a} ± 0.53	65.09 ^{b} ± 0.62
ADF	49.49 ± 3.04	50.36 ± 0.24	51.37 ± 1.01
NDF	49.91 ^{a} ± 3.83	51.40 ^{a} ± 0.53	53.97 ^{b} ± 1.19

C: control, GP1: 1% addition of dried grape pomace from daily dry matter intake (DMI), GP2: 2% addition of dried grape pomace from daily DMI, CP: crude protein, EE: ether extract, CF: crude fiber, NFE: nitrogen free extract, NFC: nonfiber carbohydrates, OM: organic matter, ADF: acid detergent fiber, NDF: neutral detergent fiber. Different letters in row indicate statistical differences (Tukey test, $p < 0.05$); data are presented as mean ± SD.

3.2. Mineral Profile

The changes in feeding are manifested in blood serum mineral profile [45]. Minerals perform a number of important physiological functions, such as the effect on acid-base balance, osmotic pressure, adrenal function, normal heart function, but also the metabolism of proteins or carbohydrates [46–48]. The difference in the P content after the GP was not statistically significant (Table 5). However, average P concentrations were higher than the upper limits in comparison as previously reported [48–51]. On the other hand, Jelinek et al. [52] found in rams similar blood serum P content from 2.49 to 2.92 mmol/L (depending on age). Identically, Chedea et al. [25] did not describe a statistically significant effect of dried GP (15% concentrations) in dairy cows on blood serum P content. The Ca content was similar, after feeding of all examined diets and in the interval according to Merck [51] (2.88–3.20 mmol/L). Ca concentrations were also comparable with data reported by Dias et al. [20] and Kovacik et al. [37], but higher in comparison with Schweinzer et al. [53]. Similarly, Chedea et al. [25] reported an effect of dried GP on Ca content in dairy cows (diet contained 15% dried GP). Iannaccone et al. [26] also reported in Fresian calves (10% proportion of dried GP meal in concentrate) a significant effect on the content of Ca. A similar ratio of Ca:P 1.07:1 (C, GP1) and 1.13:1 (GP2) was found which is in consent with previously reported data [48]. Concentrations of Mg in experimental groups were higher than upper limits 1.10 mmol/L found by Tschuor et al. [50] and 1.31 mmol/L Merck [51]. Simpraga et al. [21] determined the content of Mg 1.30–1.60 mmol/L, which was similar to GP2. The GP addition did not affect the content of Mg, which was also confirmed by Chedea et al. [25]. The Na^{+} content was after the addition of GP lower in comparison with control variant but its content was in the interval 130.00–155.00 mmol/L reported by Vrzgula et al. [48]. However, the analyzed Na^{+} values were lower than determined by Kovacik et al. [37]. The intake of GP decreased non-significantly the K^{+} content. According to Merck [51], the reference range for K^{+} is 3.90–5.40 mmol/L. The values found in our experiment were in the range reported by Tschour et al. [50] (4.60–6.50 mmol/L). The ratio of Na and K 23.81:1 (C), 24.04:1 (GP1), 26.53:1 (GP2) was found, thus similar compared to the recommendation of Vrzgula et al. [48]. The 2% GP intake increased the concentrations of Cl^{-} ($p < 0.05$), which we do not consider a negative effect, because the main problem for chlorides is mainly a decrease, which can cause digestive disorders [48]. However, in all groups, the Cl^{-} concentrations in blood serum were in physiological range according to Vrzgula et al. [48] and Tschour et al. [50], but higher compared to Merck [51]. Kovacik et al. [37] found higher concentrations of Cl^{-} compared in their study. The main factor that can influence the reduced mineral absorption in this type of dietary supplement is increased fiber intake [54], which is not confirmed by animals' in vivo studies, similar to our study.

Table 5. Biochemical wether blood parameters.

		Feeding Groups		
Parameters	**Unit**	**C**	**GP1**	**GP2**
P	mmol/L	2.89 ± 0.18	2.87 ± 0.16	2.75 ± 0.50
Ca	mmol/L	3.09 ± 1.12	3.08 ± 0.42	3.10 ± 0.77
Mg	mmol/L	1.69 ± 0.92	1.92 ± 0.96	1.32 ± 0.44
Na	mmol/L	143.08 ± 2.96	135.13 ± 8.18	140.63 ± 1.96
K	mmol/L	6.01 ± 1.16	5.62 ± 0.33	5.30 ± 0.06
Cl^-	mmol/L	105.28 ^{a} ± 1.68	106.60 ^{a} ± 0.91	108.40 ^{b} ± 1.47
GLU	mmol/L	3.90 ^{a} ± 0.30	3.17 ^{b} ± 1.05	3.26 ^{b} ± 0.35
CHOL	mmol/L	1.01 ± 0.00	1.01 ± 0.00	1.01 ± 0.00
TG	mmol/L	0.45 ± 0.06	0.53 ± 0.08	0.43 ± 0.07
TP	g/L	74.45 ± 8.18	77.25 ± 6.01	66.25 ± 15.35
ALB	g/L	33.87 ± 3.43	23.34 ± 10.15	29.41 ± 6.39
GLB	g/L	40.83 ± 9.44	53.91 ± 12.97	46.50 ± 10.64
UREA	mmol/L	6.36 ± 1.19	6.52 ± 0.86	5.63 ± 0.75
AST	µkat/L	2.02 ± 0.79	1.26 ± 0.69	1.57 ± 0.28
ALT	µkat/L	0.34 ± 0.14	0.40 ± 0.08	0.41 ± 0.04
ALP	µkat/L	3.49 ± 1.51	4.34 ± 1.24	5.16 ± 1.37
GGT	µkat/L	0.14 ± 0.08	0.20 ± 0.09	0.17 ± 0.06

C: control, GP1: 1% addition of dried grape pomace from daily dry matter intake (DMI), GP2: 2% addition of dried grape pomace from daily DMI, GLU: glucose, CHOL: cholesterol, TG: triglycerides, TP: total protein, ALB: albumins, GLB: globulins, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, GGT: gamma glutamyl transferase, different letters in row indicate statistical differences (Tukey test, $p < 0.05$); data are presented as mean ± SD.

3.3. Energetic Profile

The glucose values (Table 5) were in physiological range 2.30–4.44 mmol/L [48,50,51]. However, glucose value decreased after the addition of GP (1% GP by 18.72%; 2% GP by 16.41%), but statistically significant ($p < 0.05$) only in GP2, that was also confirmed by Iannaccone et al. [26]. The concentrations of 1% GP also non-significantly decreased glucose concentration in an experiment of Chedea et al. [25] and Kollathova et al. [8]. The decrease in glucose is probably related to the low energy value of GP [10]. Decreased glucose content is also associated with liver damage [48,55], which in our case can be refuted based on the results of liver enzymes. On the other hand, Alba et al. [27] determined statistically higher blood glucose after the addition of grape residue flour (2% from concentrate) in lactating dairy sheep compared to a recent study. The cholesterol concentrations in the wethers' blood serum were in all groups very similar. Bahrami and Chekani-Azar [42] and Alba et al. [27] found no statistically significant differences in cholesterol concentrations after GP feeding. Slightly lower cholesterol values in blood serum compared to physiological range ([49]: 1.05 mmol/L) were found. In addition to antioxidant activity, polyphenols have been shown to have several cardioprotective and atheroprotective effects, including lowering plasma cholesterol levels [26]. The concentration of triacylglycerides (TG) in GP2 was the highest but statistically non-significant. Similar results were also reported by Chedea et al. [25], where GP feeding has not affected the values of triacylgylcerides. On the other hand, Alba et al. [27] after feeding grape pomace confirmed a statistically significant increase in TG in dairy sheep as a consequence of increased fat intake from grape pomace.

3.4. Nitrogen Profile

Changes in protein, albumin, and urea levels are needed to diagnose disorders of nitrogen metabolism [48]. The highest but statistically non-significant content of total proteins ($p = 0.380$), globulin ($p = 0.548$), and urea ($p = 0.564$) in GP1 was found (Table 5). However, in the control, the highest albumin content was observed but statistically non-significant ($p = 0.154$). After the GP addition, a narrower ratio between albumin and globulin (C 0.83/1; GP1 0.43/1; GP2 0.63/1) was observed. Alba et al. [27] reported statistically significant lower TP, GLB, and urea after grape residue flour (2% from concentrate)

addition in lactating dairy sheep in comparison with the present study. Alba et al. [27] also determined similar results after GP feeding on albumin (statistically non-significant). Bahrami and Chekani-Azar [42] found no significant effect of GP on the content of total proteins. The concentration of total proteins, albumin, globulin, and urea were in the reference range [21,56]. In contrast to our results, Panev et al. [57], Carlos et al. [58], and Jelinek et al. [59] reported lower average total proteins in wethers, in Morada Nova sheep, as well as generally in sheep.

3.5. Enzymatic Profile

Enzymatic profile indicators (AST, ALT, ALP) (Table 5) were in physiological range according to Tschour et al. [50]. Determination of enzyme activity is necessary in order to exclude hepatopathy. Furthermore, AST and ALT values were comparable with Rahman et al. [60]. The GGT values were under the limit recommended by Tschour et al. [50], Lepherd et al. [61], and Shek Vugrovecki et al. [24], but in accordance with reference values according to Al-Hadithy et al. [62]. After the GP feeding non-significant lower AST ($p = 0.512$) values and higher ALT ($p = 0.490$), ALP ($p = 0.124$) and GGT ($p = 0.857$) values were observed. Similarly, Chedea et al. [25] did not confirm the effect of GP feeding in dairy cows (diet contained 15% dried GP) on AST, ALP, and GGT. Iannaccone et al. [26] also did not find the effect of GP addition in calves (10% dried GP meal in concentrate) on AST and ALT values. In the study of Nudda et al. [63] the effect of grape seeds addition (300 g per day) on sheep AST and ALT parameters was not found but statistically significant higher ALP and lower GGT were observed. Comparable with the present study, a decrease in AST values in the lambs after feeding of GP (5%, 10%, and 20% in dry matter) with the lowest value in variant with 5% addition, was reported by Jin et al. [64].

4. Conclusions

The GP addition to the ruminants' daily diet increased the digestibility of nutrients without the negative effect on the biochemical profile of animals. The digestibility of crude protein, NFC, NDF, and OM in wethers was significantly higher at a higher dose of dried grape pomace (2% of GP). The addition of GP into the daily diet did not affect the nitrogen, enzymatic, mineral, and energetic profile of wethers blood serum except Cl^- and glucose (2% of GP). Dried grape pomace in an amount of 2% diet dry matter can be considered as a suitable source of nutrients in sheep feeding, which in addition should improve the digestibility of diet crude protein.

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Institutional Review Board Statement: The conditions of animal care, manipulations and use corresponded with the instructions of the Ethics Committee of the Slovak University of Agriculture in Nitra, Protocol No. 48/2013. According to the State Veterinary and Food Administration of the Slovak Republic, the given study is evaluated as inexperienced agricultural practices that do not fall under the legislation of Government Regulation of the Slovak Republic 377/2012 of 14 November 2012, laying down requirements for the protection of animals used for scientific or educational purposes.

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Review

Biogenic Selenium Nanoparticles in Animal Nutrition: A Review

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Abstract: Selenium still represents a matter of debate in the scientific community. Bionanotechnology has introduced a whole new perspective on selenium use in animal nutrition. In recent years, attention has been focused on selenium nanoparticles prepared by chemical synthesis. Societal pressure directs research in a "greenway" that is more eco-friendly. Biogenic selenium nanoparticles thus represent a new space for research in the use of this new form of selenium in animal nutrition. Recent research shows that biogenic selenium nanoparticles have low toxicity, improve antioxidant status, and increase the body's immune response. However, their benefits may be much greater, as numerous in vitro studies have shown. In addition, biogenic selenium nanoparticles possess antimicrobial, antifungal, and anticancer activities. Further research should answer questions on the use of biogenic selenium nanoparticles as a feed supplement in individual categories of livestock, and their safety in terms of long-term supplementation.

Keywords: nanoparticles; selenium; animal nutrition

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1. Introduction

The element selenium was first described by the Swedish chemist Jöns Jacob Berzelius in 1818, who found this element in the mud at the bottom of a sulfuric acid preparation. At that time, it was believed that selenium was toxic [1]. Early research on this element was focused mainly on its toxicity as in the 1930s, it was found that selenium caused the poisoning of livestock (the so-called "alkali disease"), especially in areas with a high amount of selenium in the soil [2]. In agricultural soils, Se exists in two bioavailable inorganic forms as selenate or selenite; plants are able to uptake selenium in these two forms and convert them to organoselenium compounds such as selenocysteine (SeCys) and selenomethionine (SeMet) [3]. Plants are the main source of Se for grazing and forage-eating animals such as cattle, horses, sheep, goats, and swine. Forages in which Se levels exceed 5 mg/kg should be considered hazardous for the health of livestock [4]. Some plant species are considered selenium hyperaccumulators (e.g., *Astragalus* spp. and *Senecio* spp.); they tend to easily take up selenium from the soil and accumulate it in high concentrations (1000–15,000 mg Se/kg dry matter) into their tissues [3]. Long-term ingestion of plants or fodder with contents of Se above 1 mg/kg in dry matter (DM) can cause chronic Se toxicity (i.e., selenosis) in livestock [3,5–7]. Young animals tend to be more susceptible to selenium poisoning.

Until the 1950s, selenium was considered to be toxic to humans and animals. However, perceptions of selenium significantly changed in 1957 when Schwarz and Foltz stated that the addition of Se prevented liver necrosis in rats [7]. A few years later, it was found that selenium is incorporated into leucocytes in dogs; this finding indicated the role of

Se in immune function [8]. In 1973, scientists discovered that Se is a component of the enzyme glutathione peroxidase (GPx), which is a selenoprotein that detoxifies harmful organic hydrogen peroxides [9]. In Eukaryotes, over 26 distinct selenoproteins have been identified. However, their functions are not completely understood. All selenoproteins that are known to play a role in oxidoreductase intervening enzymes are implicated in multiple metabolic pathways, e.g., the maintenance of intracellular redox status, free radical scavenging, and repair of oxidized lipids [10]. These discoveries were the beginning of more extensive studies on the role and importance of this element for human and animal health and nutrition. Studies are still ongoing, although selenium is currently recognized as an essential micronutrient that performs multiple functions (e.g., anticancer, joint health, immune resistance, and antioxidant properties) in the growth and functioning of living animal cells and human bodies. As an element of GPx and thioredoxin reductase enzymes, selenium protects the biomolecules against reactive oxygen species (ROS) and free radical damage. Antioxidants can reduce the harmful effects of ROS on animal organisms [11]. The biological activity of Se depends on its chemical form. Selenium compounds commonly exist in four oxidation states in nature: selenate (Se^{+6}), selenite (Se^{+4}), elemental selenium (Se^{0}), and selenide (Se^{-2}). The inorganic forms of Se (i.e., selenates and selenites) are soluble in water and, usually, they present in this form in water, or they can be found in different minerals [12]. They are known to be toxic to biological systems even in low concentrations [13]. In contrast, Se^{0} is essentially nontoxic and highly insoluble in water; it rarely occurs in its elemental state. In the form of organic bindings, Se occurs as selenides [12], and these compounds are considered to be the most stable [14].

2. The Importance of Selenium in Animal Nutrition

Trace elements play an essential role in animal diet. Selenium is one of the critical nutritional factors necessary for the normal functioning of the immune system [15] and maintenance of health, growth, and various biochemical–physiological functions [16]. Numerous scientific investigations have demonstrated that a deficiency in Se could lead to serious disruptions in an animal organism such as liver necrosis, muscular dystrophy, pancreatic fibrosis, mastitis, cystic ovaries, and dysfunction of the thyroid metabolism [17–19]. The symptoms of selenium deficiency have been reported in monogastric animals and ruminants. In young ruminants, such as calves and lambs, Se (and also vitamin E) deficiency often leads to the commonly named "white muscle disease" (WMD) or nutritional muscular dystrophy [20–22]; in older ruminants, a low selenium state is associated with poor reproductive performance, unthriftiness, placental retention, and impaired immunity [18]. In monogastric animals (swine, poultry, and horses), Se deficiency leads to the damage of vital organs, such as the liver, kidney, and pancreas, and to WMD, "Mulberry heart disease" (MHD), lower immune responses, and increased susceptibility to viral infections [18,23]. Selenium deficiency can cause a variety of reproductive disorders in animals (e.g., damage of embryonic development, infertility, retained placenta in dairy cattle, abortions, and a decrease in egg production in laying hens) [24,25]. Selenium deficiency is related to oxidative stress, which refers to the production of a large amount of ROS in the body. ROS can damage cells and tissues and adversely affect organs and their functions. According to the results presented in publications in the field of human medicine, the Czech Republic was ranked among the countries with a low selenium intake, those which had populations that were found to have selenium concentrations below the European average. Thus, sufficient Se supplementation in animals tends to be important not only to maintain the good health and performance of animals themselves but also to increase its supply to the human population through higher selenium content in milk and meat. Selenium plays an important role in maintaining the good health of the mammary gland and, thus, has an impact on milk quality. Selenium deficiency is associated with increased intramammary infections in dairy cattle. When evaluating the occurrence of Se deficiency in cattle by examining the blood of 879 cows in 34 regions in the Czech Republic, selenium deficiency was found on 50% of tested animals and 54% of the farms. Studies from Slovenia and

Ireland have reported similar findings [26,27]. Selenium deficiency is related to various Se concentrations in soils in different regions. Extensive monitoring of Se concentration in soil, plants, animal feed, and blood in 30 farms in different regions in Kosovo showed a low concentration of Se in soil (under 500 μg/kg) and plants (under 50 μg/kg); among all minerals measured in animals blood, the larger deficiency was found for Se [28]. Selenium deficiency in livestock is often related to low Se content in forage and pasture. Compared to the control group, calves supplemented with selenium-fortified hay had higher Se blood concentration and improved body weight and immune response upon vaccination [29]. Nordic countries (e.g., Sweden and Finland) are generally considered to be selenium-poor areas (<0.125 mg/kg) [30–32]. Currently, almost all crop fertilizers in Finland contain Se in the chemical form of sodium selenate (15 mg Se/kg). In areas such as China and North America, where irrigated soils contained excessive Se concentrations (>1 mg/kg), it led to high Se concentrations in surface waters, causing the phenomena of Se pollution, ecological damage, and human diseases [33]. Possible toxic effects for humans and animals as a result of the excessive Se contention in water may be a future challenge. Currently, there is no regulation concerning Se supplementation in animals; however, the National Research Council (NRC) provides guidance. The daily dietary requirements of Se in cattle recommended by NRC are 100 μg/kg of DM for beef cattle and calves, and 300 μg/kg DM per day for dairy cows [34]. Poultry Se requirements range between 150 and 200 μg/kg DM; some diets also include 300 μg/kg DM [35] (Table 1). Current regulations in the US allow up to 300 μg/kg DM of dietary addition of Se in poultry diet, and for the European Union, the total maximal level of dietary Se inclusion is up to 500 μg/kg DM [36]. The dietary Se requirements for swine ranges from 150 μg/kg DM for finishing pigs and sows to 300 μg/kg DM for weaning pigs [37]. Even though selenium is important for many physiological functions in the body, a high dietary level of Se can cause toxicity. Doses of Se which cause acute toxicity in different animal species are represented in Table 2. Signs of acute Se toxicity may vary with the concrete amount of Se consumption or administration, the chemical form of Se, animal age, and species [38], but they usually follow death within 2–5 h after acutely toxic Se consumption or injection of Se [39]. Selenium can be found in all agroecosystems, such as soils, rocks, and water. Acute oral selenium poisoning usually occurs with exposure ranging from 1 to 10 mg/kg bw depending on the species (Table 1), age, and Se chemical form. Young animals are more susceptible to acute Se toxicosis with dosages of 0.2–0.5 mg/kg bw. Parenteral Se products can cause acute toxicity and death at dosages of 1 mg/kg bw [39].

Table 1. Selenium daily nutritional requirement (supranutritional) and acute toxic levels in various animal species.

Animal Species	Selenium Daily Nutritional Requirement (μg/kg DM)	Se Dose (mg/kg Body Weight-BW) Inducing Acute Toxicity	Reference
Dairy cattle	300	3.0	[34]
Beef cattle	100	3.0	[34]
Sheep, goat	100–300	0.5	[40,41]
Swine	150–300	1.2	[37]
Horse	100–200	3.0	[42]

Table 2. List of dietary selenium sources in animal nutrition.

Selenium Sources	Description	Reference
Inorganic	• Cost effective; • Relatively nontoxic; • Lower bioavailability in comparison with organic Se and SeNPs; • Commonly supplemented in injections or mineral salt complexes; • Absorbed by simple diffusion in the intestinal tract.	[43]
Sodium selenite/selenate	Inorganic Se salts are frequently used as feed supplements because they are cost effective and relatively nontoxic; they are most commonly supplemented in the form of injections or mineral-salt complexes. However, they were reported to have lower bioavailability and lower transfer to animal products compared to organic Se and SeNPs.	

Table 2. *Cont.*

Selenium Sources	Description	Reference
Organic	• Naturally derived by plants; occurs in feeds; • Better bioavailability in comparison with inorganic Se; • Less toxic than inorganic Se; • Commonly supplemented in the form of selenium-enriched yeasts (SYs); • Absorbed by active transport in the intestinal tract.	[44,45]
Selenomethionine (SeMet); Selenocysteine (SeCys)	SeMet is often supplemented in the form of SYs. Organic Se sources have shown better bioavailability and improved Se storage in animal tissues compared to inorganic.	
Selenium nanoforms	• Prepared via 3 different routes: chemical, physical, and biological; • High Bioavailability; • Less toxic compared to inorganic and organic Se; • Environmentally friendly; • Mechanism of absorption and distribution in tissues is not totally known.	[46–48]
SeNPs prepared using a chemical/physical method; biogenic SeNPs prepared via green synthesis	Chemically synthesized SeNPs are less toxic to animals than inorganic and organic Se sources, but they are not environmentally friendly due to the toxic chemicals produced during the NPs' preparation. Biogenic NPs are more stable, more eco-friendly, and less toxic.	

3. Selenium Supplementation

Selenium can affect the immune and antioxidant systems of animals through GPx and selenoproteins with various biological functions. Dietary Se can be supplemented in animals from two important sources: organic and inorganic. Organic Se is more bioavailable than inorganic Se [Table 2]. In recent years, the application of Se in nanoforms (Table 2) has attracted more attention, mainly due to the possibility of using Se in a zero-oxidation state (Se^0), which presents low toxicity and better bioavailability compared to other oxidation states [49]. Moreover, supplementation of NPs can improve the delivery and absorption of the trace elements in animals and humans while causing no environmental damage.

3.1. Selenium Absorption and Bioavailability

The efficiency of Se absorption is affected by the form of dietary selenium and differs between ruminants and non-ruminants [50]. It has been found that organic forms of Se are actively absorbed in the intestinal tract via an amino acid transport mechanism, unlike inorganic Se, which is absorbed by a simple diffusion process [51]. Selenomethionine (SeMet) is essential for humans and animals and cannot be synthesized in the body [52]. Selenocysteine (SeCys) is a structural component (cofactor) of selenoenzymes; therefore, it is important for their catalytic activity. These selenoenzymes play a key role in redox homeostasis in mammals; their active component is SeCys which is synthesized in the body de novo [53]. GPx and other selenoenzymes are the major Se-containing antioxidants in the body that help to neutralize ROS [54]. The expression of selenoproteins is specific to various tissues and depends on Se availability in feed [55]. Selenoproteins help animals resist oxidative stress, which can be caused by, heat, decreased productive performance, and various diseases (e.g., mastitis and intramammary infections in dairy cattle). The concentration of GPx is typically analyzed in blood plasma to assess selenium deficiency and to evaluate antioxidant status or oxidative stress [56]. In the case of Se deficiency, GPx concentration tends to be low. When oxidative stress is high and Se contention in feed is limited, L-SeMet is released from the proteins due to the protein turnover and provides Se for the production of selenoproteins. SeCys, the same as inorganic Se, does not play a significant role as a nutritious Se source [57]. SeMet represents the storage form of Se in animal and human tissues [58]. Dietary selenomethionine can be incorporated into muscle protein in place of methionine and become a rich store of selenium; it represents a highly available substrate for many proteins and can substitute methionine in the protein structure [58,59]. All dietary Se sources, except for organic L-SeMet present in SYs or naturally derived in plants, are primarily metabolically transformed to selenide in the liver and then used for SeCys synthesis. Non-ruminant animals are not able to

synthesize selenomethionine from inorganic forms of selenium [60] but can convert it to another essential amino acid—selenocysteine [16]. The mineral (inorganic) forms of Se are reported to have some disadvantages such as relatively high toxicity, low transfer to animal products (milk, eggs, and meat), and the inability to facilitate Se storage in the body [16,61]. In ruminants, microbial digestion by ruminal microorganisms (RMOs) proceeds before digestion in the abomasum and small intestine [62]. The ruminal microbes reduce most of supplemented inorganic Se to unabsorbable selenium sources (selenide and elemental selenium), thereby decreasing Se bioavailability to 20–25% [51]. The absorption of inorganic Se in the form of sodium selenite in the small intestines of monogastric animals and poultry is approximately 80%, while in ruminants, this range is only 29%, and for organic Se in monogastric species and poultry, it is greater than 90% [19,63]. This difference appears to be the result of as reduction in dietary Se forms by microbes in the rumen. The organic forms of selenium naturally occur in plant-based feedstuffs, and selenized yeast (SY) are selenoaminoacids (selenomethionine and selenocysteine), which contain a selenol group in place of the sulfur-containing thiol group [18]. SeMet is considered a metabolically effective organic Se and is traditionally supplemented in the form of selenized yeast (SY), which has been grown in a high selenium medium. In animals' intestinal tracts, proteins from SYs are broken down into small peptides and then amino acids. Thus, SeMet can be absorbed in the intestine the same way as amino acid methionine and build selenium deposition in the body. However, the binding of selenium by microbial cells highly depends on the concentration of Se in the cultivation medium and cultivation conditions [64]. Selenium can be found in all cells and tissues, but the level of Se and its distribution in the body tissues is influenced by the dietary Se form and intake. After oral selenium supplementation (organic SY and inorganic sodium selenite), the Se uptake in sheep was only 34%, whereas, in pigs, it was 85% [51]. Some studies demonstrated increased Se concentrations in meat after SY dietary inclusion compared to inorganic Se [65,66]. In their study, Paiva et al. [66] demonstrated the increase in muscle Se in lambs supplemented with organic Se (0.2, 0.4, and 0.8 mg/kg DM) compared to the inorganic Se form. Selenium content in the muscle was higher with more Se inclusion in the diet linearly. Organic Se sources demonstrated a higher capacity to accumulate Se in muscles than inorganic Se. Hepatic GPx activity was found to be higher in animals supplemented with sodium selenite (SS), which refers to the metabolic pathway of inorganic Se. Sodium selenite biotransformed to selenide for further GPx synthesis more quickly. Similar results were obtained by Steen et al. [67]. Se concentrations in the muscles and blood of lambs receiving organic Se were significantly higher compared to the inorganic group. Improved beef meat quality parameters (e.g., color stability) and increased Se concentration in the muscles of Charolais bulls supplemented with organic Se (0.2 mg/kg DM) for 60 days were observed by Grossi et al. [57]. The selenium concentration in the meat of Nellore cattle was higher in animals supplemented with organic Se (0.3, 0.9, and 2.7 mg/kg DM) compared to organic Se [68]. Results obtained by Hall et al. [69] and Galbraith et al. [70] also showed better bioavailability of the organic forms of Se compared to the inorganic form. An increase in Se in the blood serum and GPx activity in lambs was demonstrated after organic and inorganic Se (0.15 mg/kg DM) supplementation; no significant difference between the organic and inorganic Se forms was observed [71]. Various species of ruminant animals (i.e., dairy cows, beef cattle, calves, and lambs) were supplemented with the ten times maximum permitted Se (in the organic form of SY) dosage of 0.568 mg/kg DM, and there was no adverse health effect observed. Moreover, it was observed to increase Se in the blood and milk samples [72]. The digestive system of animals is more adapted to the organic form of Se, which naturally occurs in feeds, and it has better assimilation [73]. Organic Se in high doses can also be toxic, but SeMet does not produce free radicals when reacting with glutathione. However, the molecular mechanism of selenium toxicosis is not well understood and indeed needs further investigation. Selenium supplementation (SY and SS at the dose 0.4 mg/kg DM) increased GPx activity, regardless of the selenium source [74]. Some other studies also did not confirm the greater biological effects of organic Se [75–77].

Postpartum and pregnant animals are generally more susceptible to Se deficiency and often require Se supplementation due to oxidative stress, which can be caused by pregnancy itself. Dietary organic Se supplementation in postpartum mice enhanced Se deposition in the liver, blood, and mammary gland, in addition to an increase in GPx activity [78]. In dairy cattle, oxidative stress during pregnancy and postpartum can increase intramammary diseases and mastitis cases. Long-term Se deficiency may also lead to thyroid-related diseases such as autoimmune thyroiditis [25]. Se supplementation in pregnant and lactating ewes showed a significant increase in the Se concentration in the blood and an increase in thyroid hormones compared to the control group [79]. These results suggest that organic Se supplementation can improve the antioxidant status of pregnant animals. More efficient transfer of Se from organic Se dietary sources to dairy cattle milk in comparison with inorganic Se was demonstrated [80–82]. Due to the fact of these controversial results, additional investigations in this research area are strongly recommended.

Many studies have described the bioavailability of different chemical forms of selenium [51,56,63,64], e.g., diets enriched with the organic Se increased Se concentrations in animal tissues compared to animals supplemented with inorganic Se.

The selenium-enriched microalgae, *Chlorella vulgaris*, was used as an organic source of dietary Se [83,84]. The effects of the supplementation of sodium selenite and selenized microalgae biomass on fish mortality, growth, and the accumulation of Se in the muscles and liver were observed in common barbel [83]. The results showed more accumulation and bioavailability of Se in muscles and liver in barbel supplemented with Se-enriched microalgae than animals supplemented with inorganic sodium selenite. Marounek et al. [85] also observed better accumulation of Se in tissues of rabbits supplemented with organic Se yeasts and Se microalgae. Similar results were obtained by Hassan et al. [86,87]. Reports on the concentration of SeMet in *Chlorella vulgaris* are controversial. De Alcantara et al. [88] found that 70% of intracellular Se in *Chlorella vulgaris* is in the form SeMet.

In contrast, Neuman et al. [89] found 24–30% of SeMet from the total amount Se accumulated in algae cells, but the concentration of SeCys in algae cells was higher (48.76%). Supplementation in animals with Se-enriched microalgae can benefit from the presence of antioxidants, vitamins, and other biologically active compounds. On the other hand, microalgae cultivation is costly, and the accumulation of intracellular SeMet can be various depending on the cultivation method and conditions.

3.2. Selenium and Vitamin E

The most studied beneficial health effect of Se has been studied in connection with vitamin E. Vitamin E and Se have interrelated functions in animals and human organisms. Inadequate amounts in the diet leads to similar adverse effects. The synergetic interaction between Se and vitamin E can enhance GPx synthesis, an important part of the antioxidant pathway in the body. Selenium deficiency is often characterized by low concentrations of both Se and vitamin E [90]. Thus, optimum Se and vitamin E levels are necessary to minimize the oxidative damage of cells and tissues in the body [12]. Supplementing dairy cows with low doses of both vitamin E and Se (Se injection 1 mg/kg bw; vitamin E 32 g/d) reduced the duration of clinical mastitis symptoms by 62%, in cows supplemented only with vitamin E mastitis, the duration of symptoms was reduced by 44%, and in cows supplemented with Se by 46% [91]. These results demonstrate the beneficial interaction of Se–vitamin E (SeE) dietary inclusion in reducing the duration of clinical mastitis, which can improve dairy cows' production. Zahrazadeh et al. [92] evaluated the influence of SeE injection (0.5 mg of Se in the form of sodium selenite; 56 IU of vitamin E) on body condition, lactation performance, and oxidative status in dairy cows. SeE injections showed a beneficial effect on body score, antioxidative parameters, and lactating performance in Holstein cows. The mechanism of the beneficial effect of vitamin E and SeE supplementation on mastitis is not fully understood, and this needs further research. Hogan et al. [93] observed that vitamin E deficiency was associated with a reduction in neutrophils that had

bactericidal activity and increased mastitis. Ali et al. [94] reported that SeE injections (75 mg of vitamin E per day per animal; 2800 mg of Se) improved the reproductive performance of lambs compared to vitamin E supplementation alone. In another study, Se supplemented alone had a better effect on the semen quality of boars than the group supplemented with SeE [95]. Kappel et al. [96] did not observe the improvement in reproductive efficacy in cows injected with SeE (680 IU of vitamin E and 50 mg of Se in the form of sodium selenite). The daily nutritional requirement of vitamin E for adult cattle recommended by the National Research Council (NRC) is 15–60 international units (IUs). The daily nutritional need for nursing calves ranges between 40 and 60 IUs [34]. Supplementation of 0.3 mg/kg bw of organic Se and 100 mg/kg of vitamin E improved the immune status of broiler chickens, but there was no difference found compared to the animal group supplemented with Se alone [97]. In recent years, only a few studies have been related to the toxic effects of vitamin E supplementation. In general, vitamin E is considered to be one of the least toxic of the vitamins [37]. A study on higher doses (500, 1000, and 2000 mg/kg bw) of vitamin E oral supplementation over 30 days in albino rats showed a negative effect on the liver and kidneys. Still, no deaths were recorded [98]. Hale et al. [99] reported on the toxicity of intravenous vitamin E (33.5 mg/kg bw) supplementation in neonatal piglets. Rapid intravenous injections of vitamin E resulted in sepsis and abnormal pulmonary function. Further studies are needed to investigate the possible undesirable effect of vitamin E supplementation and to determine appropriate dosage levels.

4. Selenium Nanoparticles in Animal Nutrition

An appropriate animal diet and living environment play a key role in animal health and performance. Thus, optimizing these factors is important for increasing rearing efficiency, which can positively determine the quality of production of animal origin. Over the last decade, nanotechnology has received the attention of many researchers due to its promising agricultural and food applications. Nanotechnology provides new "intelligent" solutions in animal nutrient delivery and health protection, and, indeed, it has the large potential to improve animal production systems [100]. This interest is mainly caused by the unique physicochemical properties of nanoparticles (NPs), which refers to their small size (1–100 nm), high stability, hydrophobicity, and large surface area. NPs' hydrophobicity is important for good dispersion in water or serum and is also required to enhance their interaction with cell membranes [101]. The NPs' size affects the cellular intake and allows them to easily pass through the stomach wall and diffuse into body cells quicker than common elements with larger particle sizes. The in vitro absorption of NPs with a diameter of 0.1 μm was found to be higher than 1 and 10 μm NPs [102]. The thickness of gastric mucus layers (total mucus), which continuously cover the gastrointestinal tract's (GIT) surface, varies from 200 μm in the small intestine to 480–800 μm in the large intestine [14] and could allow the transport of NPs through the layer. According to Corbo et al. [103], NPs, especially nanominerals (e.g., Se and Zn), have a higher surface-area-to-volume ratio, providing more surface area for contact with the mucosal tissues and cells. Better absorption of NPs into the mucosal surface increases the particle residence time in the GIT. When a nanomineral is introduced into a biological medium, such as blood or mucus, proteins adsorb on its surface, giving it a unique "biological identity", a so-called protein corona, which can have an impact on the NPs' distribution as well as their potential toxicity [104]. Nanoparticles have been used in animal nutrition for their antibacterial, antifungal, and antioxidant properties as well as probiotics and to maintain general animal performance and health. The antimicrobial activities of metallic NPs (e.g., ZnO, CuO, and AgNPs) and SeNPs have been demonstrated by different researchers [105–108].

Selenium nanoparticles (SeNPs) are nano-sized (generally <60 nm in diameter) elemental selenium particles with excellent nano-properties [109]. For the NPs' synthesis, there are two main strategies used: bottom-up (including chemical vapor deposition, hydrothermal and solvothermal methods, chemical reduction, and green synthesis) and top-down (including mechanical milling, laser ablation, etching, sputtering, and electro-

explosion). The top-down strategy involves the mechanical breaking down of the bulk material into nanostructured materials. In contrast, the bottom-up method uses chemical reactions to break bulk into several parts to form NPs [110]. Methods of NPs synthesis can also be divided into physical, chemical, and biological (the so-called "green way" or "green synthesis"). The chemical methods of nanoparticle synthesis are the most common approaches commercially employed in various areas of NP applications. Concurrently, plenty of research indicates a potential environmental threat of nanotechnology related to NP toxicity [111–115]. The chemical approach to NP synthesis is related to the use of toxic chemicals, which are hazardous to humans and the environment [116]. Designing NPs via a green route using biological and eco-friendly materials reduces the negative environmental impact [117].

Various studies have investigated the possibilities of using selenium nanoparticles as a new source of selenium (Table 3). For instance, sodium selenite NPs coated with methacrylate polymers were orally supplemented with ruminants, improving selenium absorption [62]. Shi et al. [118] stated that dietary nano-sized Se improved Se content in the blood and tissues and enhanced ruminal fermentation and feed utilization in sheep, which were fed a basal diet supplemented with 0.3, 3, and 6 g/kg DM of nano-Se. Kojouri et al. [119] reported the positive effect of dietary SeNPs inclusion (0.1 mg/kg DM for 60 days) on the antioxidant activity and weight gain of young lambs. In another experiment, the inclusion of 1 mg/kg DM of nano-sized Se into sheep's diet exhibited a better antioxidative effect after 20–30 days of supplementation [120]. Xun et al. [121] also reported enhanced rumen fermentation and feed conversion efficiency in sheep supplemented with 4 mg/kg DM of nano-sized Se compared with selenium yeast (SY). In another experiment, supplementation with 0.5 mg/kg DM of nano-sized Se improved hair follicle development and promoted growth in Cashmere goats [122]. Experiments with nano-Se inclusion in broiler chicken diets conducted by Gangadoo et al. [48,123] demonstrated improved gut health and general animal performance; the best results in both experiments were obtained with an SeNP supplementation of 0.9 mg/kg DM with no toxic effect occurring. Previous studies have demonstrated the benefits of using SeNPs in broiler feed, with increased absorption and diffusion of material into organs and tissues, increased antioxidant capacity, and meat quality.

In contrast, Wang et al. [124] did not observe any beneficial effect of SeNP supplementation in terms of enhancing the oxidative status in broilers, but Se improved the survival rates. Gulyas et al. [125] reported changes in the proteome profile in chickens after SeNPs supplementation. These results could be related to the specific patented method of NP preparation used in this study. Several studies reported improvements in the growth performance [126–128], intestinal health [129,130], and antioxidant status [131] of aquatic animals supplemented with SeNPs. Se supplementation alleviated the antioxidant balance and enhanced kidneys cells' resistance to oxidative damage in grass carp [132]. In another study, SeNP supplementation improved intestinal health, feed utilization, and growth performance in Nile tilapia [130]. The enhancement of the growth performance and feed efficiency after SeNP supplementation (0.4–0.8 mg) in Nile tilapia was also observed by Ibrahim et al. [133]. Markedly, the nanoform of Se can enhance growth performance in fish. The recommended dosage of SeNP dietary inclusion ranges from 0.15 to 4 mg/kg depending on the fish species [134].

Table 3. Effect of SeNP supplementation on animal health and performance.

Experimental Animals	Element	Dose	Toxicity	Major Effect	Reference
Dorset sheep	SeNPs	0.3, 3, and 6 mg/kg DM fed for 75 days	No information	Improved Se content in blood and tissues and enhanced ruminal fermentation and feed utilization	[118]
Small tail Han sheep	SeNPs	0.3, 3, and 6 mg/kg DM fed for 75 days	No information	Improved Se content in blood and tissues and enhanced ruminal fermentation and feed utilization	[118]

Table 3. *Cont.*

Experimental Animals	Element	Dose	Toxicity	Major Effect	Reference
Tan sheep	SeNPs	0.3, 3, and 6 mg/kg DM fed for 75 days	No information	Improved Se content in blood and tissues and enhanced ruminal fermentation and feed utilization	[118]
Neonatal lambs	Sans	0.1 mg/kg DM fed for 60 days	No information	Improved weight gain on the 14th and 28th day; enhanced antioxidant parameters	[119]
Lori–Bakhtiari sheep	SeNPs	1 mg/kg DM diet for 10 days	SeNPs were found to be less toxic than SS	Improved antioxidant parameters compared to the experimental group fed with sodium selenite (SS)	[120]
Sheep	SeNPs	4 mg/kg DM	No information	Enhanced rumen fermentation and feed conversion efficiency compared with the group fed with 4 mg/kg DM selenized yeast (SY)	[121]
Cashmere goats	SeNPs	0.5 mg/kg DM	No information	Improved the hair follicle development and promoted growth	[122]
Khalkhali goats	SeNPs	0.5 mg per animal per day	No information	SeNP inclusion improved Se status in goats (increased Se in blood, colostrum, and milk) compared to SS and SeMet	[82]
Makuei sheep	SeNPs	0.1 mg/kg of live weight	No information	Reduced oxidative stress and enhanced weight gain compared to the group supplemented with SS	[135]
Male rats	SeNPs	0.5, 1.5, 3.0, and 5.0 mg Se/kg for 28 days	Damage of the liver parenchyma and intestinal epithelium in animal groups fed with 1.5, 3, and 5 mg Se/kg	Increased Se content in the blood compared to the control group	[136]
Mice	SeNPs	4, 40, and 400 µg/kg of bw	Dietary SeNPs showed less toxicity compared to inorganic SS and sodium hydroselenite but was more toxic than SY; subacute toxicity was observed with administration of 400 µg/kg bw	Inorganic Se forms showed higher toxicity in comparison with SeNPs and organic SY	[137]
Swiss albino mice	SeNPs	2 mg/kg of bw was administrated	Organic and inorganic Se forms showed less toxicity at the same dosage of 2 mg/kg of bw	SeNPs improved antioxidant protection of cells compared to inorganic forms	[47]
Male rats	SeNPs	0.0, 0.2, 0.4, 0.8, 2.0, 4.0, or 8.0 mg Se/kg of bw were administrated for 14 consecutive days	Doses greater than 2.0 mg Se/kg of bw induced chronic toxicity	Supranutritional levels (0.2, 0.4, and 0.8) of SeNPs did not show toxic effect	[138]
Male Sprague–Dawley (SD) rats	SeNPs	0.0, 0.2, 0.4, 0.8, 2.0, 4.0, or 8.0 mg Se/kg of bw were administrated for 2 weeks	Doses greater than 4.0 mg Se/kg of bw induced chronic toxicity, damaging effect	Supranutritional levels (0.2, 0.4, and 0.8) of SeNPs had a positive effect on reproductive function (promoted sperm motility)	[139]
Male SD rats	SeNPs	0.0, 0.2, 0.4, or 0.8 mg Se/kg of bw were administrated for 2 weeks	No toxic effect was observed	Improved antioxidant capacity in the liver and kidney; beneficial effects on immune and antioxidant capacity (dose of 0.4 mg had the best response)	[140]
Male SD rats, Buffalo rats	SeNPs	0.0, 2, 4, or 8 mg Se/kg of bw were administrated for 2 weeks	SeNP administration over 4.0 mg Se/kg bw caused a toxic effect on liver	Impaired the antioxidant capacity in serum with the administration of non-lethal doses	[141]
Female and male rats	SeNPs (20 nm)	0.05, 0.5, or 4 mg Se/kg bw/day for 28 days for female rats; 4 mg Se/kg bw/day for male rats	Nanoparticle-specific toxicity of Se did not occur; no histological changes in the liver occurred	Lowered body weight at all doses of SeNPs	[142]

Table 3. *Cont.*

Experimental Animals	Element	Dose	Toxicity	Major Effect	Reference
Male rats	SeNPs	0.5, 1.5, 3.0, and 5.0 mg Se/kg were administered for 28 days	Tested doses did not have a significant toxic effect on liver, kidney, or spleen	Enhanced Se content in blood compared to the control group	[143]
Mice	Biogenic SeNPs 50–80 nm synthesized using *Lactobacillus casei*	-	No toxic effect occurred	Protected the intestinal barrier function against oxidative damage	[144]
Male mice	Biogenic SeNps 80–220 nm synthesized using *Bacillus* sp. MSh-1	oral administration of 0, 2.5, 5 10, and 20 mg kg^{-1} of Se NPs for 14 consecutive days	A dose of 20 mg/kg showed toxicity	Less toxic effect compared to synthetic SeNPs	[107]
Male mice	Biogenic SeNPs produced using yeast strain *Kluyveromyces lactis GG799*	0.2, 0.6, and 6 mg/kg	No toxic effect occurred	Attenuation of oxidative stress, intestinal inflammation, and intestinal barrier disfunction	[145]
Ross 308 broiler male chicken	SeNPs	0.3, 0.9, and 1.5 mg Se/kg were fed for 28 days	No toxic effect in tissues occurred	Improved the gut microflora environment; the best performance demonstrated a 0.9 mg/kg Se concentration	[123]
Broiler male chicken	SeNPs	0.3, 0.9, and 1.5 mg Se/kg were fed for 28 days	No toxic effect in tissues occurred; no damaging effect on intestinal morphology	Dietary SeNP inclusion showed comparable results with organic Se at the best dose of 0.9 mg/kg (improved absorption in the duodenum)	[48]
male Arbor Acres broilers	SeNPs	0.0, 0.3, 0.5, 1.0, or 2.0 mg/kg of diet	No toxic effect occurred	0.3–0.5 mg/kg optimum doses improved meat quality, immune function, and antioxidant status. Liver and muscle Se contents increased with SeNP supplementation	[146]
Broiler chicken	SeNPs	0.3 mg/kg of diet	No toxic effect occurred	Enhanced Se and vitamin E concentrations in breast muscles, improvement of antioxidant and immune properties	[147]
Broiler chicken	SeNPs	4.25 mg/kg DM	No toxic effect occurred	Changes in proteome profile indicated dietary stress from SeNPs supplementation	[125]
Ross 308 broiler chicken	SeNPs	0.1, 0.2, 0.3, 0.4, or 0.5 mg/kg of diet	No toxic effect occurred	Dietary SeNPs improved weight gain, feed conversion ratio, and growth performance	[148]
Ross 308 broiler male chicken	SeNPs	0.1 or 0.4 mg/kg of diet	No toxic effect occurred	Improved antioxidant status, better production performance, and immune system response	[149]
Ross broiler chicken	SeNPs	0.3, 0.45, or 0.6 mg/kg of diet	No toxic effect occurred	Increased body weight gain, improved feed conversion ratio, and meat quality	[150]
Broiler chicken	SeNPs	0.5, 0.8, or 1.2 mg/kg of diet	No toxic effect occurred	SeNPs dietary inclusion improved performance and immune system better than diets included SeMet and vitamin E	[151]
Broiler chicken	Biogenic SeNPs produced using the bacteria *Pantoea agglomerans*	-	No toxic effect occurred	Protective effect against oxidative and immune stress	[152]

Compared to selenite and selenate, SeNPs are more biocompatible and less toxic to animal organisms [14]. Nevertheless, high doses or long-term supplementation of SeNPs may lead to adverse effects in animal organisms and can be toxic. Several in vivo studies were conducted to measure NPs toxicity. Urbankova et al. reported that SeNPs supplementation had fewer negative effects in rats compared to the standard form. In contrast, supranutritional doses of SeNP administration (0.2, 0.4, and 0.8 mg/kg of body weight) showed a positive effect on reproductive functions and immune and antioxidant capacity. Other experiments on mice and rats supplemented with SeNPs demonstrated the hepatotoxic effect of SeNPs, which were also confirmed by further histological examination [47,62,137,138,142]. Damage to the liver parenchyma and intestinal epithelium in rats was reported after 0.5, 1.5, 3, and 5 mg/kg DM of SeNP supplementation [136]. The authors suggest that short-term SeNP supplementation can be safer and more beneficial in specific treatments. This unfavorable effect could be related to the tested animals' metabolisms, biological characteristics, and the correlation between animal weight and the dosage of NPs administrated. The toxicity of NPs can largely vary among different species [153]. SeNP hyperaccumulation in *Pangasius hypophthalmus* liver, brain, and muscles was observed after SeNP supplementation (2.5–4 mg/L), which caused oxidative stress and toxicity in fish [154]. In another study, SeNP (100 µg Se/L) supplementation in *Oryzias latipes* enhanced oxidative stress caused by the hyperaccumulation of Se in the liver [155].

Based on the studies mentioned above, SeNP supplementation can have many health benefits (e.g., improved production performance, growth, feed efficiency, antioxidant status, and immune status) when present in animal diets compared to inorganic Se sources. Nevertheless, high doses of SeNPs can cause the hyperaccumulation of Se in tissues and oxidative stress or toxicity. Therefore, SeNPs should be included in animal diets in optimum doses to formulate nutritionally balanced feeds. The mechanism of nano-sized Se conversion remains unclear, and the gut microbiota is thought to play a key role in this process. The application of SeNPs showed promising results in improving the oxidative status of the cell induced by a reduction in glutathione (GSH), superoxide dismutase (SOD) levels [156], and GPx activities [157]. Whereas the great advantage of SeNP application compared to sodium selenite can be increased availability of the element [135], on the other hand, this advantage could be turned into a disadvantage through uncontrolled SeNP penetration across cellular membranes, which might be harmful to animal health. According to Surai et al. [35], the metabolism and assimilation of nano-sized Se could be disadvantageous in the animal diet when Se's main mechanism of biological activity is mediated *via* selenoprotein synthesis. Moreover, the effect of dietary SeNPs on gut health and the formation of the accumulated nano-sized Se in animal tissues after supplementation is still unknown and needs further investigation. Furthermore, the topic of whether SeNPs supplementation may increase Se stores in the body remains unanswered.

4.1. Green Synthesis of SeNPs

Over the past decade, the biological method of producing NPs has become an emerging trend in nanotechnology and was developed as a sustainable way to overcome the disadvantages of chemical-based NP synthesis (e.g., high cost and toxic chemicals usage) [104]. Green synthesis provides a new possibility to synthesize NPs via an eco-friendly approach using simple unicellular or multicellular biological entities (e.g., bacteria, fungi, yeast, algae, and plant tissues) as natural reducing and stabilizing agents. The biologically synthesized nanostructures offer substantially different properties such as good adhesion, tribologically good properties, optical and electrical properties, and many promising applications. In NP synthesis, reducing and capping agents play an important role in impacting useful NP properties such as size, morphology, stabilizing, and protecting the NPs' surface, preventing aggregation and uncontrolled growth [158]. Chemical components (i.e., polyethylene glycol, formaldehyde, polyethyleneimine, and polyacrylic acid) used as capping, reducing, stabilizing agents, or solvents in the procedures of chemical NP synthesis are hazardous and extremely toxic [159,160]. To be easily utilized in the living

systems and not cause cellular toxicity, capping agents should be nontoxic, biodegradable, biocompatible, biosoluble, and well dispersed [161]. Green capping agents (e.g., amino acids and polysaccharides) are environmentally friendly. They may lead to designing NPs with unique morphologies and sizes, which can improve, for example, drug delivery via NPs, thereby enhancing NPs' antifungal, antiviral, and antibacterial activity. Nutrients in the form of nanoparticles can be encapsulated in nanocapsules and carried via GIT into the bloodstream and then into body organs, where they enhance the bioavailability of delivered nutrients [162]. Biological synthesis was successfully used to produce different metal NPs, such as AuNPs, FeNPs, and AgNPs [160,163–166]. Green synthesis was also employed to produce SeNPs [167–170], and their antimicrobial, antifungal properties, and cytotoxicity were tested in various in vitro studies.

4.2. Antimicrobial Potential of SeNp Produced via Green Synthesis

Antimicrobial resistance (AMR) represents a major global problem, which significantly affects human and animal health. The wide use of antibiotics as growth promoters in animal farming has caused the development of increased antibiotic resistance in numerous bacterial strains. As a consequence, in 2003, the use of antibiotics in livestock diets was banned in the EU [171]. AMR adversely affects animal health, leading to the poor quality of products of animal origin and economic losses. Therefore, finding a new solution to overcome antibiotic usage is strongly needed. In recent years, nanotechnology enabled the manufacture of effective antimicrobial agents from nano-scaled materials, particularly metals. Many studies confirmed the antioxidant, antibacterial, anticancer, and antifungal activities of metallic NPs [86,172–174]. Whereas selenium nanoparticles have attracted scientific interest primarily as a result of research into their anticancer properties; this nanomaterial's antibacterial potential has recently been identified. NPs have a large surface area, which increases the area of interaction with pathogenic microorganisms. Furthermore, due to the nano size, they are more likely to enter bacterial surfaces.

Although most of the studies on the antimicrobial potential of biogenic SeNPs were conducted in vitro (Table 4), the results of these studies showed noticeable antibacterial, antifungal, and anticancer SeNPs activities against many important humans and animal pathogens and their potential for future applications in nanomedicine and veterinary. Furthermore, SeNPs produced through the green way show lower cytotoxicity, greater bioavailability, and reactivity than inorganic and organic Se, which makes them an attractive candidate for future therapeutic applications. The therapeutic effect of biogenic SeNPs (2.5, 5.0, and 10.0 mg) was also confirmed in an in vitro experiment in a mouse model infected with *Toxoplasma gondii* with no cytotoxicity observed [107]. Based on in vitro studies (Table 4), SeNPs represent a viable approach to inhibit bacterial growth without using antibiotics and to overcome the drawbacks of synthetic methods that employ toxic chemicals. Interesting results were obtained by Cremonini et al. [175] who demonstrated the significantly better antibacterial activity of biogenic SeNPs in comparison with chemically produced NPs.

Table 4. Effects of biogenic SeNPs tested in vitro with potential use in nutrition.

Biological Organism Used for NPs Synthesis	NPs Characterization (Size, Shape)	Applied Dose	Pathogens/Cells	Effect	Reference
Cyanobacteria *Anabaena* sp.	Spherical NPs 5–50 nm	50 µg/mL	Gram-positive and Gram-negative strains of *Staphylococcus aureus* and *Escherichia coli*	• Antibiofilm and antimicrobial activity • Cytotoxicity (apoptosis) in HeLa cells	[176]
Lactobacillus casei ATCC 393	50–80 nm	8 µg Se/mL	The porcine jejunal epithelial cell line (IPEC-J2)	• ↑ Antioxidant activity • No cytotoxicity in IPEC-J2 cell	[177]

Table 4. *Cont.*

Biological Organism Used for NPs Synthesis	NPs Characterization (Size, Shape)	Applied Dose	Pathogens/Cells	Effect	Reference
Citrus fruit (*Citrus limon*, *Citrus paradise*) extracts	100–800 nm	10 and 12 mM	*E. coli*, *Micrococcus luteus*, *Bacillus subtilis*, and *Klebsiella pneumoniae*	• ↑ Antimicrobial effect	[178]
Mushroom extract	8 nm	0.5–1.5 µM	Gram-negative *E.coli*	• ↑ Antimicrobial activity • No cytotoxicity in prostate cancer cell lines • ↑ Antioxidant activity	[179]
Brown alga *Sargassum swartzii*	21 nm	10 mg/mL	*V. parahaemolyticus*	• ↑ Bacterial inhibition	[180]
Bacillus licheniformis	110 nm	2 mg/mL	Cancer cells	• ↑ TNF; induced cell death; • ↑ ROS	[181]
Yeast *Magnusiomyces ingens* LH-F1	Spherical; average size 87.82 nm	No information	Gram-positive *Arthrobacter* sp. W1, Gram-negative *E. coli* BL21	• Inhibition against Arthrobacter but not against *E. coli*	[182]
Trichoderma harzianum JF309	-	400 µg/mL	*Alternaria* toxins, *Fusarium verticillioide*, and *F. graminearum*	• Antifungal effects against Alternaria toxins; fumonisin B1; deoxynivalenol • No cytotoxicity in human cells	[183]
Zingiber officinale	100–150 nm	250 µg/mL	*E. coli*, *Klebsiella* sp., *Pseudomonas* sp., *Staphylococcus aureus*, and *Proteus* sp.	• ↑ Antimicrobial activity • ↑ DPPH	[184]
Ceropegia bulbosa tuber's aqueous extracts extract	277.5 nm	25, 50, 75, and 100 µL/mL	*E. coli* and *B. subtilis*	• ↑ Antimicrobial activity • ↑ Antilarval activity • ↑ Cytotoxicity in the human breast cancer cells at dose 34 µg/mL; anticancer efficacy • No cytotoxicity in human cells	[185]
SeNPs synthesized by the green method (no information about organism used for preparation)	60 nm	0.3, 0.4, and 0.5 mg/mL	*E. coli* and *Candida albicans*	• ↑ Antimicrobial activity at all SeNPs concentrations	
The aqueous filtrate of *Spirulina platensis*	Average size 79.40 nm, spherical shape	5 and 10 mM	*C. albicans*, *Klebsiella pneumoniae*, and *Salmonella abony*	• No cytotoxicity in liver and kidney cells • ↑ Antimicrobial activity	[186]
Escherichia coli, *Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus*, and *S. aureus*	90–150 nm	25–250 µg/mL	*E. coli* and *S. aureus*	• ↑ Antimicrobial activity • No cytotoxicity in human cells	[187]
Gram-negative *Stenotrophomonas maltophilia* and Gram-positive *Bacillus mycoides*	160.6 nm for G+; 170.6 nm for G−	50, 100, and 250 µg/mL	*C. albicans*, *C. parapsilosis*, and *Pseudomonas aeruginosa*	• ↑ Antimicrobial activity against P. aeruginosa • ↓ Antimicrobial activity against Candida sp. • No cytotoxicity	[175]

Table 4. *Cont.*

Biological Organism Used for NPs Synthesis	NPs Characterization (Size, Shape)	Applied Dose	Pathogens/Cells	Effect	Reference
SeNPs coated with the antimicrobial polypeptide (ε-poly-l-lysine)	80 nm	5, 10, 25, and 50 µg/mL	*Enterococcus faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, and *K.*	• ↑ Antimicrobial activity compared to traditional antibiotics • No cytotoxicity in human cells	[188]

↑ higher level; ↓ lower level; G+ gram-positive bacteria; G- gram-negative bacteria.

The strengths, weaknesses, opportunities, and threats (SWOT) analysis for a brief overview of advances and weaknesses of SeNP application in animal nutrition is proposed in Figure 1.

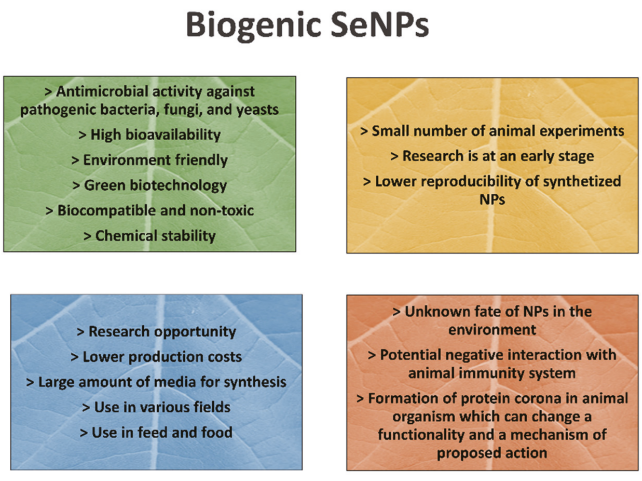

Figure 1. The strengths, weaknesses, opportunities, and threats (SWOT) analysis of SeNP application in animal nutrition.

4.3. Synthesis by Plants and Microorganisms

Live plants; plant tissues; and extracts from the plant leaf, latex, root, seed, and stem, or the whole plant have also been used to synthesize NPs, as they act as stabilizing or reducing agents [189,190]. Due to their genetic variability, plants possess many interesting metabolites, such as phenolic compounds, alkaloids, and sterols, that can serve as excellent biocapping and/or reducing agents. In NPs biosynthesis, plant polyphenols, which possess hydroxyl reducing groups, are usually used as stabilizing and reducing agents. Hydroxyl groups of biologically active plant compounds can also act as a capping agent by depositing on the NPs' surfaces. Polyphenols and proteins may play a key role in reducing selenium ions to their element and stabilizing the SeNPs' form [191]. Polysaccharides may effectively improve the NPs' stability and morphology [192]. The different preparation methods of the plant extract from the same plant tissue may also significantly affect the shape, size, and distribution of NPs [193].

Singh et al. [193] used different *Zingiber officinale* rhizome extract preparation methods and obtained NPs with different properties. The significant advantage of plant-mediated NP synthesis is the inexpensiveness of culture compared to synthesis using microorganisms. In addition, it reduces the cost of microorganism isolation and further NP purification [194]. Moreover, plant-mediated NPs are stable, reproducible, environmentally friendly, and less time-consuming to produce [195]. Anu et al. [167] used *Allium sativum*

extract to produce SeNPs and synthesized NPs 40–100 nm in size, showing decreased cytotoxicity compared to chemically produced SeNPs. SeNPs mediated from various plant extracts (e.g., *Diospyros Montana*, *Murraya koenigii, Ephedra aphylla,* and *Thymus vulgaris*) were reported to have antifungal, anticancer, and antimicrobial activity [108,168,196,197]. Green synthesis of SeNPs is commonly achieved by reducing selenate/selenite in the presence of bacterial proteins and plant extracts containing various metabolites such as phenols, flavonoids, alcohols, and proteins. Many microorganisms (e.g., *Herbaspirillum* sp., *Bacillus arseniciselenatis*, *B. selenitireducens*, and Comamonas testosteroni) have been observed to reduce toxic selenate and selenite into the nontoxic element selenium through aerobic or anaerobic conditions [189,198,199]. Microbes can produce NPs either intra- or extracellularly via different bioreduction processes using various microbial enzymes [190]. Microbial NP synthesis includes two reduction processes (reduction from selenate to selenium trioxide and then to elemental selenium), catalyzed by selenite and selenate reductases [200].

The study conducted by El-Saadony et al. [201] showed that SeNPs synthesized using *Lactobacillus paracasei* had an antagonistic effect against pathogenic fungi and significantly inhibited the growth of *Candida* and *Fusarium* species, which are the most known animal pathogenic species. Moreover, the diameter of obtained SeNPs ranges from 30 to 50 nm. In comparison, a previous study by Sasidharan and Balakrishnaraja [202] synthesized SeNPs by bacteria species (*Lactobacillus casei; Streptococcus thermophilus; Bifidobacterium; Lactobacillus acidophilus; Lactobacillus helveticus; Klebsiella pneumoniae*), but the disadvantage was the size of the produced NPs ranged from 50 to 550 nm. SeNPs synthesized using various cyanobacteria extracts (e.g., *Nostoc sphericum*, *N. punctiforne*, *Spirulina pratensis,* and *Athrospira indica*) showed good antioxidant activities and are recommended for future use as food supplements [203].

SeNPs can play an important role in eliminating microbial infections and, thus, improving animals' growth and performance. SeNPs can inhibit both Gram-negative and Gram-positive bacteria by interrupting microbial biofilm [204] and possess significant antifungal activity by inhibiting spore germination [153]. The antifungal activity of SeNPs was tested mostly by in vitro experiments, and more extensive research in this field is needed. Shakibaie et al. [106] demonstrated a good potential of using bacteria *Bacillus* sp. for SeNPs synthesis. SeNPs prepared using these bacteria were orally administered to male mice, and biogenic SeNPs showed significantly less toxicity than synthetic SeNPs and SeO_2.

Nevertheless, the reason for such a difference is not clear. Some in vivo experiments with biogenic SeNP dietary inclusion showed an improved oxidative status in tested animals without toxic effects [125,153,154]. Shirsat et al. [205] demonstrated a protective effect against the oxidative and immune stress of biogenic SeNPs synthesized using the bacteria *Pantoea agglomerans* in broilers' diets. Song et al. obtained promising results, which used yeast *Kluyveromyces lactis GG799* for SeNPs production. SeNPs demonstrated no toxicity in mice. Moreover, dietary supplementation with 0.6 mg/kg Se effectively attenuated oxidative stress, intestinal inflammation, and intestinal barrier dysfunction. However, these experiments are only a few, and further investigation of the impact of biogenic NPs on animals' performance and production is required.

4.4. Synthesis of SeNPs by Marine Algae and Microalgae

Marine algae generally contain a wide spectrum of biologically active compounds such as polysaccharides, proteins, PUFA, various pigments, and antioxidants. Considering this spectrum, it predestines them to diverse commercial applications [206]. Marine algae may represent a novel nanotechnological solution that could facilitate the application of new alga-mediated NPs in medicine and animal nutrition. Some algae (e.g., *Chlorella vulgaris, Sargassum wightii, Spirogyra insignis, Chondrus crispus, and Tetraselmis kochinensis*) were used for the synthesis of metallic NPs such as Ag and Au NPs [207–210]. SeNPs synthesized via *Spirulina pratensis* showed antibacterial activity against foodborne microorganisms

(*Staphylococcus aureus* and *Salmonella typhimurium*), but antibacterial activity increased with NP size reduction [211]. Aqueous extract of algae *Sargassum angustifolium* was used for biosynthesis of SeNPs, which were finally examined on antibacterial activity. Algae-coated SeNPs showed better antibacterial activity against *Vibrio harveyi* compared to uncoated SeNPs [212]. Algal cell walls are mainly composed of polysaccharides, natural polymers containing monosaccharides linked with glycosidic bonds. In recent years, the application of diverse algal polysaccharides (e.g., alginate and laminarin) has been reported [213]. Developing drug delivery systems using seaweed polysaccharides has received special attention in the scientific community due to the important field of biomedical research. Algal polysaccharides were successfully used for coating NPs as a stabilizing agent. The hydrophilic surface of functional groups on polysaccharides (e.g., hydroxyl, sulfate, and carboxyl groups) allows them to easily interact with biological tissues. Therefore, algal polysaccharides can serve as an excellent template for NP synthesis in modern nanotechnology.

Colloidal stability is frequently an issue that requires significant consideration since high agglomeration levels have been recorded in some situations [214]. The use of algae in NP production is also limited due to the lack of understanding of the synthesis mechanism. Studies regarding the employment of marine algae for SeNP production are still ongoing. It is believed to have a wide potential in the synthesis of biogenic NPs with interesting new properties.

5. Conclusions

The role of selenium in animals is reviewed and discussed. Selenium deficiency can lead to many diseases in animals, as can selenium overdose. The bioavailability of Se depends on the chemical form of supplementation and animal species exposed to a Se-enriched diet and requires further investigation. Nanotechnology offers novel "intelligent" solutions in animal nutrition, health protection, and animal production systems. There are many applications of NPs in animal production, but the results of various studies that have evaluated the effects of dietary SeNP inclusion are ambiguous. The biological synthesis of SeNPs using bacteria, plants, and algae shows a great opportunity for further application in various fields as well in animal nutrition and production. Thus, further detailed studies in this field are required to achieve more optimized green methods of NP synthesis and application in animal diets.

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Effects of Knotweed-Enriched Feed on the Blood Characteristics and Fitness of Horses

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Abstract: The aboveground biomass of dry knotweed was administered daily to large groups of young (1- to 3-year-old) stallions of the Czech Warmblood, Czech-Moravian Coldblood and Silesian Norik breeds, fed individually for 4 and 6 months in two successive winter experiments. Their fitness was compared with control groups consisting of equally numerous subgroups comparable in age, breed, body mass and initial blood parameters. The effects of knotweed on the horses' fitness were evaluated based on changes in blood characteristics. Even if administered in small amounts, 150 g per day, knotweed could (1) increase the thrombocyte numbers, (2) increase the globulin content (thus improving the horses' immunity, which is desired in large groups of animals), (3) stimulate lipid metabolism in cold-blooded horses and (4) decrease the concentration of cholesterol. The long-lasting effect of knotweed on both the urea and triglyceride–cholesterol ratio presumably reflected, between the two experiments, the temporary protein starvation of horses on pastures with poor quality of grass in a dry summer.

Keywords: stallion; bioactive compounds; immunity; lipids; cholesterol

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1. Introduction

Many countries have been trying to eradicate knotweed due to its invasiveness, although it has many practical uses, including improving the health of animals through feed supplementation. This report suggests that it could be used as a feed supplement for horses, thereby improving their fitness. This study was part of a larger project in which knotweed had been administered to several animal species, the health of which improved.

Japanese knotweed (*Reynoutria japonica*, syn. *Polygonum cuspidatum*), including its hybrid (*R.* × *bohemica*), has been thoroughly studied from a range of perspectives, both as an "enemy" of native flora due to its invasiveness and as a plant with many beneficial uses, including positive health effects [1–4]. As a source of resveratrol and other substances with anti-ageing and other positive effects on both animals and humans, the entire knotweed plant can also be expected to have positive effects on the health of horses. Resveratrol has already been used in horses with positive results. Resveratrol is, however, extracted mainly from roots and rhizomes; the aboveground portions of knotweed, which contain equally powerful antioxidants, deserve more thorough investigation [5]. Neochlorogenic acid was identified as an efficient antioxidant in the aboveground knotweed biomass [6]. High amounts of carotenoids in knotweed leaves are comparable to those in spinach [7]. In addition, knotweed plants produce a significant number of bioactive constituents, namely, phenolic substances derived from resveratrol, such as piceid, piceatannol, astringin and emodin, which are also found in aboveground biomass albeit in smaller quantities than in rhizomes and roots.

Among the bioactive components of knotweed, resveratrol and, more recently, piceid (also called polydatin) have been thoroughly studied. Resveratrol was found to modulate many different pathways, as it binds to numerous cell signalling molecules, modulates cell regulatory genes, activates transcription factors, suppresses pro-inflammatory genes' expression and inflammatory biomarkers, induces antioxidant enzymes and inhibits protein kinases and the expression of angiogenic and metastatic gene products. It thus has strong potential as a treatment for inflammatory, cardiovascular, pulmonary and age-related diseases such as cancer, diabetes and even Alzheimer's disease. These chronic illnesses and neurological and autoimmune diseases are accompanied by the dysregulation of multiple cell regulating pathways and are connected with inflammation. Resveratrol targets sirtuin, adenosine monophosphate kinase, nuclear factor-κB, inflammatory cytokines, anti-oxidant enzymes along with cellular processes such as gluconeogenesis, lipid metabolism, mitochondrial biogenesis, angiogenesis and apoptosis. Immunity is regulated by its interfering with immune cell regulation, pro-inflammatory cytokines' synthesis and gene expression [8]. Similarly, piceid regulates lipid metabolism, helps treat cardiovascular diseases [9] and diabetic cardiomyopathy via its anti-inflammatory and anti-oxidative effects [10], preserves mitochondrial function in the central nervous system, offers a therapeutic option for Spinal cord ischemia/reperfusion injury [11], decreases the levels of reactive oxygen species in neurons from the ischemic cortex, ameliorates oxidative stress and mitochondria-dependent apoptosis [12], fights neurodegenerative diseases including Alzheimer's, Parkinson's, cognition/memory dysfunction, brain/spinal cord injuries, ischemic stroke and miscellaneous neuronal dysfunctionalities [13], promotes a radiosensitising effect on osteosarcoma cancer cells, reduces clonogenic survival of tumor cells and induces osteogenic differentiation, alone and in the presence of ionising therapy [14]. Emodin has recently garnered increased interest, with refs. [15,16] reporting its antiviral and anti-inflammatory activities against SARS-CoV-2, for the reason that it is one of the components of the Chinese medicine LQF, the Lianhua-Qingwen formula based on 11 herbs with 61 compounds that is used for the prevention and treatment of viral diseases, including coronavirus disease 2019 (COVID-19).

Three resveratrol analogue glucosides, namely, piceid, piceatannol glucoside and resveratroloside, were found to have antibacterial effects [11], and their bioactivity was comparable to that of resveratrol, which is released in the gut due to glucoside hydrolysis. Piceid exhibited more powerful effects than resveratrol against hepatitis B virus [17,18]. Piceatannol had even more efficient anti-inflammatory, immunomodulatory, anti-proliferative, anti-leishmanial, and anti-leukaemic activities than resveratrol. Resveratroloside competitively inhibited α-glucosidase, thus alleviating postprandial hyperglycemia in diabetic mice [19]. Crude extract from knotweed roots and rhizomes inhibited such troublesome bacteria as *Staphylococus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella anatum* [20,21].

It might therefore be expected that there are even greater health effects of knotweed plants than there are of their individual components.

In horses, resveratrol has already been used with positive results. Inflammation is accompanied by increased enzymatic activity of the granulocytic enzyme myeloperoxidase (MPO), resulting in the production of the highly oxidative acid HOCl [22,23]. Resveratrol was shown [24] to substantially decrease HOCl production and mitigate inflammation in horses. Inhibitory effects of resveratrol on equine neutrophil myeloperoxidase were described [25,26]. It was found that four weeks of supplementation with 1 g/day resveratrol in old horses decreased the inflammation-induced production of cytokinin both in vitro and in vivo, indicating that resveratrol has substantial potential for the treatment of acute and chronic inflammatory horse diseases [27]. Resveratrol was also reported to reduce the gene expression of inflammatory mediators, thus allowing even old horses to move freely in training and competitions [28,29]. Resveratrol has been administered to performance horses in a number of food supplements, such as Equithrive Joint® and Resverasyn®, with the aims of reducing the effects of laminitis and slowing ageing.

Metabolic efficiency in animals decreases with age, leading to increased levels of creatine kinase and glucose. A decrease in both creatine kinase and glucose levels in horses receiving resveratrol and hyaluronic acid (EquithriveJoint®) was found [30]. EquithriveJoint® was administered to old, lame horses and a decreased serum malondialdehyde (MDA) concentration and modulated serum levels of glutathionperoxidase (GPx), catalase and superoxiddismutase (SOD) were found, indicating protective effects of EquithriveJoint® against oxidative stress and ageing [31]. A beneficial effect of EquithriveJoint® on the performance of horses with hindlimb lameness treated with triamcinolone was also demonstrated [32]. Supplementation with these plant-derived phenolics in old horses was suggested [33] to lower the doses of nonsteroidal anti-inflammatory drugs and thus reduce their side effects. Since laminitis and other diseases impair older horses, the effects of resveratrol on the health of young horses are unclear.

Based on the above evidence, there is good reason to assume that knotweed would help treat horse health issues in a similar way to resveratrol. Although the diseases occur mainly in older horses, the aim of this project was to find out whether knotweed can also improve the fitness of young horses. Reports on the health effects of supplementation with knotweed herbs in animals are scarce; a drug containing knotweed (Praziver®) helped cure equine helminthiasis [34].

Knotweed is not only one of the best sources of resveratrol, its derivatives, carotenoids [7] and neochlorogenic acid [6], but it also contains other substances, the effects of which are still not well known. A conservative approach to the use of knotweed as a dietary supplement in animals thus persists, although there have been many reports of various animal species grazing on it without problems. It is also on the list of safe plants for fodder, as it was introduced to Europe for use as feed for domesticated animals. Aboveground parts of knotweed are consumed by humans in some areas, e.g., in Japan and North America [6].

This study thus aimed to fill the knowledge gap regarding the effects of knotweed on the health status of young horses. We performed two consecutive experiments, during which the dry aboveground knotweed biomass was administered as a dietary supplement to a large number of young stallions.

2. Materials and Methods

2.1. Horses and Husbandry

Most of the horses involved in the two experiments belonged to the warmblood breed known as the Czech Warmblood, while the minority of coldblood horses belonged to two breeds, the Czech-Moravian Belgian Horse, a breed constituted from the Czech Coldblood and Moravian Coldblood, and the Silesian Norik. These horses were kept at the Regional Stud Farm Tlumačov, Czech Republic, which provides breeding services, rears foals and young stallions and performs early testing and training of stallions. Most of the stallions are sold at the age of 2–3 years, and only the selected ones are further kept for breeding and training. In October 2021, i.e., 2 years after the experiments, those warmblood horses that had been involved in the experiments and later sold to the horse keepers in the Czech Republic who got them involved in sport activities, were tracked in the records of the Czech Equestrian Federation: https://www.jezdectvi.org/kone (accessed on 25 October 2021).

In winter, the horses were kept in individual boxes on a deep straw bed and fed individually with hay, with a feed supplement and with an experimental mixture, which were always consumed without leftovers. From May to October/November they stayed on pastures at grass only. In boxes, only the 3-year-old stallions were under training, receiving 30 min of daily exercise. All of the horses were healthy and under veterinary control.

2.2. Experimental Design

Two feeding experiments in two successive winters were run with 74 and 62 young stallions. Experiment 1 lasted from 10 January 2018 to 10 May 2018, Experiment 2 from 12 November 2018 to 14 May 2019. In Experiment 1, there were 15 horses born in 2015, 24 horses born in 2016 and 35 horses born in 2017. From those 24 horses born in 2016 and

35 horses born in 2017, 11 and 27 horses, respectively, were also included in Experiment 2, according to the rule that the knotweed-fed horses from Experiment 1 were also knotweed-fed in Experiment 2 to avoid affecting the control horses with recent treatment. In addition, 24 new horses born in 2018 were included in Experiment 2. Each group of 1–3 years old stallions (foals) was divided in both seasons into two subgroups based on age, body weight and blood test values. The resulting experimental groups were not significantly different before the experiments in any of the baseline parameters, including weight, blood test results, age, breed and/or parentage. The health status of the stallions was monitored by testing blood samples.

2.3. Feed and Feeding Regimen

All stallions were fed individually in boxes, receiving hay ad libitum, a regular feed supplement and the experimental mixture, which were always consumed without leftovers. As they were kept on a deep straw bed, straw also became an occasional component of their diet.

Each horse was fed daily with:

(1) Local grass hay. The real consumption of hay per day, per horse by the 1-year-old, 2-year-old and 3-year-old warmbloods was 4, 5.5 and 8 kg hay, respectively; by the 1-year-old and 2-year-old coldbloods, it was 6 and 10 kg hay, respectively. For the nutrient content, see Table 1;

(2) A regular feed supplement for foals and stallions in amounts covering their nutrient requirements with respect to their age and breed, i.e., 1.5 and 2.0 kg for 1-year old, 2.0 and 2.5 kg for 2-year old and 2.5 and 3.0 kg for 3-year old warm- and cold-blooded horses, supplied by a regional feed producer RenoFarmy (Troubky, Czech Republic), specially prepared for the horse breeding farm Tlumačov to meet the feeding requirements of the young, 1–3-year-old stallions kept there. The feed supplement was fed to them, together with grain oats. For the composition and amounts of the individual components of the feed supplement and grain oats, see Table 1;

(3) 0.5 kg of the experimental mixture prepared and supplied by Dibaq (Helvíkovice, Czech Republic). For the composition and nutrient contents, see Table 1.

Table 1. Amounts, composition and nutrient contents of the feed for 3-year-old warmblood horses.

	Unit	Grass Hay	Feed Supplement		Experimental Mixture for Control Horses	Experimental Mixture for Knotweed-Fed Horses		Salt	Control Horses, Total	Knotweed Fed Horses, Total
			Oat Grain	Supplementary Feed		KnotWeed	Rest of Mixture			
INTAKE	kg/day	8.00	0.25	2.50	0.50	0.15	0.35	0.03	11.28	11.28
ENERGY	MJ/day	56.90	3.10	28.20	6.50	1.18	4.63	0.00	94.70	94.01
Protein	g/day	752.00	29.50	432.50	51.50	18.97	35.6	0.00	1265.50	1268.57
Lysine	g/day	24.00	1.00	17.50	1.70	0.73	1.19	0.00	44.20	44.42
Calcium	g/day	26.40	0.20	22.50	0.22	1.2	0.15	0.00	49.32	50.45
Phosphorus	g/day	25.60	0.80	15.00	1.97	0.37	1.37	0.00	43.37	43.14
Magnesium	g/day	12.80	0.30	5.00	0.42	0.39	0.29	0.00	18.52	18.78
Sodium	g/day	1.60	0.10	4.00	0.04	0.07	0.03	10.00	15.74	15.80
Selenium	mg/day	0.80	0.05	0.71	0.01	0.01	0.01	0.00	1.57	1.58
Copper	mg/day	56.00	1.50	18.30	1.78	0.77	1.25	0.00	77.58	77.82
Zinc	mg/day	206.40	8.80	255.00	10.73	5.86	7.46	0.00	480.93	483.52

The amounts of the feed supplement and composition of the experimental mixture for individual horses respecting their breed, age, weight and condition were determined according to the nutrient requirements recommended by the NRC (National Research Council, 2007), based on experience with the development of similar feeds for horses enriched with bioactive compounds of plant origin, such as Fitmin herbs Regeneration, Fitmin herbs Calmer, or Fitmin Bronchial, see https://www.fitmin.cz (accessed on 30 October 2021).

The control group of horses (not fed knotweed) received 500 g/day/individual barley pellets with soya oil and beet sugar; horses from the treatment group (fed knotweed) received a mixture of dry aboveground knotweed biomass and barley with soya oil and beet sugar (500 g/day/individual). Each horse from the treatment group received daily 150 g of dry knotweed biomass containing 0.087 g of emodin, 0.028 g of resveratrol, 0.554 g of oxyresveratrol and 0.139 g of piceid. Oxyresveratrol, which is resveratrol with an additional -OH group, and piceid, which is resveratrol glucoside, split into glucose and resveratrol in the gut, thus contributing to the overall amount of available resveratrol administered daily to each individual horse, which ultimately amounted to 0.63 g. As some of these horses had been involved in both experiments, the stallions that had already received knotweed in Experiment 1 continued receiving it in Experiment 2.

There was a warm and dry season between the two experiments, from May to November 2018, during which the horses only fed on grass from pastures.

2.4. Measured Characteristics

2.4.1. Analysis of Resveratrol, Piceid, Astringin and Piceatannol in Knotweed Biomass

Finely ground (10 mm sieve) samples (500 mg) of dry aboveground biomass were extracted with 10 mL 60% ethanol, as it was the most efficient extractant for both resveratrol and its glucosides. Prior to analysis, the samples were filtered with syringes (NY, 0.2 μm). The extracts were analysed by a validated HPLC-UV method. Instrument: Shimadzu NEXERA with PDA detection 306 nm; column: Phenomenex synergi Hydro-RP 80A, 250 mm × 4.6 mm, 4μm (30 °C); flow rate: 1.5 mL.min^{-1}; mobile phases: A–10 mM ammonium acetate at pH 4.15 using acetic acid, B-acetonitrile, concentration gradient from 7 to 90%. Standards: Sigma-Aldrich, Piceid: 98%, Resveratrol: 99%, Emodin: 98%, Astringin: 99%, Piceatannol: 99%. Mixed calibration was used for quantification. Validation report called *Determination of selected biologically active substances present in knotweed by HPLC/UV method for AKP*, has been deposited in the Library of VUOS in Pardubice Rybitví as SOP 568, VU 4661, 2007.

2.4.2. Blood Testing: Haematology and Biochemistry

At the beginning and end of each experiment, before the horses were released into pastures, they were weighed, and blood samples were drawn on the stud farm in the early morning for biochemical and haematological tests. Blood was drawn from the jugular vein and collected in a vacuum tube. One millilitre of blood was collected in a tube with EDTAK3 and was used for haematological tests, and 10 mL was collected in a tube with pro-coagulation gel and used for biochemical tests. The samples were kept cool during their rapid transport to the laboratory. Blood samples from Experiment 1 were analysed at the veterinary clinic in Brno, where they were analysed with a BC-2800 Vet haematological analyser (Mindray, Shenzhen, China) in horse mode. White blood cells were counted under a microscope in blood smears stained according to Pappenheim (May-Grünwald, Giemsa-Romanowski) in 200-cell subsamples. Blood samples from Experiment 2 were analysed in the Medila laboratory for biochemical parameters, and the haematological samples were analysed in the veterinary laboratory of the veterinary clinic Štrossovka s.r.o., Pardubice, using an automated haematological counter. The blood smears were simultaneously fixed and stained with Hemacolor®. The cellular elements (neutrophils, eosinophils, basophils, monocytes, lymphocytes and activated lymphocytes) were counted with the LeukoCounter system, and the individual populations of leukocytes were expressed as percentages of 200-cell subsamples. The following parameters were measured: total protein, albumin, urea, creatinine, cholesterol, glucose and triglyceride levels; AST, ALT and ALP activity; haemoglobin content (as the mean corpuscular haemoglobin (MCH), which is calculated by dividing the total weight of the haemoglobin by the total number of red blood cells in a particular volume of the blood sample and gives an average concentration of haemoglobin in each red blood cell throughout the sample); plateletcrit (PCT); and leucocyte, erythrocyte, thrombocyte, lymphocyte, neutrophil and monocyte counts. The albumin results could

not be compared between the two experiments because different methods were used to determine the levels in Experiment 1 (photometry) and Experiment 2 (electrophoresis). As all biochemical laboratories in the Czech Republic use the same methods, maximum divergence should not exceed 20% and low bias due to lab change is to be expected.

2.5. Statistical Data Evaluation

The data were statistically evaluated with ANOVA (STATISTICA v.12, Dell Inc., Tulsa, OK, USA). One-way ANOVA (factor: experimental treatment—control or knotweed-fed) or two-way ANOVA (factors: experimental treatment, horse age or horse type) was applied. The mean differences between the control and knotweed-fed horses were measured. If homoscedasticity was not met, the Kruskal-Wallis test was used. Multivariate redundancy analysis (RDA) was performed using Canoco 5.0 (Biometris, Plant Research International, Wageningen, The Netherlands) [35]. Biochemical and haematological characteristics and their differences between the beginning and the end of the experiments were used as response data. Experimental treatment (control or knotweed-fed) was used as the explanatory variable.

3. Results

3.1. Blood Characteristics—Overview

Since body weight was not affected by knotweed in either experiment, only blood characteristics are dealt with here. Biochemical and haematological characteristics measured at the end of the two experiments are presented in Table 2. Besides the blood characteristics of significance described below, there were also a higher number of monocytes in knotweed-fed horses but only in Experiment 1.

The parameters shown in Figure 1 are the biochemical (Figure 1a) and haematological (Figure 1b) characteristics measured in the blood samples drawn at the end of Experiment 1, explaining the variability due to treatment—the closer a vector of a certain characteristic towards "Fed knotweed", the more significantly would "feeding with knotweed" influence this characteristic. The similar direction of vectors indicates correlation among characteristics.

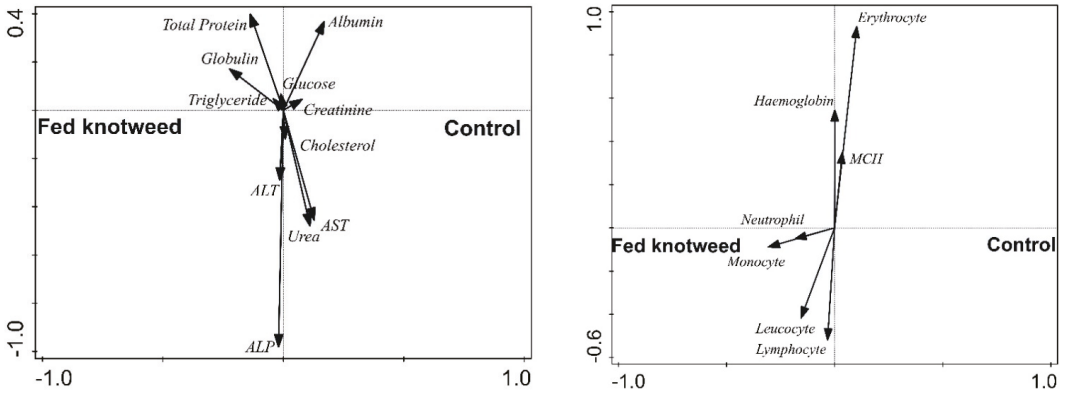

Figure 1. (**a**) Diagram showing the relationship of the biochemical data in Experiment 1. Treatment explained 1% of the variability in the biochemical data. Vectors pointing in the same direction show characteristics with positive correlations; arrows heading towards a type indicate that this type positively affects these characteristics. (**b**) Diagram showing the relatedness of the haematological data of Experiment 1. Treatment explained 1.5% of the variability in the haematological data. Vectors pointing in the same direction show characteristics with positive correlations; arrows heading towards a type indicate that this type positively affects these characteristics.

Table 2. Biochemical and haematological characteristics of horse blood from the end of Experiment 1 and of Experiment 2. Mean ± SEM. Significant differences between control and knotweed-fed horses are marked by different letters in superscript.

		Experiment 1		Experiment 2	
Biochemical Characteristic	**Unit**	**Control Horses**	**Knotweed-Fed Horses**	**Control Horses**	**Knotweed-Fed Horses**
Total protein	g/L	60.696 ± 0.581	61.787 ± 0.710	61.281 ± 0.754	60.743 ± 1.274
Albumin	g/L	36.083 ± 0.313	35.405 ± 0.377	34.452 ± 0.668	33.823 ± 0.605
Globulin	g/L	24.612 ± 0.573	26.381 ± 0.744	26.829 ± 0.703	26.920 ± 1.258
Urea	mmol/L	5.500 ± 0.141	5.324 ± 0.169	5.171 ± 0.162	5.537 ± 0.279
Creatinine	µmol/L	110.482 ± 1.981	108.787 ± 2.264	87.484 ± 1.734	88.467 ± 1.718
Cholesterol	mmol/L	2.307 ± 0.045	2.307 ± 0.054	2.061 ± 0.053	2.110 ± 0.056
Glucose	mmol/L	6.344 ± 0.192	6.399 ± 0.226	5.842 ± 0.207	5.804 ± 0.182
Triglyceride	mmol/L	0.328 ± 0.019	0.337 ± 0.018	0.274 ± 0.016	0.291 ± 0.016
AST activity	µkat/L	5.168 ± 0.062	5.053 ± 0.092	5.755 ± 0.189	5.703 ± 0.319
ALT activity	µkat/L	0.213 ± 0.006	0.215 ± 0.011	0.202 ± 0.010	0.210 ± 0.009
ALP activity	µkat/L	3.042 ± 0.148	3.077 ± 0.153	3.575 ± 0.219	3.774 ± 0.292
Haematological Characteristic	**Unit**	**Control Horses**	**Knotweed-Fed Horses**	**Control Horses**	**Knotweed-Fed Horses**
Haemoglobin	g/L	108.500 ± 2.015	108.649 ± 2.178	113.194 ± 2.940	111.233 ± 2.721
PCT	%			0.084 ± 0.003 ^{b}	0.100 ± 0.004 ^{a}
MCH	pg	13.150 ± 0.139	13.095 ± 0.148	14.074 ± 0.117	13.910 ± 0.148
Erythrocyte	10^{12}/L	10.471 ± 2.278	8.267 ± 0.124	8.001 ± 0.173	7.950 ± 0.152
Thrombocyte	10^{9}/L			161.839 ± 5.665 ^{b}	189.367 ± 7.769 ^{a}
Leucocyte	10^{9}/L	8.228 ± 0.227	8.749 ± 0.293	8.781 ± 0.319	9.047 ± 0.351
Lymphocyte	10^{9}/L	4.604 ± 0.188	4.680 ± 0.186	5.591 ± 0.286	5.756 ± 0.317
Neutrophil	10^{9}/L	3.362 ± 0.130	3.771 ± 0.223	2.830 ± 0.261	2.871 ± 0.223
Monocyte	10^{9}/L	0.028 ± 0.007 ^{b}	0.055 ± 0.007 ^{a}	0.187 ± 0.022	0.157 ± 0.023

Additional variability is explained by the horse type and age, revealed in more detailed analyses of the individual characteristics described below. Only significantly different blood parameters are reported in the following section.

3.2. Blood Characteristics of Significance

3.2.1. Total Protein

The stallions fed knotweed had a significantly larger decrease in the amount of total protein in the oldest age group (born in 2016) than the control stallions (Experiment 2, Figure 2).

3.2.2. Globulin and Albumin/Globulin Ratio

Blood tests also revealed a statistically significant effect of knotweed on the globulin level in Experiment 1. Stallions fed knotweed had a higher level of globulin than horses in the control group ($p = 0.05$), and the difference was more distinct among warm-blooded horses (Figure 3).

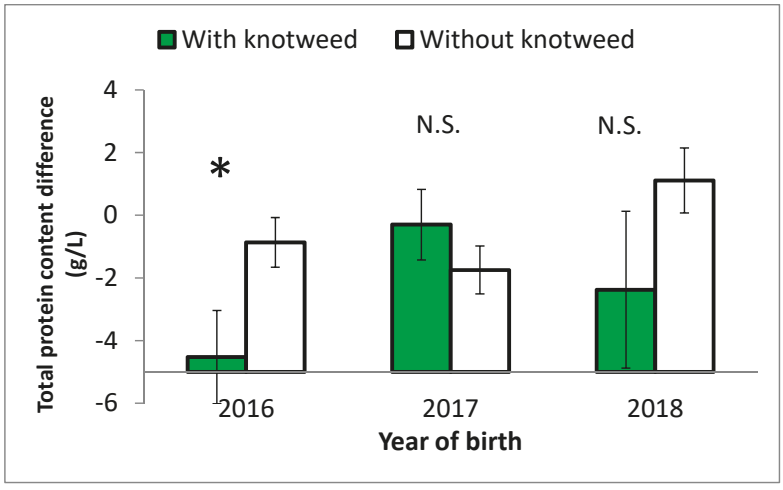

Figure 2. Differences in the total protein content in equine blood between the beginning and the end of Experiment 2, shown for stallions of the three age groups (born in 2016, 2017 and 2018) fed and not fed knotweed. Means ± SEM. Statistically different values at $p \leq 0.05$ are marked with *. N.S. = non-significant difference.

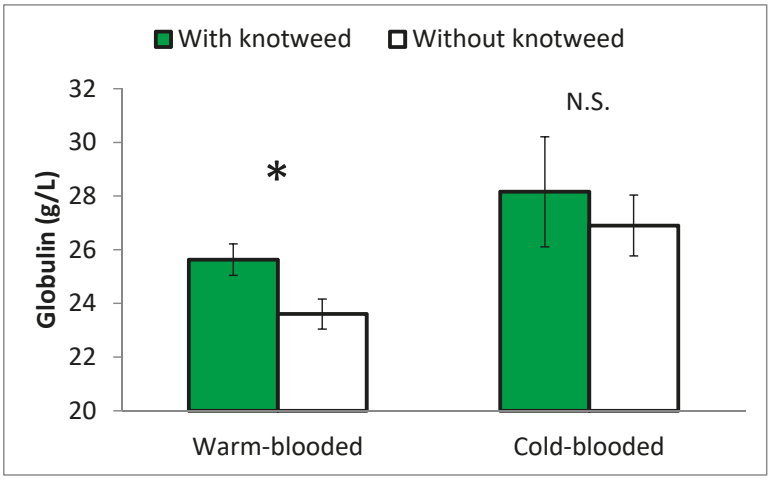

Figure 3. Globulin content in the blood of stallions of all ages. The comparison was between warm-blooded and cold-blooded stallions fed with and without knotweed, Experiment 1. Means ± SEM. Statistically different values at $p \leq 0.05$ are marked with *; N.S. = non-significant difference.

Additionally, the albumin/globulin ratio was significantly affected by knotweed supplementation ($p = 0.04$); the effect of knotweed was significant in warm-blooded horses (Figure 4).

3.2.3. Thrombocytes, Plateletcrit (PCT) and Mean Cell Hemoglobin (MCH)

In Experiment 2, the stallions fed knotweed also produced more thrombocytes ($p = 0.006$) and PCT ($p = 0.002$) than the control stallions. The MCH increase in the stallions in the control group during Experiment 2 was more pronounced than that in the stallions fed knotweed ($p = 0.02$).

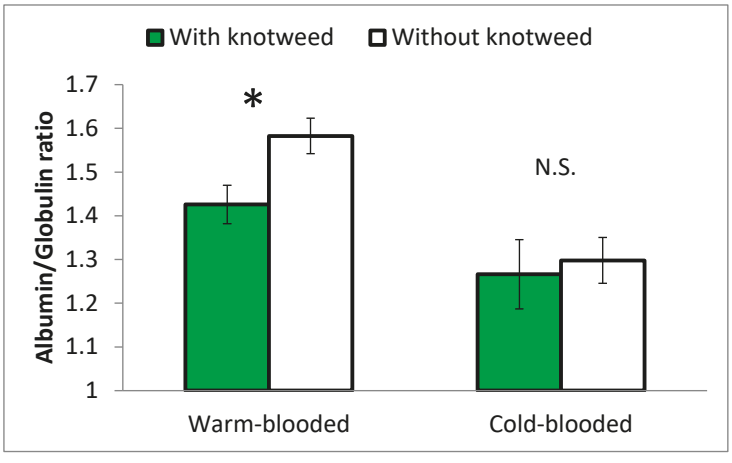

Figure 4. Albumin/globulin ratio in the blood of warm-blooded and cold-blooded stallions fed with and without knotweed, Experiment 1. Means ± SEM. Statistically different values at $p \leq 0.05$ are marked with *; N.S. = non-significant difference.

3.2.4. Activity of ALP

ALP activity decreased more in the blood of knotweed-fed horses than in the blood of the control horses ($p = 0.05$). If the warm-blooded and cold-blooded breeds were compared, the decrease was significant only in cold-blooded stallions (Figure 5).

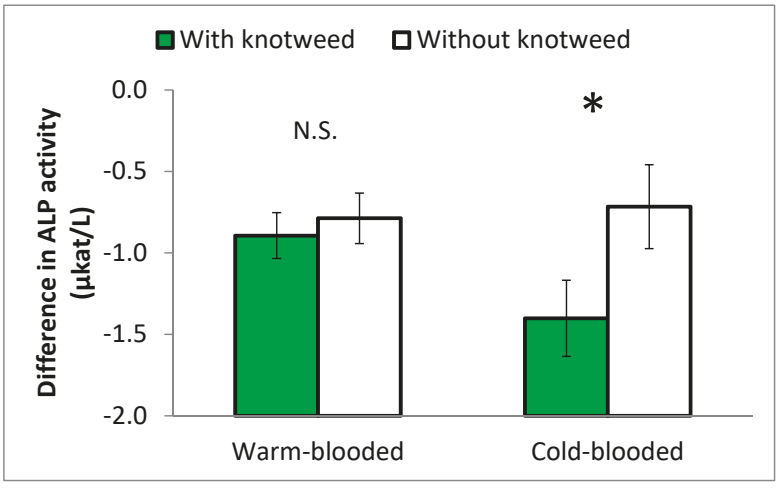

Figure 5. Differences in equine ALP activity between the beginning and the end of Experiment 1 among horses fed and not fed knotweed. The warm-blooded and cold-blooded breeds were compared. Statistically different values at $p \leq 0.05$ are marked with *. N.S. = non-significant difference.

This effect was significant in Experiment 1 but not in Experiment 2, in which a number of ALP values were missing.

3.2.5. Triglycerides

The level of triglycerides was higher in the cold-blooded horses fed knotweed than in the control ones (Figure 6).

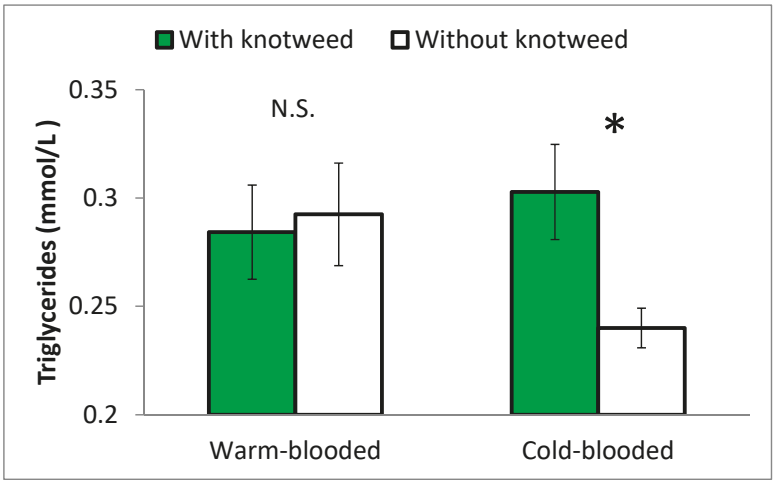

Figure 6. Levels of triglycerides at the end of Experiment 2 in the blood of horses fed and not fed knotweed. Warm-blooded and cold-blooded horses were compared. Statistically different values at $p \leq 0.05$ are marked with *. N.S. = non-significant difference.

3.2.6. Cholesterol

Both 2- and 3-year-old stallions fed knotweed showed a decrease in blood cholesterol levels during Experiment 2 while those not fed knotweed experienced an increase. The statistical difference between these two groups was significant at $p = 0.022$. However, when the warm-blooded and cold-blooded were compared separately, the effect of knotweed on cholesterol was only significant in the cold-blooded horses (Figure 7).

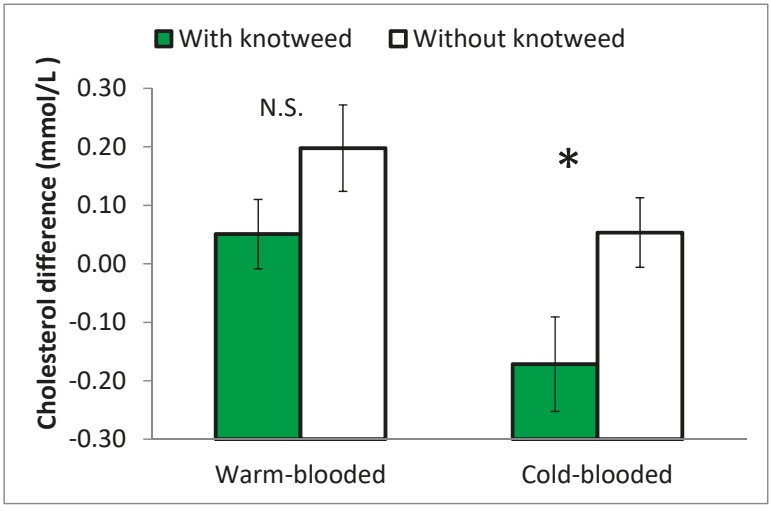

Figure 7. The differences in the blood cholesterol levels of 2- and 3-year-old stallions fed with and without knotweed from the beginning to the end of Experiment 2, comparing warm-blooded and cold-blooded. Statistically different values at $p \leq 0.05$ are marked with *. N.S. = non-significant values.

3.2.7. Urea

There was no difference in the urea blood levels between the knotweed-fed and control horses at the end of either of the two experiments. However, urea measured before the start of Experiment 2 was significantly higher (5.96 mmol L^{-1}) in the 2- and 3-year-old horses, i.e., those who had been receiving knotweed in the previous winter and spring in Experiment 1, compared with the respective control horses (5.51 mmol L^{-1}).

3.3. Sports Carrier of the Experimental Horses

Owing to a delay between the experiments and this report, it was possible to find out which warm-blooded horses were already in use in sports and what their first sport results were. By 20 October 2021, roughly equal numbers of control (14) and knotweed-fed (13) horses out of 26 in each category had already been registered for horse jumping and dressage competitions, and the control and knotweed-fed horses jumped over 104 and 109 cm high obstacles (in average; $p = 0.3$) and had been involved in 7 and 13 competitions ($p = 0.15$), respectively. These data are only of limited informative value as many other factors are involved, including socioeconomic ones. However, it is obvious that knotweed intake in feed restricted neither the fitness nor the performance of horses but rather supported it.

4. Discussion

4.1. Biochemistry and Haematology

Among the effects of knotweed on horse health, the following aspects are worth noting.

4.1.1. Total Protein, Globulins and Albumin/Globulin Ratio

The concentration of total protein represents the sum of the protein fractions in plasma: of albumin and all the fractions of globulins. Compensations occur between proteins in plasma because decreases or increases in their concentrations are common [36,37], which means that the concentration of total protein is totally dependent on the concentrations of these two groups of proteins.

Globulins are mainly involved in the immune response, transport of substances and coagulation [38]. In equine plasma, globulins account for a large proportion of the total protein. Five different groups of globulins are recognized: alpha 1 and alpha 2 globulins, beta 1 and beta 2 globulins, and gamma globulins. Altogether they account for 40–50% of the serum protein content, the remaining 50–60% of the plasma protein content is albumin. The albumin/globulin ratio is a convenient means of comparing values among horses.

In this study, concentrations of globulins in the blood of knotweed-fed horses were higher and the A/G ratio was lower than in the blood of control horses. All the values were within physiological limits and no pathology was observed in any of these experimental horses. Increased levels of globulins are often accompanied by decreased levels of albumins. Low levels of albumin may result from the decreased production of albumin or the increased loss of albumin via the kidneys, gastrointestinal tract, skin or extravascular space, or from increased catabolism of albumin [31]. Here, non-significantly lower level of albumin could indicate decreased absorption of proteins in the gut in the presence of knotweed, resulting in globulin increase, as globulins take over some of the functions of albumin. The increase of globulins suggests that the immunity of the horses was under non-specific pressure; the immunity of control horses was reduced, and their antibody levels decreased. On the contrary, the antibody levels in the stallions fed knotweed did not decline.

Increasing the plasma globulin concentration is desirable but difficult, as there are only a few means of doing so. Knotweed supplementation thus represents a suitable method.

4.1.2. Thrombocytes

Thrombocytes are non-nuclear blood elements that are crucial in the onset of blood clotting. Their number in the blood depends on their production in the bone marrow, consumption and losses. They account for the smallest proportion of blood cells. As their

average lifespan in horse blood is 4–6 days [39], their population size sensitively reflects both their production and their destruction. They contain many substances involved in inflammatory reactions, blood coagulation and other specific reactions. It is impossible to assess immune function based on the thrombocyte count, as their absolute numbers do not correlate with thrombocyte function. Therefore, although stallions fed knotweed produced more thrombocytes than the control stallions in Experiment 2, the informative value of such a finding is limited with regard to the effect of knotweed supplementation on immunity. On the contrary, the number of thrombocytes decreased in the blood of knotweed-fed pigs in our previous study [40]. The different types of knotweed biomass in the feed supplements, i.e., stems and leaves in the supplement for horses, and rhizomes and roots in the supplement for the pigs (varying in their contents of bioactive substances, namely carotenoids, resveratrol, piceid and emodin), might help explain this difference and serve as a hint for further research. The decrease in thrombocyte numbers in pigs could be explained by high resveratrol content in knotweed roots [41,42] while the increase in thrombocyte numbers in horses could be due to other bioactive compounds in knotweed leaves and stems, such as carotenoids. It was reported that tomato puree rich in carotenoids improved mice health and increased their thrombocyte numbers in mice exposed to toxic fluorides [43]. The cellular membranes of thrombocytes in the carotene-deficient blood of people infected with the dengue virus suffered from dryness, which led to the death of these cells [44]. Carotenoids were not targeted in this study, however; their considerable content in knotweed leaves has been reported [7] and they deserve further study.

PCT, plateletcrit, is a number describing ratio between the volume of thrombocytes and plasma. A higher thrombocytes number means a higher PCT [42]. In this study, PCT was higher in the blood of knotweed-fed horses than in the blood of control ones.

4.1.3. Activity of Alkaline Phosphatase (ALP)

ALP is a non-specific metallo-enzyme (Mg, Zn), a hydrolase removing phosphate group from various proteins and nucleotides and hydrolyzing inorganic phosphate, produced in liver, bones, intestines, kidneys and placenta. Only activities of ALP isoenzymes from liver and bones, both coded by the same gene, could be detected here because the other ALP isoenzymes survive in the blood only for a short amount of time. ALP from bones is typical for young individuals whose bones are growing. When the growth slows down the activity of ALP physiologically decreases, being associated with mineralisation of bones [45]. The ALP, which was lower in the blood of knotweed-fed horses than in the blood of control horses in this study, was probably not produced in the liver but in the bones as there are no statistically significant changes in any other parameters connected with the liver (AST, GMT, bilirubin). It means that the decrease in ALP activity was associated with the physiological process of adolescence. In this study, the ALP decrease was enhanced by knotweed and correlated positively with urea ($r = 0.411$, $p = 0.01$) in knotweed-fed horses in Experiment 1, indicating a positive effect of knotweed on nitrogen use from the feed, as discussed below in Section 4.1.6. The ALP values from all the horses were within physiological limits [46].

4.1.4. Triglycerides

Triglycerides are another group of lipids involved in lipid metabolism that accounts for the majority of the lipids found in adipose tissue. They are synthesised primarily in adipose tissue, the liver, small intestine, and mammary glands. Circulating concentrations of triglycerides in normal animals reflect the balance in triglyceride absorption by the small intestine, synthesis/secretion by the hepatocytes and uptake by the adipose tissue. This balance is affected by the concentration of fat in the diet and by the production of hormones such as insulin and glucagon [47]. The triglyceride levels were significantly elevated in cold-blooded stallions to such an extent that the levels in cold-blooded and warm-blooded stallions were equal (Figure 6), which indicates that knotweed stimulated lipid metabolism

in cold-blooded stallions but not in warm-blooded horses whose metabolic activities were already high.

Many studies ascribed the lowered concentration of triglycerides in the blood of different species to different active compounds from knotweed [48–52]. This study found the opposite: an increase of triglyceride concentration in the blood of knotweed-fed cold-blooded horses. The mechanism behind the increased triglycerides is not clear. This increase could be due to the different physiologies of horses or to specific active compounds from knotweed, or a combination of both. The anatomy and physiology of digestion is partly different in horses than in all of the others species, including humans, that have been tested in previous studies. A horse has no gallbladder. It means that bile is constantly passing from the liver directly to the intestine through bile duct. Pancreatic juice also constantly flows directly into the intestine. The production or release of gall and pancreatic juice in horses is not dependent on the food in the intestine or on the amount of lipids in feed like in other mammals [53]. If some active compounds from knotweed, such as piceatannol, could change the amount of bile acids in the gall, different amounts of lipids from food could be absorbed. Epicatechin-3-O-gallate from knotweed could increase the concentration of triglycerides in the blood by blocking lipase [48,54]. The horses in this study were fed a common diet without higher amounts of lipids and statistically significant results were within physiological ranges. We cannot say whether the triglyceride levels were increased due to the absorption from food or the release from adipose tissues. Answers to these questions are beyond the scope of this study. However, it shows an interesting potential of knotweed supplementation to support the breeding and performance of cold-blooded horses.

4.1.5. Cholesterol

Cholesterol is a fatty substance either obtained from food in the gut or produced in the liver when needed. It is a necessary component of all cell membranes and a fundamental component of many molecules, such as steroid hormones; it is also involved in the transport of lipids between different tissues in the body and the blood [47]. This study revealed more considerable reductions in the concentrations of blood cholesterol in the cold-blooded knotweed-fed horses. There are many studies describing different mechanisms by which resveratrol decreased the cholesterol concentration in blood, e.g., by reducing the basal and insulin-induced glucose conversion to total lipids in white adipose tissue [55]. There are also studies which found no effect of resveratrol on cholesterol concentration in horse blood [30]. Again, it seems that different active compounds are behind these diverse effects of knotweed. Another study [50] reported that a piceatannol-enriched diet decreased the cholesterol concentration in blood due to lowering its absorption in the intestine resulting from higher excretion of bile acids to the intestine. Piceid is also known for decreasing cholesterol concentrations [49].

All of the animals involved in the experiments reported here were healthy; no lipid metabolism disorders were noted. Nevertheless, their metabolisms were improved due to the reduction in cholesterol concentration. Knotweed, owing to its content of resveratrol possessing hepatoprotective effects, can help prevent steatosis, which is an abnormal retention of fat in an organ, usually the liver [56,57].

4.1.6. Urea

The urea measured in the blood of horses that had received knotweed in the previous winter and spring was higher than the urea in the blood of the control horses. All of these horses spent the extremely hot and dry summer between the two successive experiments on pastures and only fed on grass. It was reported [58] that changes in grass quality in the growth season and its protein (nitrogen) content correlate with blood urea level but do not affect the blood protein in horses on grass. The decreased grass quality resulted in lower blood urea content by up to half of its normal level [59]. In this study, the higher urea level

in earlier knotweed-fed horses could indicate their better protein availability as all of the horses were on the same grass.

4.1.7. Urea, ALP, Triglycerides and Cholesterol Interactions

Urea was also positively correlated with ALP decreasing with horse weight, the decrease being enhanced by knotweed in Experiment 1. In Experiment 2, ALP was negatively correlated with cholesterol ($r = -0.435$, $p = 0.03$) in the control horses but not in the knotweed-fed horses, revealing the role of ALP in the metabolism of phosphorus associated with cholesterol presumably as phospholipids, i.e., amphiphatic molecules enabling the water-solubility of cholesterol. Similarly, the cholesterol and triglycerides showed a non-significant correlation in the horses fed knotweed compared to the horses not fed knotweed, where a highly significant correlation was found ($p = 0.001$).

When all of the age categories were evaluated together, the same knotweed effect was noted at the end of both experiments: the control horses not fed knotweed had a highly significant correlation between triglycerides and cholesterol ($p = 0.01$ in Experiment 1 and $p = 0.001$ in Experiment 2), but in the blood of all of the knotweed-fed horses, there was a non-significant correlation between triglycerides and cholesterol in either experiment. When only 1- and 2-year-old horses from Experiment 1 were taken into account, reappearing as 2- and 3-year-old horses in Experiment 2, a significant correlation was found only in the horses not fed knotweed before the start of Experiment 2. This reveals a strong influence on this ratio of 3-year-old horses, i.e., the heaviest ones.

These urea, ALP, cholesterol and triglyceride interrelations indicate the effect of knotweed on the protein and lipidic (lipoprotein, phospholipid) metabolism of horses. Several authors [59,60] have reported largely increased triglyceride content due to the seasonal food deprivation of only grass-fed equines. Others [61,62] have discerned three major classes of lipoproteins in equine plasma: very low density lipoproteins (VLDL, 24 per cent of the total plasma lipoprotein mass) rich in triglycerides and cholesterol and poor in protein (apolipoprotein B-100 and apoB-48), cholesterol-rich low density lipoproteins (LDL, 15 per cent) with three discrete subfractions, and protein-rich HDL with the dominant protein apoA-I (HDL, 61 per cent), all composed of lipids and apoliproteins in different proportions. Mass spectrometry revealed that the apolipoproteins contain etw. 400–500 amino acids. These might serve for protein synthesis as one of the means of lipoprotein utilisation.

Several papers [59,63] report that in horse serum (plasma), the cleavage of VLDL mainly releases triglycerides, while LDL largely releases cholesterol. In this study, the loss of correlation between triglycerides and cholesterol in knotweed-fed horses as compared with control ones could be explained if different types of lipoproteins were cleaved in knotweed-fed horses than in control horses not fed knotweed.

A similar loss of (negative) correlation in knotweed-fed horses, which was recorded between cholesterol and ALP, would suggest that, besides apolipoproteins, phospholipids as solubilising components and ALP targets are associated with the cholesterol particles.

The low quality of pasture grass later in the summer might be perceived as a period of mild fasting, producing higher blood triglyceride levels [59,60]. However, all of the changes measured in the blood plasma were within normal limits. Ref. [64] states that equine energy metabolism evolved in sparse grassland environments and has been adapted to the intake of a low energy diet with large quantities of roughage rich in cellulose and lignin, and that equids shift between the glucose-oriented metabolic pattern of non-ruminants and a metabolism similar to that of the ruminants when fed with a roughage-based diet. Since the blood glucose level is maintained within a narrow limit, preservation of its supply depends on gluconeogenesis, and protein catabolism increases to provide amin acids as precursors for glucose synthesis. However, more detailed studies are needed to reveal which metabolic processes are affected by knotweed in horse diets.

Meanwhile, we assume that even in earlier knotweed-fed horses, the higher urea levels indicating higher protein availability, together with a change in the ratio of lipidic substances, might have resulted from the long-lasting effects of knotweed on the metabolism of

lipoproteins. Different types of lipoproteins would be metabolised with varying intensities, and the apolipoproteins released, together with other internal sources, might help horses overcome the periods of protein starvation on dry pastures lacking high quality grass.

5. Conclusions

The results obtained in this study indicate that knotweed consumption helps (1) improve the immunity of horses by increasing their blood globulin levels, (2) stimulate lipid metabolism in cold-blooded horses and (3) lower cholesterol levels. These effects have been found in young and healthy horses and are expected to be more pronounced in older horses.

Because knotweed has the potential to stimulate the release of fat from hepatic cells and thus speed up liver regeneration processes, it could reduce the risk of adverse health consequences in horses with impaired lipid metabolism.

The sporting success of the horses involved in the experiments was monitored. Half of the foals from both the control and knotweed-fed groups have been in use in sports in the Czech Republic since 2018, i.e., 2 years after the second experiment. The knotweed-fed horses achieved slightly better results than control horses in jumping competitions.

6. Patents

This study is a part of a more extensive project resulting in the registration of the Utility Model "Compound feed containing knotweed for game, domestic and farm animals".

Author Contributions: Conceptualisation, P.M., M.K. and J.N.; methodology, M.K., P.M., T.F., Š.V. and J.N.; formal analysis, T.F.; investigation, M.K. and Š.V.; data curation, T.F.; writing—original draft preparation, M.K.; writing—review and editing, all authors; visualisation, T.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Mendel University in Brno, 21 October 2021. Feeding experiments are not applicable according to the law of the Czech Republic.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available from the third author.

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Effect of Sainfoin (*Onobrychis viciifolia*) Pellets on Rumen Microbiome and Histopathology in Lambs Exposed to Gastrointestinal Nematodes

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Abstract: Our study analyzed the ruminal fermentation and microbiome, hematological profile, and abomasal histopathology of lambs experimentally infected with a gastrointestinal nematode (GIN) and fed sainfoin pellets (SFPs; 600 g DM/d/animal) for 14 d. Twenty-four lambs infected with *Haemonchus contortus* were divided into two separated groups: animals fed meadow hay (control) and animals fed SFPs. The ruminal contents, fermentation parameters, and microbiome in vitro and in vivo were determined using molecular and microscopic techniques. Ruminal contents in the SFP group indicated smaller populations of *Archaea* ($p < 0.001$), *Methanomicrobiales* ($p = 0.009$), and lower methane concentrations in vitro ($p = 0.046$) and in vivo ($p = 0.030$) than the control group. The relative abundance of *Butyrivibrio fibrisolvens* quantified by real-time PCR was higher in the lambs with the SFP diet ($p = 0.05$). Haemonchosis affected the number of red blood cells of the lambs ($p < 0.001$). The lambs in the SFP group had a higher percentage of damaged abomasa glands than did the control group ($p = 0.004$). The consumption of SFPs by GIN-infected lambs may affect ruminal methanogens and subsequently decrease methane emission without undesirable changes in the ruminal microbiome or the health of the animals.

Keywords: bacteria; flavonoids; hematological profiles; methane concentration; plant bioactive components; ruminal fermentation; sheep

1. Introduction

Parasitic infection in ruminants, mainly by gastrointestinal nematodes (GINs), influences the intensity of emissions of greenhouse gases and substantially increases the yield of methane emission compared to uninfected animals [1,2]. Promising new nutraceuticals containing bioactive components in ruminant nutrition, however, could have both anthelmintic and anti-methanogenic properties [3,4]. Plant additives with bioactive components can modulate the bacterial, archaeal, and eukaryotic populations in the rumen by interactions

between diet and the microbes because the microbiome plays a crucial functional role in nitrogen use, fermentation, and methane concentration [5].

Plant bioactive components such as flavonoids and condensed tannins (CTs) in feeds have the potential to reduce environmental methane pollution from ruminants by complex bioactivity occurring simultaneously in plants and animals [6]. The main bioactive components in the tanniferous legume sainfoin (*Onobrychis viciifolia*) are flavonoids and CTs formed by the polymerization of flavan-3-ols, with high proportions of prodelphinidins (70%) and procyanidins (30%) [7]. Tanniferous forages are rich in prodelphinidins, have higher antiparasitic activity, and have the effect of reducing methane emissions [8–10]. Sainfoin also reduces the degradation of feed proteins without affecting the digestibility of the nonprotein fraction, thereby increasing the flow of non-ammonia nitrogen and essential amino acids into the small intestine and reducing urinary nitrogen losses [11,12]. The ability of sainfoin to reversibly bind proteins leads to a reduction in GIN parasitism in small ruminants [13,14]. Many studies have focused on the use of sainfoin for its nutritional and anthelmintic effects, but it also contains beneficial flavonoids with similar mechanisms of action as tannins and similarly interferes with the biology of GINs [15]. Combining CTs with quercetin or luteolin identified synergistic anthelmintic effects between tannins and flavonoid monomers [16]. The production of sainfoin pellets (SFPs) at high temperatures and pressure does not affect their bioactivities associated with antioxidative properties [17]. Based on previous studies [18,19], we hypothesized that SFPs would also contribute to desired changes in the ruminal microbiome and histopathology in lambs loaded with parasites.

Analyses of ruminal microbiomes and histopathological observations are needed to identify the possible consequences of bioactive components used in the nutrition of parasite-laden lambs. Our aim was to (1) identify the main flavonoids and phenolic compounds of the SFPs and (2) determine the ruminal fermentation and microbiome, hematological profile, and histopathology of the abomasum of lambs infected with GINs during consumption of SFPs for 14 d.

2. Materials and Methods

2.1. Ethics Statement

This study was conducted following the guidelines of the Declaration of Helsinki and national legislation in the Slovak Republic (G.R. 377/2012; Law 39/2007) for the care and use of research animals. The experimental protocol was approved by the Ethical Committee of the Institute of Parasitology of the Slovak Academy of Sciences on 22 November 2020 (protocol code 2020/21).

2.2. Animals, Diets, and Experimental Design

We housed 24 male lambs (Improved Valachian) 3–4 months of age with an average initial body weight of 15.0 $\pm$ 2.07 kg in common stalls for 7 d for a period of adaptation and another 7 d for acclimatizing to feeding, with free access to water. The lambs were obtained from a commercial farm (PD Ružín–Ružín farm, Kysak, Slovakia) where they were also housed during the experiment. Each animal was fed daily meadow hay (MH) *ad libitum* and 300 g dry matter (DM) Mikrop ČOJ, a commercial concentrate (MIKROP, Čebín, Czech Republic). The number of animals used in the experiment was assigned following VICH GL13 guidelines (Veterinary International Committee on Harmonization—Efficacy of anthelmintics: specific requirements for ovine). At the beginning of the experiment—day (D) 0, all parasite-free lambs were infected orally with approximately 5000 third-stage larvae of the MHCo1 strain of *Haemonchus contortus* susceptible to anthelmintics [20]. A modified McMaster technique [21] with a sensitivity of 50 eggs per gram (EPG) of faeces was used for detecting *H. contortus* eggs on D30. The lambs were divided into two groups of twelve animals each (one stall per group) on D30 after infection, when all parasites had matured to the adult stage: control animals fed MH (control, MH, 600 g DM/d/animal) and animals fed sainfoin pellets (SFPs, 600 g DM/d/animal). Both groups continued to be fed commercial concentrate (300 g DM/d/animal). All lambs were positive with a mean

EPG of 9405 ± 4584 in the SFPs group and a mean of 11420 ± 372 in the control group. SFPs were obtained from a commercial source (NATURE'S BEST, EQUOVIS GmbH, Münster, Germany). This feeding scheme continued for 14 d. The lambs were weighed at the end of the experiment and had an average final body weight of 18.3 ± 3.22 kg. All animals were killed at the end of the experiment following the rules of the European Commission (Council Regulation 1099/2009) for slaughtering procedures [22].

2.3. Experiment In Vitro

In vitro gas fermentation technique (IVFT) has been widely used to evaluate the nutritive value of feeds for ruminants and to assess the effect of different nutritional strategies on methane (CH_4) production. Therefore, IVFT using batch-culture incubations of buffered ruminal fluid incubated at 39 °C for 24 h under anaerobic conditions was used [23]. Control animals were donor animals for control groups and SFPs animals were donor animals for SFPs groups for the in vitro experiment. At the end of the experiment the ruminal contents (RCs) were taken from each lamb of each group immediately after slaughter in the abattoir, packed in prewarmed flasks and transported to the laboratory. RCs were pushed through four layers of gauze and pooled in equal volumes based on control and SFP groups. The pooled RCs were purged with CO_2, mixed with McDougall's buffer [24] in a 1:2 ratio, and dispensed in volumes of 35 mL into fermentation bottles (120 mL) containing 250 mg (DM basis) of substrate. The meadow hay or SFPs were used as the substrates of a ration with commercial concentrate (800:200, *w*/*w*) as the components of the diets for the controls and SFP groups for in vitro experiment. Commercial concentrate, MH, and SFPs were ground using a grinder (Molina, MIPAM, České Budějovice, Czech Republic) and sieved through 0.15–0.40 mm screens. The in vitro experiment had a completely randomized design using the two diets (control and SFP) in fermentations with the two inocula of ruminal fluids (control and SFPs), with three replicates (three incubation bottles) for each diet and inoculum. The in vitro experiment was repeated three times within three consecutive days ($n = 3 \times 3$).

2.4. Chemical Analysis of the Dietary Substrates

The chemical compositions of the dietary substrates (Table 1) were analyzed using standard methods [25,26].

Table 1. Chemical compositions of the dietary substrates.

Substrate	DM (g/kg)	NDF (g/kg DM)	ADF (g/kg DM)	CP (g/kg DM)	N (g/kg DM)	Ash (g/kg DM)
Meadow hay	885	640	423	84	14	84
Concentrate	888	231	130	211	34	104
SFPs	918	460	357	121	19	100

DM, dry matter; NDF, neutral detergent fiber; ADF, acidic detergent fiber; CP, crude protein; N, nitrogen; SFPs, sainfoin pellets.

2.5. Analysis of Bioactive Compounds

SFPs were ground to a fine powder, and 100 mg were extracted three times with 80% MeOH at 40 °C for 60 min. The extracts were evaporated to dryness and were then dissolved in 2 mL of Milli-Q water (acidified with 0.2% formic acid) and purified by solid-phase extraction using an Oasis HLB 3 cc Vac Cartridge (Waters Corp., Milford, CT, USA) as was previously described [27]. Bioactive compounds were analyzed by ultrahigh resolution mass spectrometry (UHRMS) on a Dionex UltiMate 3000RS system (Thermo Scientific, Darmstadt, Germany) with a charged aerosol detector connected to a high-resolution quadrupole time-of-flight mass spectrometer (Compact, Bruker Daltonik GmbH, Bremen, Germany). Phenolic acid and flavonoids were identified chromatographically on a Kinetex C18 column (2.1 × 100 mm, 2.6 µm, Phenomenex, Torrance, CA, USA), with mobile phase A consisting of 0.1% (*v*/*v*) formic acid in water and mobile phase B consisting of 0.1% (*v*/*v*) formic acid in acetonitrile, as was previously described [19]. Stock solutions of hyperoside and chlorogenic acid were prepared in 80% MeOH at concentrations of 2.5 and 3.6 mg/mL,

respectively, and kept frozen until used. Calibration curves for these two compounds were constructed based on seven concentration points (from 500 to 3.6 µg/mL). Hyperoside was used to calculate the number of flavonoids identified in the extract, and chlorogenic acid was used for phenolic acids, using Bruker QuantAnalysis 4.3 software (Bruker Daltonik GmbH, Bremen, Germany). All analyses were performed in triplicate.

2.6. Basic Ruminal Fermentation Analysis

RC samples from the in vitro and in vivo experiments were collected for determining pH, methane, volatile fatty acids (VFAs), ammonia concentrations, in vitro DM digestibility (IVDMD), and population of ruminal microorganisms (bacteria, protozoa, and methanogens). Concentrations of methane in vitro and VFAs were determined by gas chromatography on a PerkinElmer Clarus 500 gas chromatograph (Perkin Elmer, Inc., Shelton, CT, USA) [28]. Methane concentration in vivo was calculated by measuring the molar proportions of the VFAs in the rumen as: 57.5 mol glucose = 65 mol acetate + 20 mol propionate + 15 mol butyrate + 60 mol CO_2 + 35 mol CH_4 + 25 mol H_2O [29]. The concentration of ammonia-N was determined using the phenol-hypochlorite method [30].

2.7. Rumen Microbial Quantification

Samples for counting ciliate protozoa from the RCs were fixed in equal volumes of 8% formaldehyde, and the protozoa were counted and identified microscopically [31]. Total bacteria, *Archaea*, *Methanobacteriales*, and *Methanomicrobiales* from the in vitro experiment and *Archaea* and *Methanobacteriales* from the in vivo experiment were quantified using fluorescence in situ hybridization as described previously [32]. DNA for quantifying bacteria was isolated from the ruminal samples using a Mini Bead-Beater (BioSpec, Bartlesville, OK, USA) to lyse the cells [33] followed by purification using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). DNA concentrations and qualities were measured using a NanoPhotometer R NP80 (Implen GmbH, München, Germany). Total bacteria, *Streptococcus bovis*, *Butyrivibrio proteoclasticus*, *B. fibrisolvens*, *Fibrobacter succinogenes*, *Megasphaera elsdenii*, *Ruminococcus albus*, *R. flavefaciens*, and the genera *Prevotella and Lactobacillus* were quantified by real-time PCR using the PCR primers [34–39].

2.8. Hematological Parameters

Samples of blood were collected from the jugular vein of each animal on D0, D23, D30, D37, and D44. Basic hematological parameters were determined using an Abbott CELL-DYN 3700 hematological analyzer (Global Medical Instrumentation, Inc., Ramsey, NJ, USA).

2.9. Histopathology

Samples of fresh abomasal tissues were washed in a phosphate buffer (0.1 M, pH 7.4), put in plastic containers, and fixed in a 10% buffered FA solution as pieces of tissue spread on a flat piece of polystyrene as previously described [19]. The fixed material was processed using a series of reagents in the following sequence: 75% alcohol for 1 h, 90% alcohol for 1 h, 95% alcohol for 1 h, 100% alcohol 3 times for 1 h. Then, the material was cleared in xylene 3 times for 1 h. The material was infiltrated in paraffin 3 times for 1 h 20 min. The described steps took place in a tissue processor (Excelsior AS Thermo Scientific, Runcorn, UK). Afterward, specimens were embedded in Paraplast PLUS paraffin blocks (Leica, Buffalo Grove, IL, USA), which were then cut with a rotary microtome into sections 2.5 µm thick. Slides with a paraffin section were automatically stained with hematoxylin and eosin (Varistain Gemini Thermo Scientific, Runcorn, UK). An Axio Lab. A1 microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss Axiocam ERc 5s digital camera was used for histological evaluation. Photographs were analyzed and recorded using ZEN 2.3 (blue edition) software (Carl Zeiss Microscopy GmbH, Oberkochen, Germany, 2011).

2.10. Statistical Analysis

Data for the fermentation parameters and microbial populations were analyzed using an unpaired *t*-test (GraphPad Prism 8; GraphPad Software, Inc., San Diego, CA, USA). Two–way analyses of variance were used for analyzing the hematological parameters as models representing the two animal groups (control and SFPs) and sampling days (D0–D44). The effects included in the model were treatment (T), time, and the interaction between them (T × time). Results were considered significant at $p < 0.05$.

3. Results

3.1. Phytochemicals

The phytochemical substances in the SFPs consisted of 32.56 g/kg DM flavonoids, 4.68 g/kg DM phenolics, and 0.37 g/kg DM others (glycosides, hydroxy fatty acids, and a derivative of cinnamic acid, Table 2).

Table 2. The main phytochemicals in the MeOH extract of the sainfoin pellets.

No.	RT (min)	UV (nm)	m/z [M-H]$^-$	MS ^{2} Main Ion	MS ^{2} Fragments	Formula	Compound	mg/g DM
1	2.4	261,296	153.018	109.0291		$C_7H_6O_4$	3,5-Dihydroxybenzoic acid	0.26
2	2.6	261,296	153.018	109.0291		$C_7H_6O_4$	2,4-Dihydroxybenzoic acid	0.29
3	4.6	245sh,348	175.0601	157.0488	131,115	$C_7H_{12}O_5$	3-Isopropylmalic acid	0.28
4	5.1	248sh,326	353.0884	191.0557		$C_{16}H_{18}O_9$	Trans-5-caffeoylquinic acid	0.37
5	5.9		367.1034	193.0501	173.134	$C_{17}H_{20}O_9$	3-*O*-Caffeoyl-4-*O*-methylquinate	0.24
6	6.2	282,316	239.0563	179.034	195,221,149	$C_{11}H_{12}O_6$	Derivative of cinnamic acid	0.09
7	6.7	269,348	771.1993	609.1451	462,301	$C_{33}H_{40}O_{21}$	Quercetin 3-rutinoside 7-galactoside	0.26
8	8.6	269,345	355.1042	161.0227	193.179	$C_{16}H_{20}O_9$	Trans-feruloylglucose	0.25
9	9.4	269,340	625.1400	316.0221	117.163	$C_{27}H_{30}O_{17}$	Myricetin-3-rutinoside	1.91
10	9.5	280	325.0937	119.0497	117.163	$C_{15}H_{18}O_8$	8-β-Glucopyranosyloxycinnamic acid	3.27
11	9.8	266,346	623.1262	285.0408	447	$C_{27}H_{28}O_{17}$	Luteolin 4′-glucoside 7-galacturonide	1.80
12	9.9	262,345	739.2094	284.0327	572,255,178	$C_{33}H_{40}O_{19}$	Kaempferol 3-(2″-rhamnosylrutinoside)	0.32
13	10.2	265,349	339.1092	145.0276	163	$C_{16}H_{20}O_8$	TPCA [1]	0.27
14	10.5	256,354	609.1462	300.0281		$C_{27}H_{30}O_{16}$	Rutin	18.92
15	10.8	269,354	463.0912	300.0269	271,151	$C_{21}H_{20}O_{12}$	Quercetin-4′-glucoside	1.06
16	10.9	269,343	637.1414	299.0567	284.2337	$C_{28}H_{30}O_{17}$	MHGB [2]	2.03
17	11.0	264,346	447.0930	285.0397	269,209,251	$C_{21}H_{20}O_{11}$	Kaempferol 3-*O*-glucoside	0.64
18	11.4	265,344	593.1527	285.0408	151,327,178	$C_{27}H_{30}O_{15}$	Kaempferol 3-*O*-glucoside [3]	1.87
19	11.6	254,354	623.1626	315.0515	151,243,271	$C_{28}H_{32}O_{16}$	Isorhamnetin 3-*O*-rhamnoglucoside	3.75

[1] Trihydroxy-6-[4-(3-oxobutyl)phenoxy]oxane-2-carboxylic acid; [2] 2-(3-Methoxy-4-hydroxyphenyl)-5-hydroxy-7-[2-*O*-(beta-D-glucopyranuronosyl)-beta-D-glucopyranosyloxy]-4H-1-benzopyran-4-one; [3] Kaempferol 3-*O*-glucoside-7-rhamnoside.

3.2. Ruminal Fermentation In Vitro

Methane concentration was significantly lower ($p = 0.046$), but IVDMD ($p < 0.001$), total VFA concentration ($p = 0.011$), and *n*-valerate concentration ($p = 0.017$) were significantly higher, in the SFP than the control group (Table 3). *Archaea* and *Methanomicrobiales* populations were smaller in the SFP than the control group ($p < 0.001$ and $p = 0.009$, respectively).

Table 3. Effect of SFPs on ruminal fermentation and microbial populations in vitro.

	Control	SFP	SEM	*p*
Ammonia N (mg/L)	270	282	15.5	0.849
Methane (mmoL)	1.15	1.02	0.063	0.046
pH	7.60	7.45	0.041	0.064
IVDMD (g/kg DM)	311	447	18.0	<0.001
Total VFAs (mmoL/L)	31.5	39.9	1.76	0.011
Acetate (mol%)	65.5	66.1	0.997	0.768
Propionate (mol%)	17.7	17.1	0.799	0.711

Table 3. *Cont.*

	Control	SFP	SEM	*p*
n-Butyrate (mol%)	11.0	11.2	0.239	0.764
iso-Butyrate (mol%)	0.861	0.617	0.158	0.455
n-Valerate (mol%)	2.25	2.56	0.066	0.017
iso-Valerate (mol%)	2.50	2.27	0.115	0.318
Caproate (mol%)	0.150	0.181	0.019	0.430
Acetate: propionate	4.02	4.10	0.353	0.923
Total protozoa (10^3/mL)	6.65	8.7	6.612	0.438
Total bacteria (10^8/mL)	4.02	3.9	0.164	0.721
Archaea (10^6/mL)	3.93	2.64	0.240	<0.001
Methanobacteriales (10^6/mL)	1.53	1.48	0.024	0.363
Methanomicrobiales (10^5/mL)	1.56	1.42	0.027	0.009

IVDMD, in vitro dry matter digestibility; VFAs, volatile fatty acids.

3.3. Ruminal Fermentation and Microbiota in the Lambs

The SFPs significantly affected ($p = 0.030$) methane concentrations in the lambs (Table 4). Total protozoa, expressed as a count per gram of wet RC (wRC) in the lambs, did not differ significantly ($p = 0.05$) between the groups. The populations of *Archaea* ($p < 0.001$), *Methanomicrobiales* ($p = 0.009$), and *Methanobacteriales* ($p < 0.001$) were significantly smaller for the SFP than the control group. The relative abundances of *S. bovis*, *B. proteoclasticus*, *F. succinogenes*, *M. elsdenii*, *R. albus*, *R. flavefaciens*, and the genera *Prevotella* and *Lactobacillus* did not differ significantly between the groups ($p = 0.05$). The relative abundance of *B. fibrisolvens* was significantly higher in the SFP than in the control group ($p = 0.006$). The relative abundance of the 16S rRNA gene was expressed as an arbitrary unit (AU) relative to the total abundance of bacterial genes of the control group.

Table 4. Effect of SFPs on ruminal fermentation and microbial populations in the lambs.

	Control	SFP	SD	*p*
Ammonia N (mg/L)	205	199	79.1	0.849
Methane (mmoL)	3.17	2.30	0.903	0.030
pH	6.36	6.37	0.575	0.974
Total VFAs (mmoL/L)	68.2	70.2	27.71	0.847
Acetate (mol%)	74.6	73.3	4.33	0.462
Propionate (mol%)	11.9	10.5	4.42	0.425
n-Butyrate (mol%)	11.1	13.5	3.96	0.123
iso-Butyrate (mol%)	0.06	0.06	0.140	0.946
n-Valerate (mol%)	1.33	1.60	0.828	0.414
iso-Valerate (mol%)	0.89	0.77	0.595	0.633
Caproate (mol%)	0.20	0.34	0.473	0.457
Acetate: Propionate	7.19	7.91	0.529	0.507
Total protozoa (10^5/g wRC)	6.7	8.2	5.61	0.508
Archaea (10^7/mL)	7.71	4.79	1.84	<0.001
Methanobacteriales (10^7/mL)	3.74	2.90	0.672	<0.001
Methanomicrobiales (10^7/mL)	3.50	2.50	0.463	0.009
Total bacteria (AU)	1.0	1.01	0.110	0.863
S. bovis (AU)	1.0	1.04	0.145	0.525
B. proteoclasticus (AU)	1.0	1.06	0.161	0.325
B. fibrisolvens (AU)	1.0	1.13	0.124	0.006
F. succinogenes (AU)	1.0	0.97	0.311	0.832
M. elsdenii (AU)	1.0	0.94	0.258	0.587
R. albus (AU)	1.0	1.10	0.175	0.160
R. flavefaciens (AU)	1.0	0.97	0.147	0.594
Genus *Prevotella* (AU)	1.0	0.96	0.299	0.729
Genus *Lactobacillus* (AU)	1.0	1.04	0.126	0.500

wRC, count per gram of wet ruminal content; AU, arbitrary unit.

3.4. Hematological Parameters

The complete red blood cell (RBC) hemograms of each infected animal identified clinical signs of haemonchosis such as anemia after D23 (Table 5). The RBC count, level of hemoglobin (HGB), hematocrit (HCT), and mean corpuscular volume (MCV) were not significantly influenced by treatment or the interaction between treatment and time ($p = 0.05$). Time significantly affected all RBC parameters ($p < 0.001$). Total leukocytes, neutrophils, and lymphocytes were not significantly influenced by treatment, time, or the interaction between them ($p = 0.05$, Table 6). Treatment and time significantly affected the counts of monocytes ($p = 0.05$) and basophils ($p = 0.05$ and $p = 0.01$, respectively), and time significantly affected the counts of eosinophils ($p < 0.001$).

Table 5. Effects of SFPs on the red blood cell parameters of the lambs.

	Day	Control	SFP	SD	*p*		
					Treatment (T)	Time	T × Time
RBC (T/L)	0	11.37	11.63	1.176	0.133	<0.001	0.681
	23	7.68	7.10	1.289			
	30	7.22	6.56	1.382			
	37	7.15	6.55	1.373			
	44	7.00	6.83	1.276			
Hemoglobin (g/L)	0	111.7	115.5	11.62	0.326	<0.001	0.462
	23	74.6	68.9	11.30			
	30	71.5	64.8	12.89			
	37	72.0	67.1	14.28			
	44	69.5	71.7	14.29			
Hematocrit (g/L)	0	0.263	0.265	0.0251	0.208	<0.001	0.458
	23	0.187	0.171	0.0261			
	30	0.182	0.165	0.0313			
	37	0.185	0.174	0.0339			
	44	0.178	0.187	0.0336			
MCV (fL)	0	23.2	23.0	1.86	0.281	<0.001	0.354
	23	24.5	24.4	2.11			
	30	25.4	25.3	2.25			
	37	26.0	26.7	2.04			
	44	25.6	27.4	2.29			

RBC, red blood cell; T/L, 10^{12} per liter; MCV, mean corpuscular volume; SD, standard deviation.

Table 6. Effects of SFPs on the total leukocyte and differential counts of the lambs.

	Day	Control	SFP	SD	*p*		
					Treatment (T)	Time	T × Time
Total leukocyte (G/L)	0	8.68	8.92	2.280	0.159	0.225	0.951
	23	8.00	9.25	2.515			
	30	7.24	7.81	4.475			
	37	7.29	7.71	1.705			
	44	6.55	7.90	2.047			
Neutrophils (G/L)	0	3.18	3.10	1.662	0.361	0.304	0.923
	23	3.13	3.66	1.589			
	30	2.69	2.84	1.064			
	37	2.63	2.68	1.095			
	44	2.51	3.01	1.526			

Table 6. *Cont.*

	Day	Control	SFP	SD	*p*		
					Treatment (T)	Time	T × Time
Lymphocytes (G/L)	0	2.23	2.22	1.313	0.899	0.606	0.987
	23	1.95	2.13	0.977			
	30	2.35	2.19	1.045			
	37	2.61	2.50	1.186			
	44	2.21	2.18	1.214			
Monocytes (G/L)	0	2.38	2.55	1.288	0.047	0.024	0.977
	23	2.10	2.48	1.055			
	30	1.87	2.30	0.983			
	37	1.67	1.92	0.606			
	44	1.44	1.93	0.686			
Eosinophils (G/L)	0	0.093	0.096	0.0596	0.200	<0.001	0.395
	23	0.046	0.041	0.0193			
	30	0.065	0.033	0.0356			
	37	0.043	0.033	0.0177			
	44	0.039	0.041	0.0225			
Basophils (G/L)	0	0.803	0.945	0.7947	0.028	0.002	0.930
	23	0.778	0.953	0.6667			
	30	0.275	0.445	0.2770			
	37	0.336	0.575	0.4151			
	44	0.349	0.751	0.5041			

SD, standard deviation; G/L, 10^9 per liter.

3.5. Histopathology

The changes in the abomasal tissues of the lambs inoculated with *H. contortus* were mild and included damage to epithelial cells and inflammatory infiltration (Table 7). The percentage of damaged glands ($p = 0.004$) was significantly higher, and mucosal hypertrophy (not significant, $p = 0.05$) was marginally higher, in the SFP than in the control group. Glandular dilatation and submucosal edema were similar in both groups.

Table 7. Effects of SFPs on the histopathology of the abomasal tissues of the lambs.

Effect	Control	SFP	SD	*p*
Hypertrophy of mucosa (%)	15.4	30.8	3.31	0.375
Epithelial cell damage (%)	100	100	0.00	–
Hyperplasia of mucus-producing cells (%)	7.7	0	1.51	0.327
Dilatation of glands (%)	30.8	30.8	3.62	0.999
Damage of glands (%)	0	46.2	3.31	0.004
Inflammatory cell infiltration (%)	100	100	0.00	–
Submucosal edema (%)	30.8	38.5	3.73	0.695

4. Discussion

Multidisciplinary studies (agronomic, nutritional, parasitological, and chemical) have identified many benefits to animal health of tanniferous forages and legumes used as feed for ruminants [40,41]. Sainfoin generally contains mainly CTs (approximately 42–50 g CTs/kg DM), which have been well studied [42–44]. The consumption of sainfoin disturbs various stages of parasitic life cycles, mainly due to its high tannin content [45]. The anthelmintic activity of CTs, however, can be increased by the addition of flavonoids, which also interfere with the biology of GINs [15,16]. We therefore focused on the analysis of flavonoids and phenolic acids.

Quantitative UHRMS analyses of the bioactive components in the SFPs identified more than 32.0 g/kg DM flavonoids and 4.5 g/kg DM phenolic acids. The main flavonoid was rutin (18.92 mg/g DM), which has multiple pharmacological activities with metabolic

health benefits [46]. Rutin can alter the ruminal microbiome and reduce the population of methanogenic bacteria [47]; and adding rutin (3.0 mg/kg) to feed dairy cows for 11 weeks improved the efficiency of carbohydrate fermentation in the rumen and the ability to synthesize protein [48]. Our results with SFPs indicated reductions in methane concentrations and *Archaea* population sizes of 11 and 33% and 27 and 38% in in vitro and in vivo treatments, respectively. Ruminal contents in the SFP group indicated also smaller populations of *Methanomicrobiales* (in vitro and in vivo) and *Methanobacteriales* (in vivo) than the control group. In another study, dietary supplementation with dry fumitory, mallow, wormwood, and chamomile with flavonoids (0.4–12.2 g/kg DM) for 70 d had no anti-methanogenic effect in lambs [19]. The anti-methanogenic effect of SFPs observed in our study may have been due to the direct effect of either the CTs [6,49] or rutin [50] or both on methanogenesis in the SFP group. Data on reducing methane concentration by rutin supplementation in vitro and in vivo, however, have been inconsistent [51–53]. Acceptably low methane emissions can therefore be achieved by a suitable choice of the vegetative stage of sainfoin [54]. The stimulation of gas concentration by rutin (50 mg/g DM) in ruminal fermentation in vitro led to an increased CO_2 concentration and a decreased methane concentration, probably because rutin is a substrate for nonmethanogenic microbiota [50]. However, the consumption of SFPs by infected lambs for 14 d in our experiment affected ruminal methanogens and consequently reduced methane emission without adverse changes in the ruminal microbiome.

The relative abundance of *B. fibrisolvens* in our study was higher in the SFP than the control group, because the replacement of MH by SFPs was probably associated with the increased demand for microbial degradation of fiber in the SFP group. Other bacterial species were not significantly affected, probably due to the relatively short SFP treatment. *B. fibrisolvens* plays an important role in the ruminal fermentation of polysaccharides that participate in cellulolytic processes in the rumen but do not have an autonomous cellulolytic capacity [55]. Changes in the relative abundance of *B. fibrisolvens* suggest that the SFPs did not negatively affect ruminal fermentation (e.g., total and individual VFAs). A diet with plant bioactive compounds can affect the ruminal microbiome, the kinetics of fermentation, and the response and adaptation to anti-methanogenic compounds, sometimes leading to inconsistent efficacy of phytochemicals [56–58]. We cannot sufficiently confirm the effect of SFPs on ruminal fermentation in lambs, because the SFPs were consumed for only 14 d. We also observed no effects of short-term SFP feeding on total protozoal counts in vitro and in vivo. The in vitro experiments on the effects of CT fractions of differing molecular weights on *Leucaena leucocephala* identified a lowering of the total number of ciliate protozoa with changes in counts of community members [59]. Similar effects were observed in vivo after long-term feeding of lambs with extracts of *Acacia negra* and *Uncaria gambir*, sources rich in CTs [60]. The feeding of dry leaves of *L. leucocephala* (12–36% of DM intake), however, did not affect ruminal protozoan, bacterial, or archaeal populations in crossbred heifers [61]. Supplementation of rutin for three weeks in dairy cows (3.0 mg/kg diet) did not significantly decrease counts of ruminal protozoa [49]. The amount and composition of the CTs and the length of treatment are likely the main factors influencing the effects of CT on the ruminal microbiome. In our experiment, even feeding only SFPs did not affect the protozoan counts. We can speculate about the amount of intake of CTs in the diet of the control group. Several studies have reported the CT compositions of various kinds of forages and MH from permanent pastures [6,62]. Unfortunately, we did not measure the CT content of the control MH diet.

Our study confirmed a significant reduction in RBC count, HGB level, and HCT from D23 in both groups of lambs infected with *H. contortus*, consistent with our previous results [20,28,63]. The reductions were likely due to damage caused by the GIN parasites, but SFPs as a replacement for hay did not affect the RBC parameters. The intensity and duration of hematological disorders depend on the nutritional status of infected sheep because protein-enriched diets induce resistance to infection associated with the improved regenerative capacity of bone marrow [64]. In addition to the nutritional status of the

host, differences in RBC disorders during GIN infection may be affected by the species of nematode, the severity of the infection, the iron stores and bodily reserves of the host, and the susceptibility of the host breed [65]. The basophil level in our study was higher in the infected lambs of the SFP group. Basophils are generally relatively rare and short-lived cells and probably played an important role in the immune response in the SFP group to GIN infection in our experiment [66]. The number of monocytes, eosinophils, and basophils was differentially affected by the time after infection, consistent with the blood variables during a subclinical *H. contortus* infection [67].

The development of GINs in host abomasa causes pathology, with mucosal damage and gastropathy with protein loss, followed by inflammatory immune responses of the host [68]. Our observations included microscopic changes in the abomasa, such as mucosal hypertrophy, damage to epithelial cells, mucus-producing cell hyperplasia, glandular dilatation, glandular damage, inflammatory cell infiltration, and submucosal edema. A roughened and hyperemic abomasal mucosa with enlarged glands and globular leukocytes have been described in lambs infected with *H. contortus* [69]. In our experiment, glandular damage in the SFP group differed significantly between the experimental groups, but the other changes in the abomasal mucosa were essentially the same for both infected groups. Mucosal hypertrophy was also more pronounced but not significant in the lambs fed with SFPs. An increased percentage of abomasal injuries and mucosal hypertrophy in the SFP group was attributed to regeneration, which is more common in abomasal tissue due to herbal treatment [70]. The histopathological changes in lambs in our previous study infected with *H. contortus* and supplemented with dry *Artemisia absinthium* and *Malva sylvestris* were predominantly on the mucosal membrane, with inflammatory cell infiltrates (mainly lymphocytes and macrophages with a mixture of eosinophils, plasma cells, and mast cells) [70]. Subclinical *H. contortus* infections generally damage the abomasal mucosa, which was very similar in the two infected groups, i.e., with and without SFPs.

5. Conclusions

Replacing MH with SFPs affected the composition of methanogenic bacteria in the rumens of the lambs and consequently reduced methane emissions, thus helping to reduce the environmental burden of methane and minimizing the adverse effects on animal health. From this point of view, the use of SFPs can be very useful in good agricultural practice. This study, to the best of our knowledge, is the first on the effect of SFPs on the ruminal microbiome and the abomasal histopathology of lambs loaded with parasites.

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Trace Mineral Solubility and Digestibility in the Small Intestine of Piglets Are Affected by Zinc and Fibre Sources

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Abstract: Findings that Zn and fibre source affected the nutrient apparent total tract digestibility (ATTD), made us hypothesize that interactions could occur affecting the apparent digestibility of Zn and trace elements (TEs) interacting with Zn in the digestive tract. Therefore, the study investigated the effects of Zn and fibre sources on the apparent digestibility and solubility of TEs (Zn, Cu, Fe, Mn) and pH in the small intestinal segments of 40-days-old piglets. In vitro solubility of TEs was estimated using a simulated digestion assay. Feed supplementation with potato fibre (PF) affected the ATTD of all TEs and dry matter as well as mineral solubility in the ileum and/or jejunum without any effect on pH in the small intestine. Intake of PF enhanced Zn and Cu absorption ($p < 0.01$), but significantly decreased ATTD of Fe and Mn ($p < 0.001$). Diet supplementation with Zn glycinate decreased Zn absorption in the gut ($p < 0.01$) and affected the solubility of other TEs in the different digestion phases. Although in vitro solubility of TEs does not provide a good prediction of mineral bioaccessibility, using a combination of in vitro and in vivo methods can enable prediction of the trace mineral absorption.

Keywords: trace minerals; fibre; zinc; apparent digestibility; bioaccessibility; piglets

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1. Introduction

The bioavailability of trace elements (TEs) depends on their bioaccessibility, absorption, retention, and role in animals [1,2]. Whereas bioavailability of TEs in vivo is determined by the mineral fraction, which is eventually absorbed into the systemic circulation and utilised by animals, in vitro studies define TE bioaccessibility as the fraction of minerals that is soluble in the gastrointestinal tract (GIT) and available for absorption [2,3]. Trace elements are essential for living animals to maintain their health and performance, but the generally high presence of phytate in cereal-based feedstuffs decreases the absorption of minerals from the gut and their bioavailability [4]. In the digestive tract, divalent minerals chelate with phytic acid to form soluble and/or insoluble mineral-phytate complexes. These complexes are resistant to hydrolysis by phytase activity, and for this reason, the absorption and bioavailability of trace elements are decreased [5]. In addition, pH is an important factor influencing phytate and mineral complex solubility. During passage through the small intestine, at intestinal pH (pH 6.5) the mineral complexes become insoluble and are precipitated to decrease mineral absorption due to de novo complexation [5,6], whereby the trace minerals most affected by phytate are Zn and Fe.

Zinc as a component of many metalloenzymes performs multifarious physiological roles and is involved in almost every metabolic pathway of the body [7]. Zn compounds positively affected the growth performance, intestinal morphology, and regulation of the

gut microbiota; therefore, a therapeutic dose of ZnO is used to decrease the post-weaning diarrhoea frequency in piglets [8,9]. Due to a total ban on the therapeutic use of ZnO that will be introduced from June 2022 in the European Union [10], the evaluation of different Zn sources and Zn lower doses in pig nutrition is very required and challenging. Although the allowed maximum of 150 mg Zn/kg in complete feed in the EU is an acceptable Zn level to maximize the pig performance and effectively counteract diarrhoea [11], it is necessary to improve Zn efficiency and absorption by feed composition in order to maintain animal health, welfare and productivity together with the substantial reduction of environmental pollution from animal husbandry.

Because of the different chemical properties of various Zn feed additives, solubility and interaction of TEs within the GIT may vary. Zinc sulphate together with zinc oxide are the most commonly used Zn source in pig nutrition [12]. $ZnSO_4$ has been frequently used as a standard to compare the bioavailability of Zn from different sources [13,14], but this soluble source of Zn could more readily interact with phytates and other ligands in the GIT, resulting in lower Zn absorption [15–17]. On the other hand, different chemical structures and properties of Zn organic chelates, more stable Zn complexes, could protect Zn from interaction with other minerals and feed compounds in the gut [2,18], thus making Zn more absorbable [13,19]. Moreover, some Zn organic sources could be absorbed from the intestine in an intact form [18]. Although phytate seems to be the most effective inhibitor of mineral absorption [15], other components such as dietary fibre, lignin, and polyphenolic compounds may bind minerals and affect their bioavailability as well [15,20]. Generally, fibre and compounds associated with fibre in cereal products reduced the mineral absorption in animals [21].

There are two general mechanisms for the inhibition of Zn absorption in the GIT, chelation precipitation (formation of insoluble complexes with phytic acid, dietary fibre, tannins) and competitive inhibition between minerals [22,23]. Feed supplementation with Zn and fibre from different sources (inorganic or organic Zn, and cellulose or potato fibre) could influence mineral absorption during the passage of feed through the small intestine. Our previous results indicated positive effects of potato fibre (PF) supplementation on nutrient digestibility [24]; therefore, we hypothesized that both dietary sources could also affect the apparent digestibility of TEs due to changes in pH and mineral solubility in the GIT. We focused on solubility and absorption of Zn and other interfering TEs in the small intestine as their main absorption site. In this study, we investigated the effect of Zn and fibre sources on the apparent digestibility of TEs (Zn, Cu, Fe, Mn) in the total digestive tract and in the small intestinal segments of piglets as well as pH in the gut. Moreover, we investigated in vitro solubility of TEs from the dietary treatments in a simulated digestion assay, and TE in situ solubility from the small intestinal digesta (duodenum, jejunum ileum) of piglets as a potential indicator of mineral bioaccessibility. Our in vitro experiment was designed to compare the influence of both dietary sources (Zn sources: $ZnSO_4$ or ZnGly, fibre sources: cellulose or potato fibre) on mineral solubility in the whole GIT using a simulated three-step digestion assay (gastric, small and large intestinal phases).

2. Materials and Methods

All experimental protocols involving animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and Animal Research: Reporting In Vivo Experiments (ARRIVE guidelines). All methods and procedures reported herein were carried out in line with European Union Directive 2010/63/EU for animal experiments, and the experimental protocol was approved by the Local Animal Experimentation Ethics Committee (resolution number WAW2_21/2016, Warsaw University of Life Sciences-SGGW, Warsaw, Poland) and Polish Law on Animal Protection.

2.1. Experimental Design

2.1.1. Experiment In Vivo

Animals and Diets

A total of twenty-four Danbred × Duroc barrows (body weight 10.8 ± 0.8 kg) at the age of 40 days were randomly divided into four dietary treatment groups, each fed a cereal-based diet (Table 1) supplemented with Zn and fibre from different sources during a four-week feeding period. The experimental diets consisted of cellulose (Lonocel, Cargill Poland Ltd., Kiszkowo, Poland) and zinc sulphate monohydrate for control treatment (C), cellulose and zinc glycinate (Glycinoplex-Zn, Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) for ZnGly treatment, and potato fibre (Potex, Lyckeby Starch, Kristianstad, Sweden) with $ZnSO_4$ for PF treatment or potato fibre with zinc glycinate for PF + ZnGly treatment. All experimental diets were prepared to contain a total of 150 mg Zn/kg and up to 50 g/kg of total crude fibre content in the complete diet (as-fed basis) from different Zn and fibre sources. Chemical composition of both dietary fibre preparations was published in our previous study [24]. The analyzed total content of Zn and crude fibre (CF) in the experimental diets were 141.9 mg Zn/kg and 42 g/kg of CF for C treatment, 142.2 mg Zn/kg and 40 g/kg of CF for the ZnGly, 141.1 mg Zn/kg and 40 g/kg of CF for PF, and 142.2 mg Zn/kg and 40 g/kg of CF for PF + ZnGly treatments. The ingredients and chemical composition of the cereal-based diets are shown in Table 1.

Table 1. Ingredients and chemical composition of the cereal-based diets.

Ingredients (g/kg)	Diets [1]		Analysed Composition (g/kg)	Diets [1]	
	C	PF		C	PF
Barley	200	200	Dry matter	889	890
Wheat	450	450	Crude ash	47	49
Soybean meal	170	170	Crude protein	182	183
Yellow lupin	60	60	Crude fat	42	41
Rapeseed oil	25.5	25.5	Crude fibre	41	40
Corn starch	33	-	Sugars	41	39
Cellulose	17	-	Starch	378	379
Potato fibre	-	50	Total phosphorus	5.1	5.08
Mineral-vitamin mix [2]	4	4	Phytic phosphorus	1.90	1.68
Monocalcium phosphate	7	7	aNDFom	108	112
Calcium carbonate	19	19	ADFom	55	56
Sodium chloride	3	3	Lignin (sa)	11	10
L-Lysine	6	6	Cellulose	43	47
DL-Methionine	2	2	Hemicellulose	53	55
L-Threonine	3	3	Zn (mg/kg)	142	141
L-Tryptophan	0.5	0.5	Mn (mg/kg)	99	94
			Cu (mg/kg)	125	132
			Fe (mg/kg)	315	300
			Gross energy (MJ/kg)	16.8	16.6

[1] C—the diets containing cellulose fibre, PF—the diets containing potato fibre. [2] Provided per kg of diet: 14,680 IU vitamin A, 1835 IU vitamin D3, 138 mg vitamin E, 4.59 vitamin K3, 2.94 mg vitamin B1, 7.34 mg vitamin B2, 4.40 mg vitamin B6, 44.0 µg vitamin B12, 36.7 mg nicotinic acid, 16.0 mg calcium D-pantothenate, 1.47 mg folic acid, 147 µg biotin, 228 mg Fe, 73.4 mg Mn, 156 mg Cu, 631 µg I, 411 µg Se.

Animal Performance

All piglets were housed in individual pens with free access to feed and fresh potable water. They were kept in a 12 h light/12 h dark regimen with housing temperature maintained at 25 °C. Health monitoring of all piglets was performed daily. Individual performance of feed intake and body weight were measured weekly. At the end of the 28-day feeding period, final body weight and total feed intake were used to calculate feed conversion ratio for each dietary treatment.

Apparent Digestibility

Ten days before the end of the experiment, Cr_2O_3 was included in the diets (3 g/kg diet) as an ingestible marker for estimation of the apparent digestibility of TEs. After five days of adaptation, excreta samples were collected for five days and pooled from each animal and stored at −20 °C until freeze-drying. At the end of the feeding period, the piglets were stunned with an electric shock and exsanguinated, followed by the collection of the small intestinal content. pH values in the digesta of duodenum, jejunum, and ileum were measured using a digital pH meter. The collected digesta samples from the duodenum, the middle part of the jejunum, and ileum and pooled excreta samples were freeze-dried, ground, and stored until further analysis. Dry matter (DM) total apparent digestibility was estimated from collected pooled excreta samples. The apparent digestibility of TEs and DM was calculated using the following formula (1):

$$\text{Apparent digestibility of TEs/DM, \%} = 100 - [(Cr_2O_3 \text{ in diet} \times \text{TEs/DM in digesta and/or faeces})/(Cr_2O_3 \text{ in digesta and/or faeces} \times \text{TEs/DM in diet}) \times 100] \quad (1)$$

2.1.2. Experiment In Situ

After slaughtering the piglets from each dietary treatment group (in vivo experiment), we collected the digesta from their duodenum, jejunum, and ileum to investigate the effects of Zn and fibre source on in situ solubility of Zn, Cu, Fe, and Mn in the small intestine. Measuring the solubility of these trace elements (TEs) from the digesta samples was done using the technique of centrifugation and a method based on Kleinman et al. [25]. Briefly, 0.5 g of digesta sample from each small intestinal segment was put into a bottle together with 25 mL ultra-pure water (EASYpure II UV/UF, Werner Reinst-wassersysteme, Leverkusen, Germany). Then, the samples were shaken at 180 rpm for 60 min. After centrifugation at 6000 rpm for 10 min, the supernatants were filtered through filter papers (Whatman 541), and the soluble content of TEs in the supernatants was analysed directly by atomic absorption spectrophotometry (AAS). The in situ soluble content of TEs was calculated according to the following Equation (2):

$$\text{In situ soluble TEs, \%} = (\text{soluble TEs in digesta supernatant/total TEs in the diet}) \times 100 \quad (2)$$

2.1.3. Experiment In Vitro

In Vitro Solubility of Zn Sources

We estimated the effect of pH of buffers simulating the GIT environment on in vitro Zn solubility from both Zn supplements (zinc sulphate, zinc glycinate) at three concentrations 20, 100, and 150 mg Zn/L of buffer. Aliquot amounts of $ZnSO_4$ and ZnGly were placed into bottles with magnetic stirring and dissolved in 0.2 M glycine-HCl buffer at pH 2.0 to simulate gastric digestion and in 0.2 M sodium acetate simulating pH in the small intestine at pH 4.5 and 6.5 [26]. The mixtures were incubated in a shaking bath at 39 °C for 4 h to simulate digestion, and filtered through 541 Whatman papers for Zn analysis using AAS. All incubations were done in duplicate, and in vitro solubility of ZnSO4 and ZnGly infiltrates was calculated according to Equation (3):

$$\text{In vitro solubility of Zn source, \%} = (\text{soluble Zn content/total Zn content}) \times 100 \quad (3)$$

In Vitro Simulated Solubility of TEs

In vitro simulated solubility of Zn, Cu, Fe, and Mn from four experimental diets differing in Zn and fibre source (see detailed description of in vivo study, Table 1) were estimated using a three-step in vitro simulated digestion assay applying the method of Boisen and Fernández [27]. This assay procedure investigated the solubility of the trace elements in the simulated stomach, small and large intestine digestion.

From each experimental diet (Table 1), nine dried and ground sub-samples (500 ± 0.1 mg) were weighed into pre-weighed bottles, then 25 mL of 0.1 M phosphate buffer (pH 6.0) and 10 mL of 0.2 M HCl were added, and the mixture was stirred with a magnetic bar. To mimic the gastric phase (GP), the pH of the mixture was adjusted to pH 2.0 with 1 M HCl and 1 mL of freshly-prepared pepsin solution (25 mg of pepsin/mL; P7000, Sigma Aldrich, St. Louis, MO, USA). Then, 0.5 mL chloramphenicol (0.5 g/100 mL ethanol, C-0378, Sigma Aldrich, St. Louis, MO, USA) was added to the mixture before incubating at 39 °C for 60 min in a water bath with shaking (150 rpm).

After the gastric phase incubation, the bottles were taken out of the water bath, and to mimic the small intestinal phase (SIP), 10 mL of 0.2 M phosphate buffer (pH 6.8) and 5 mL of NaOH (0.6 M) were added to the mixture and magnetic stirring was resumed. After pH was adjusted to 6.8 (with 1 M HCl or 1 M NaOH), 1 mL of pancreatin solution (100 mg of pancreatin/mL; P1750, Sigma Aldrich, St. Louis, MO, USA) was added, and then closed bottles were incubated for 4 h at 39 °C in a water bath.

The small intestine phase incubation was followed by the last process of simulated digestion phase in the large intestine (LIP) with 10 mL EDTA (0.2 M) added and pH adjusted to 4.8 with 30% glacial acetic acid. The mixture was carefully stirred with 0.5 mL of Viscozyme multi-enzyme complex (Viscozyme L V2010, Sigma-Aldrich, St. Louis, MO, USA) to simulate microbial fermentation in the large intestine, and incubated at 39 °C for 18 h in a water bath with shaking.

During all incubation periods, the bottles were closed and mixtures were stirred slowly and constantly to simulate feed digestion. At the end of each simulated phase, the pH of the mixtures was measured and 2 mL of samples were collected for centrifuging at 4000× *g* for 20 min. The supernatants from each digestion phase were filtered through 0.22 µm filter membranes (Millex GS, Merck Millipore, Tullagreen, County Cork, Carrigtwohill, Ireland) and diluted with ultrapure water for subsequent analysis directly by means of AAS. The solubility of TEs in the filtrates was calculated according to the following Equation (4):

$$\text{In vitro simulated solubility of TEs, \%} = (\text{soluble TEs in the GP, SIP, LIP/total TEs in the diet}) \times 100, \quad (4)$$

TEs = trace elements, GP = gastric phase, SIP = small intestinal phase, LIP = large intestinal phase.

In Vitro Dry Matter Digestibility and pH

After the large intestinal phase, undigested residues from the incubated bottles were filtered in dried and pre-weighed glass filter crucibles containing Celite (Celite 545, Sigma Aldrich, St. Louis, MO, USA), then washed twice with 10 mL of 96% ethanol and 10 mL of 99.5% acetone, and the crucibles were finally oven-dried for 48 h at 105 °C. After cooling, the crucibles were weighed to estimate in vitro dry matter digestibility (IVDMD) calculated using the following Equation (5):

$$\text{In vitro digestibility of DM, \%} = [\text{DM in diet} - (\text{DM in residue - DM blank})] \,/\text{DM in diet} \times 100 \quad (5)$$

At the end of each in vitro simulated phase, pH values in buffers with undigested residues were measured with the pH electrode of a digital pH meter.

2.2. Chemical Analysis

The experimental diets and lyophilized digesta and faeces were ground in a grinding mill to pass a 0.5 mm sieve and analyzed for dry matter according to the Association of Official Analytical Chemists (AOAC) method by drying samples to a constant weight at 105 °C [28]. The same method was used for the estimation of dry matter in vitro and in vivo digestibility [28].

Trace mineral concentrations in the diets, faeces and digesta samples, filtrates, and/or supernatants were analyzed using a double-beam atomic absorption spectrophotometer (AA-7000 Series, Shimadzu Co., Kyoto, Japan). The microwave-assisted digestion method

using closed pressure vessels (MWS 4 Speedwave, Berghof Co., Eningen, Germany) was used for the decomposition of the diets, faeces, and digesta samples [29].

Cr_2O_3 concentrations in faeces and digesta samples were analyzed using the colorimetric method of Kimura and Miller [30]. pH values in the digesta of duodenum, jejunum, and ileum and in in vitro buffers and mixtures were measured with the pH electrode of a digital pH meter (WTW InoLab pH Level 2, Weilheim, Germany).

2.3. Statistical Analysis

Treatment effects on the investigated parameters were analysed according to a completely randomised 2 × 2 factorial design, with a factorial arrangement of treatments using two-way ANOVA, followed by Tukey's post hoc multiple comparisons test with individual variance computed for pair-wise comparisons, where appropriate. The statistical model included the treatment effects (fibre and zinc source) and their interaction. When the interaction was significant, Fisher's Least Significant Difference test (Fisher's LSD) was applied post hoc to determine significant differences among the means. Two-way ANOVA with the main effect was also included to estimate the effect of pH on in vitro solubility of Zn sources and the effect of the digestion phase on in vitro solubility of TEs. All statistical analyses were performed with the GraphPad Prism statistical software (GraphPad Prism version 9.0.0., GraphPad Software, San Diego, CA, USA). Differences between the mean values of the different dietary treatments were considered statistically significant at $p < 0.05$. Data are expressed as means with standard errors of the difference between column means (SE).

3. Results

3.1. Experimetn In Vivo

3.1.1. Animal performance

Feed supplementation with ZnGly increased the daily feed intake and body weight of piglets (ZnGly, PF + ZnGly treatments, $p < 0.05$). The average daily feed intake (mean ± SE) during the whole feeding period was 0.89 ± 0.05 kg, 0.99 ± 0.08 kg, 0.78 ± 0.02 kg, and 1.00 ± 0.06 kg for C, ZnGly, PF, and PF + ZnGly treatments, respectively. Feed conversion ratios ranging from 1.51 to 1.55 did not differ between the dietary treatments.

3.1.2. Apparent Digestibility

Feed supplementation with ZnGly decreased the apparent total tract digestibility (ATTD) of Zn ($p = 0.01$), but the apparent digestibility of Zn in the duodenum, jejunum, and ileum did not differ between the dietary treatments (Table 2). Intake of both PF diets increased the total apparent digestibility of Zn ($p < 0.01$) compared to the ZnGly treatments as well as DM digestibility in piglets. The highest value DM digestibility was recorded in the PF + ZnGly treatment compared to the C and ZnGly treatments (Table 5).

Intake of the PF diets increased the total apparent digestibility of Cu ($p < 0.01$), but Cu digestibility did not change in the small intestine. Interaction between Zn and fibre sources affected Cu digestibility in the duodenum ($p < 0.05$), with decreased Cu digestibility in the PF treatment compared to the PF + ZnGly treatment ($p < 0.05$).

On the other hand, PF supplementation significantly reduced Fe digestibility from the ileum as well as decreased the ATTD of Fe ($p < 0.001$), while the apparent digestibility of Fe was not changed in the other small intestine segments.

ZnGly in the diet increased duodenal apparent digestibility of Mn ($p < 0.05$). However, the Zn source affected the apparent duodenal digestibility of Mn, Mn absorption did not differ between the dietary treatments in the jejunum and ileum. The effects of both dietary sources and source interaction were observed on ATTD of Mn, whereby negative values in the parameter were found, with the lowest values in the PF + Gly treatment.

No changes in pH were found in any small intestinal segments in our in vivo experiment (Table 5).

Table 2. Apparent digestibility of Zn, Cu, Fe and Mn in the small intestine and total tract of piglets fed diets supplemented with zinc and fibre from different sources.

Apparent Digestibility, %	Dietary Treatment [1]				SE	*p*-Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
Zinc			**Zn digestibility, %**					
Total tract	29.08 ^{b}	21.81 ^{a}	30.53 ^{b}	28.56 ^{b}	1.363	**0.012**	**0.002**	0.187
Duodenum	68.47	66.03	64.92	77.06	4.758	0.351	0.441	0.142
Jejunum	60.61	54.36	58.00	57.90	4.597	0.499	0.920	0.512
Ileum	57.31	50.23	55.73	53.95	2.991	0.156	0.724	0.387
Copper			**Cu digestibility, %**					
Total tract	20.05 ^{ab}	12.94 ^{a}	23.77 ^{ab}	26.51 ^{b}	2.965	0.470	**0.009**	0.114
Duodenum	61.37 ^{AB}	55.27 ^{AB}	52.04 ^{A}	67.50 ^{B}	4.982	0.378	0.774	**0.043**
Jejunum	27.44	21.58	30.07	38.83	5.086	0.806	0.105	0.225
Ileum	31.77	24.75	33.92	35.81	7.37	0.732	0.382	0.553
Iron			**Fe digestibility, %**					
Total tract	14.42 ^{b}	16.54 ^{b}	4.255 ^{a}	6.064 ^{a}	2.226	0.391	**0.0003**	0.946
Duodenum	42.97	37.35	32.37	43.26	8.453	0.426	0.759	0.342
Jejunum	29.79	28.80	23.39	21.76	6.618	0.846	0.330	0.963
Ileum	23.40	30.70	10.86	11.19	5.278	0.480	**0.008**	0.519
Manganese			**Mn digestibility, %**					
Total tract	−21.93 ^{B}	−29.27 ^{B}	−25.59 ^{B}	−52.06 ^{A}	4.051	**0.0001**	**0.0001**	**0.0001**
Duodenum	28.73	43.09	32.61	57.06	9.292	**0.0498**	0.349	0.5934
Jejunum	16.28	10.73	10.83	20.62	10.03	0.835	0.828	0.456
Ileum	12.85	14.46	14.22	12.55	5.210	0.995	0.959	0.757

[1] C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. $^{a–b}$ Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison ($n = 6$). ^{A,B} Means within lines with different superscript letters are significantly different ($p < 0.05$) using by Fisher's LSD post hoc test.

3.2. Experiment in situ

In Situ Soluble TEs

Interaction between Zn and fibre sources affected soluble Zn concentration in digesta of each small intestinal segment (Table 3). Zn solubility in the duodenum and jejunum were not affected by any dietary sources, but the effect of both dietary sources on the soluble concentration of Zn in the ileum was observed. Due to dietary source interaction, significant decreased in situ Zn solubility was observed in the duodenum ($p < 0.01$), but soluble Zn concentration increased in the jejunum ($p < 0.05$) in the PF + Gly treatment compared to the ZnGly and PF treatments. Increased Zn soluble content was observed in the ileal digesta of piglets fed the PF diet in comparison to the other dietary treatments ($p = 0.001$).

Supplementation with PF decreased in situ soluble content of Cu in the jejunum without any significant differences between the treatments.

Zn source affected soluble Fe concentration in the jejunal digesta with significantly decreased soluble content of Fe in the ZnGly treatment to the control treatment (C). Fe solubility in the ileum was affected by both dietary sources ($p < 0.0001$). The highest value was observed in the PF treatment, while the lowest in vitro solubility of Fe was observed in the ZnGly treatment compared to others.

In situ solubility of Mn was affected by both dietary sources in the jejunum and ileum. ZnGly in the diets decreased soluble Mn in the duodenum and ileum, but ZnGly increased Mn solubility in the jejunum. Feed supplementation with PF reduced the soluble content of Mn in the jejunum, on the contrary, increased in vitro solubility of Mn was observed in the ileal digesta in the PF treatment ($p < 0.01$). However, the interaction between both dietary sources affected soluble Mn concentration in the ileal digesta.

Table 3. In situ soluble content of Zn, Cu, Fe and Mn from intestinal digesta after dietary treatments with Zn and fibre from different sources.

In Situ Solubility, %	Dietary Treatment [1]				SE	*p*-Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
Zinc			**Soluble Zn, %**					
Duodenum	40.11 ^{AB}	52.42 ^{B}	57.02 ^{B}	19.30 ^{A}	9.349	0.1847	0.3929	**0.0121**
Jejunum	31.83 ^{BC}	25.52 ^{AB}	22.18 ^{A}	34.40 ^{C}	2.586	0.2605	0.8823	**0.0009**
Ileum	19.30 ^{A}	16.50 ^{A}	31.92 ^{B}	19.20 ^{A}	2.129	**0.0007**	**0.0008**	**0.0243**
Copper			**Soluble Cu, %**					
Duodenum	50.51	54.27	49.86	43.47	3.647	0.7216	0.1267	0.1739
Jejunum	102.8	99.88	75.03	85.33	8.250	0.6088	**0.0052**	0.3608
Ileum	82.55	83.64	78.99	75.51	3.286	0.7186	0.0832	0.4910
Iron			**Soluble Fe, %**					
Duodenum	15.35	16.25	20.39	14.28	1.955	0.1924	0.4383	0.0827
Jejunum	48.62 ^{b}	30.48 ^{a}	45.21 ^{ab}	44.25 ^{ab}	4.483	**0.0389**	0.2544	0.0621
Ileum	44.94 ^{b}	29.51 ^{a}	63.42 ^{c}	46.25 ^{b}	3.450	**<0.0001**	**<0.0001**	0.8022
Manganese			**Soluble Mn, %**					
Duodenum	89.88 ^{b}	77.40 ^{ab}	79.00 ^{ab}	44.05 ^{a}	10.80	**0.0366**	0.0501	0.3072
Jejunum	39.67 ^{b}	44.77 ^{b}	27.73 ^{a}	39.44 ^{b}	2.775	**0.0032**	**0.0041**	0.2397
Ileum	15.77 ^{A}	15.58 ^{A}	25.65 ^{B}	17.83 ^{A}	1.698	**0.0009**	**0.0229**	**0.0295**

[1] C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. $^{a–c}$ Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison ($n = 6$). $^{A–C}$ Means within lines with different superscript letters are significantly different ($p < 0.05$) using by Fisher's LSD post hoc test.

3.3. Experiment In Vitro

3.3.1. In Vitro Solubility of Zn Sources

The effects of pH on Zn solubility from both Zn sources in different buffers simulating pH in gastric and small intestine digestion were estimated in vitro (Figure 1). The in vitro solubility of Zn from $ZnSO_4$ and ZnGly was affected by pH ($p < 0.0001$) and Zn source ($p = 0.003$), with the lowest Zn solubility at pH 6.8 simulating digestion in the lower part of the small intestine (Tukey's post hoc multiple comparisons test with individual variance computed for pair-wise comparisons in Supplementary materials, Figure S1, Table S1). The solubility of $ZnSO_4$ was found to be significantly higher than Zn solubility from ZnGly (two-way ANOVA with the main effect only, $p < 0.001$, Figure 1).

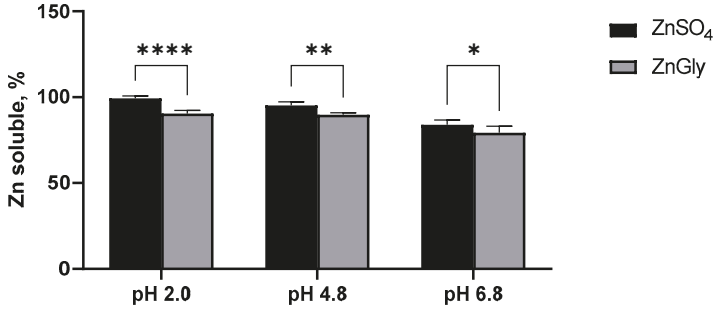

Figure 1. In vitro solubility of Zn from both Zn sources (ZnSO4, ZnGly) subjected to different buffers simulating gastric digestion in 0.2 M Gly-HCl at pH 2.0, or simulating small intestinal digestion in 0.2 M sodium acetate buffer at pH 4.8 and 6.8; Asterisks represent *p*- value classification (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

Data were analyzed using two-way ANOVA, followed by the post hoc Tukey's multiple comparisons test, which included the main effect only (pH and Zn source).

3.3.2. In Vitro Simulated Solubility of TEs

In vitro soluble or bioaccessible concentrations of TEs in the experimental diets in each digestion phase are shown in Table 4. Zn, Mn, and Cu were almost totally dissolved in the gastric digestive juice but rapidly decreased solubility of Zn, Mn, and Fe was found in the small intestine phase (SIP). The in vitro solubility of Zn, Fe, and Mn in each experimental diet was observed to be lower in the SIP than in the gastric and large intestinal phases (two-way ANOVA with main effect only, $p < 0.001$); however, Cu solubility did not differ between the simulated digestion phases.

Table 4. In vitro solubility of Zn, Cu, Fe, and Mn from the dietary treatments subjected to three-step in vitro simulated digestion assay.

In Vitro Solubility, %	Dietary Treatment [1]				SE	*p*-Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
In vitro phase	**Soluble Zn, %**							
Gastric	104.5	109.5	108.0	114.8	6.055	0.3376	0.4766	0.8778
Small intestine	3.229	2.729	1.957	3.771	1.201	0.5893	0.9250	0.3449
Large intestine	119.1	120.1	110.5	124.0	7.032	0.3102	0.7395	0.3804
In vitro phase	**Soluble Cu, %**							
Gastric	99.37 ^{ab}	110.9 ^{b}	89.99 ^{a}	93.63 ^{a}	3.986	0.0665	**0.0022**	0.3319
Small intestine	85.52	94.93	79.28	95.40	8.226	0.1293	0.7280	0.6857
Large intestine	95.14 ^{ab}	112.6 ^{b}	87.99 ^{a}	100.4 ^{ab}	5.668	**0.0119**	0.0949	0.6549
In vitro phase	**Soluble Fe, %**							
Gastric	72.24 ^{C}	60.64 ^{A}	66.84 ^{B}	68.34 ^{BC}	1.558	**0.0031**	0.4643	**0.0002**
Small intestine	4.022	2.789	3.644	4.211	0.933	0.7233	0.5796	0.3420
Large intestine	101.4 ^{B}	78.89 ^{A}	80.42 ^{A}	82.05 ^{A}	4.144	**0.0161**	**0.0381**	**0.0059**
In vitro phase	**Soluble Mn, %**							
Gastric	90.49 ^{a}	100.5 ^{b}	88.30 ^{a}	96.59 ^{ab}	2.386	**0.0006**	0.2096	0.7189
Small intestine	11.06	12.10	9.613	12.60	1.586	0.2143	0.7667	0.5434
Large intestine	97.88	103.9	101.3	103.0	3.865	0.3269	0.7445	0.5815

[1] C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. ^{ab} Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison ($n = 9$). $^{A–C}$ Means within lines with different superscript letters are significantly different ($p < 0.05$) using Fisher's LSD post hoc test.

The in vitro solubility of Zn did not differ between the dietary treatments in each phase of simulated digestion. Intake of PF diets decreased soluble Cu in the gastric phase (GP) and affected solubility of Fe in the large intestinal phase (LIP).

The Zn source influenced in vitro solubility of Cu in LIP, soluble Fe in GP and LIP, and Mn solubility in gastric digestion phases. Supplementation with organic ZnGly increased in vitro solubility of Cu in LIP ($p < 0.05$) and soluble Mn in GP ($p < 0.001$). Zn source also affected the solubility of Fe in GP and LIP; however, the interaction between Zn and fibre sources was observed in both of these digestion phases. Decreased in vitro solubility of Fe in GIP was observed in the ZnGly treatment, while the highest soluble Fe concentration in LIP was observed in the control treatment compared to other treatments.

3.3.3. In Vitro Dry Matter Digestibility and pH

Although feed supplementation with PF increased in vivo DM digestibility in piglets, in vitro dry matter digestibility (IVDMD) was not influenced by the dietary treatments (Table 5).

Table 5. Effects of dietary treatments with added Zn and fibre from different sources on pH and dry matter digestibility subjected to in vivo and in vitro digestion assay.

In Vitro, In Vivo Assay	Dietary Treatment				SE	*p*-Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
In vitro			**pH**					
Gastric phase	2.005 ^{a}	2.010 ^{a}	2.070 ^{ab}	2.090 ^{b}	0.016	0.4669	**0.0022**	0.6589
Small intestinal phase	6.753	6.750	6.753	6.737	0.004	**0.0359**	0.1232	0.1232
Large intestinal phase	4.773 ^{A}	4.793 ^{B}	4.793 ^{B}	4.773 ^{A}	0.003	0.9999	0.9999	**0.0001**
In vivo			**pH**					
Duodenum digesta	4.385	4.506	4.178	4.858	0.2749	0.1622	0.7946	0.3224
Jejunum digesta	5.505	5.160	5.378	5.505	0.1401	0.4449	0.4449	0.1078
Ileum digesta	7.325	6.818	7.257	6.867	0.2709	0.1135	0.9709	0.8317
Dry matter (DM)			**DM digestibility, %**					
In vivo assay	83.17 ^{a}	83.21 ^{a}	84.67 ^{ab}	85.31 ^{b}	0.3841	0.3900	**<0.0001**	0.4502
In vitro assay	74.78	73.93	75.35	76.25	2.404	0.9746	0.5776	0.7392

[1] C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. $^{a–c}$ Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison, means represent 6 replicates in vivo, 9 replicates in vitro (DM digestibility) or 3 replicates in vitro (pH). $^{A–B}$ Means within lines with different superscript letters are significantly different ($p < 0.05$) using Fisher's LSD post hoc test.

Different effects of Zn and fibre sources on pH values were determined: PF affected pH in the in vitro simulated GP ($p < 0.01$) and Zn source in the SIP ($p < 0.05$). The diets containing PF increased pH in the gastric phase, while ZnGly in the diets slightly decreased pH in the SIP (Table 5). Interaction between both dietary sources affected pH in LIP with increased pH in the Gly and PF treatments.

4. Discussion

Trace elements as essential feed components improve animal health and livestock productivity but feed supplementation with TEs result in a substantial excretion of heavy metals into the environment. Therapeutic use of ZnO or excessive feeding of inorganic Zn to piglets can stimulate resistance in the gut microbiota, and Zn accumulates in faeces and then in manure [31]. Replacement of inorganic feed mineral additives by more bioavailable mineral sources or using enhancers of trace mineral absorption in animal feed could reduce excretion of TEs into the environment and reduce heavy metal emissions from livestock production.

Whole-body homeostasis of Zn, Mn, Cu, and Fe is predominantly regulated by intestinal absorption [2,32]. Absorption of trace elements (TEs) does not only depend on an adequate dietary intake but is also greatly affected by its intestinal availability from the diet. Different chemical species of dietary TEs can interact with other components in the diet before reaching their absorption site and these interactions may considerably modify the metabolically available amount of the trace minerals, mineral metabolism, and their regulation [16]. Sufficient Zn supply from different organic dietary sources of Zn and from inorganic ZnSO4 showed usually no differences in metabolic utilisation in animals due to Zn homeostasis adapted to intestinal Zn content [33]. However, Zn supplementation with organic ZnGly increased Zn absorption from the GIT of Zn-deficient animals [34,35]. Our results indicated the decreased apparent total tract digestibility (ATTD) of Zn in piglets fed the diet supplemented with organic Zn glycinate compared to other treatments; however, the apparent Zn digestibility did not differ in the small intestine. Higher feed consumption of piglets fed the diets supplemented with ZnGly also increased the ingested amount of Zn that could lead to increased excretion of Zn, and as a result of Zn homeostasis regulating the absorption and excretion of Zn in the GIT [32,36], Zn apparent digestibility decreased in the ZnGly treatment. However, decreased ATTD of Zn was observed in the ZnGly treatment only. It has been known that fractional Zn absorption decreases increasing Zn content in the feed, probably due to a saturation of transport mechanisms [37]. Zn absorption is more efficient in low zinc diets [38] and relates to oral zinc intake [17]; therefore, increased feed

consumption of Zn might decrease Zn uptake by the small intestine and Zn absorption. Moreover, the importance of maintaining Zn balance is endogenous Zn excretion and Zn reutilisation by GIT which directly relates to the absorbed Zn amount [39]. Another reason for the lower ATTD of Zn from ZnGly could be the structure of zinc glycinate. The small molecular size of ZnGly tends to bind in cavities of native fibre [40] resulting in the decrease of apparent Zn digestibility, but it seems that feed supplementation with PF could eliminate the effect. Reduced absorption and retention of Zn from ZnGly were observed in pigs due to the structure and lower average bond strength between the amino acid and Zn in ZnGly [40].

Although it seems that the chemical properties of trace elements are of subordinary relevance in the absorption process of dietary TEs in the GIT, their chemical speciation is relevant for complex interactions with different chelators to promote or prevent the formation of insoluble mineral complexes [22,34]. Phytates and fibres are the main ligands binding native Zn and other trace element species in the GIT [32,41] resulting in the inhibition of Zn absorption and reabsorption by chelating ingested Zn or secreted endogenous Zn in the intestinal lumen [39]. Regardless of dietary fibre source, fibres may affect mineral absorption due to their mineral binding properties [42,43] and these interactions are important from a nutritional point of view. Cellulose is a poorly soluble and fermentable fibre source that entraps minerals to form large insoluble complexes resulting in decreased mineral bioavailability; however, most of the different fibres can also bind Zn, Fe, Mn, and Cu under in vitro conditions [44]. On the other hand, more fermentable PF can enhance the absorption of TEs by lowering pH or the production of organic acids in the GIT [45]. Although no effect of PF on pH in the gut was found in our study, we found the beneficial effect of PF feeding on apparent total tract digestibility of DM, Zn, and Cu, but ATTD of Fe and Mn was considerably decreased. Similarly, fibre source differently affected soluble content of TEs in digesta of the ileum and/or jejunum. Several ligands of different types of dietary fibres are responsible for the metal binding and the binding strength to different metal ions [41]. We can only speculate that the physicochemical properties of PF might differently affect the solubility of TEs in the GIT and their digestibility. Potato starch from potato fibre has a higher viscosity and longer texture in comparison to corn starch [46] used in the C and ZnGly diets, which could influence conditions for mineral absorption in the GIT. Different effects of digestible starch on the apparent intestinal absorption of Fe and Zn were also observed in pigs [47]. Further investigation is needed to elucidate the mechanism of action of potato fibre in mineral absorption and the different effect of PF on the digestibility of TEs.

The amount of trace minerals available for absorption (bioaccessible or soluble amount) depends on the concentration of TEs in the diet, feed composition, and the presence of ligand enhancers or inhibitors of mineral absorption in the GIT [20]. Conditions increasing the solubility of trace minerals or protecting them from interaction with inhibitors in the intestinal lumen are generally beneficial for their absorption and uptake by the apical surface of enterocytes in the small intestine [37]. On the other hand, soluble inorganic species of TEs are more sensible to form insoluble chelate compounds with various chelators at the pH of the gut lumen [48]. In our study, the reduced ATTD of Zn in the ZnGly treatment might be a result of decreased soluble Zn in the ileal digesta of pigs supplemented with ZnGly. Similarly, the highest soluble Zn content observed in the ileum of piglets fed the PF diet could lead to increased ATTD of Zn.

On the other hand, diet supplementation with PF increased the ATTD of Cu in our piglets, while in vitro solubility of Cu in the simulated gastric phase as well as in situ solubility of Cu in the jejunum decreased. Cu is primarily absorbed through the stomach and small intestine of monogastric animals and gastric acidity promotes the presence of freely-soluble Cu ions [49]. Cu solubility in the gastric phase could predict mineral availability in the rest of the gastrointestinal tract, so decreased solubility of Cu in the simulated in vitro gastric phase could be related to the reduced soluble Cu percentage in the jejunum (in situ). However, we found interactions between both dietary sources which

might influence the ATTD of Cu in piglets. It has been reported that in vitro solubility of Cu might not accurately represent the in vivo bioavailability of Cu due to either disassociation or chemical shifts in Cu along the intestinal tract of broilers [50]. In any case, it should be stressed that Zn source had no effects on either Cu solubility or Cu digestibility in the GIT of our piglets, which is an important fact concerning the competitive antagonism for absorption between those two trace minerals.

Feed supplementation with PF negatively influenced the total apparent digestibility of Fe and decreased Fe absorption from the ileum of our piglets. On the other hand, in situ solubility of Fe increased in the ileum of piglets fed the PF diet. Solubility of Fe could be affected by changes in intestinal pH in the presence of PF in the diet, but no effect of PF on pH was observed in our study. It seems that even though in situ solubility of Fe increased in the ileum, the PF treatment interactions with other feed components along the digestive tract decreased the total digestibility of Fe. Moreover, it has been identified several complexes of Fe which are soluble, but unabsorbable in the gut, using an in vitro method measuring Fe solubility to predict Fe bioavailability [51]. In vitro and in situ solubility of Fe could be affected by Zn source, because ZnGly supplementation decreased Fe solubility in both trials. Similarly, in vitro and in situ solubility of Mn was affected by both dietary sources, but the effect is inconsistent. It seems that interaction between Zn and fibre sources led to the reduced apparent digestibility of Mn. Therefore, further investigation is needed to fully understand the effects of ZnGly and potato fibre on Fe and Mn absorption and interactions, and also competitive inhibition between the minerals in the GIT.

Another important factor affecting the solubility of mineral complexes in the GIT is the pH level of its various parts. Complexes formed by phytic acid and TEs were soluble under the acidic conditions in the in vitro simulated gastric phase, and therefore the highest in vitro soluble content of each trace mineral were recorded in this phase. In contrast, the solubility of phytate complexes with Fe increases above pH 4 and they are more insoluble at gastric pH [5], resulting in reduced in vitro soluble content of Fe in GP in our study. Moreover, ZnGly in the diet even reduced Fe in vitro solubility in GP, but increased soluble Mn. It seems that PF in the diet could affect mineral solubility due to increased pH in simulated in vitro GP, but our study design meant that we did not measure pH in the stomachs of our piglets to confirm this assumption in vivo. On the other hand, increased Mn digestibility in the duodenum after ZnGly supplementation could probably be a result of higher in vitro solubility of Mn in the GP, because mineral solubility in the gastric phase then dictates the availability of those minerals in the rest of the gastrointestinal tract. In situ solubility of Mn in the small intestine segments were affected by both dietary sources.

In vitro solubility is one of the methods of assessing the bioaccessible amounts of TEs based on the determination of soluble TEs under simulated physiological conditions [1]. Although bioaccessibility of TEs evaluated by in vitro methods is an important step in the assessment of trace mineral bioavailability, more important is the amount of TEs which is absorbed into the systemic circulation, and converted to the physiologically active species [2]. In vitro methods are used to identify possible physicochemical properties of feed compounds that could contribute to explain differences in mineral absorption [51]. The prediction of bioavailability of TEs by means of in vitro digestion assay is only relative because it does not exactly mimic the in vivo digestion due to physiological and environmental factors [52], but the solubility of TEs determined in in vitro experiments might represent the bioaccessible amount of TEs in the dietary treatments for pigs. In vitro simulated digestion in the SI phase significantly decreased concentrations of soluble TEs except Cu, which could have resulted in no significant differences between the treatments in this phase. At the intestinal pH value used in simulated in vitro assay (pH 6.8) or during passage through the small intestine, mineral complexes becoming insoluble and precipitated, thus decreasing mineral absorption due to de novo complexation [5,6].

The change in pH and introduction of more enzymes in the simulated small intestinal phase activated a series of reactions leading to complexation, adsorption, and precipitation of TEs, and decreased bioaccessibility of Zn, Mn, and Fe [53]. Phytic acid in the

cereal-based diet chelated Zn, Fe, and Mn (but not Cu), forming insoluble and undigestible complexes [54]. Even though phytic acid binds Cu more strongly that Zn in vitro, complexes with Cu are soluble over a wide pH range and are less precipitated at neutral pH [55]. Moreover, no inhibitory effect of phytic acid on Cu absorption in vivo has been confirmed [56,57] nor on good solubility of Cu-phytate complexes in the intestinal lumen [58,59]. Enzyme phytases originating from plants, microorganisms, and animal tissues catalyse phytate hydrolysis to inorganic phosphate [60]. Endogenous phytases can degrade phytates, and their complexes with TEs in pig nutrition are phytases generated by the small intestinal mucosa, microbial phytase presents mainly in the large intestine, and intrinsic plant phytase derived from certain feedstuffs [61]. We assume that during our in vitro simulated digestion, hydrolysis of phytates and their complexes could take place only through intrinsic plant phytase activity, and therefore the lower in vitro soluble concentrations of TEs in the SI phase was a result of the strong proteolytic activity of proteases in the pancreatin solution at intestinal pH, which broke down the intrinsic plant feed phytases to result in increased formation of insoluble mineral-phytate complexes and their precipitation [62,63]. On the other hand, gut-derived microfloral and mucosal phytases, which are more stable at higher pH in the duodenum than intrinsic plant feed phytases, could have hydrolysed mineral-phytate complexes in the small intestine of our piglets in vivo, so the in situ solubility of TEs and also the apparent digestibility of TEs did not significantly affect the small intestinal segments of the piglets.

Our inconsistent results as well as outcomes of other studies [64] indicated that in vitro mineral solubility to predict bioavailability of TEs from their different sources may sometimes be unsatisfactory and misleading. Discrepancy and poor correlation between soluble content and total apparent digestibility of TEs could be caused by different total and soluble content of TEs in intestinal digesta collected only one-time at the end of the study (in situ soluble TEs) in comparison to the mineral concentration in pooled faeces which were collected continually 5 days (in vivo ATTD). However, it has been concluded that the variability of apparent nutrient digestibility derived after slaughtering was similar to that found with cannulated animals [65]. Moreover, except for dietary factors affecting the mineral bioavailability in vitro and in vivo, there are various physiological factors influencing the trace mineral digestibility in animals. Since the physiological factors including animal species, sex, age, state of growth or physiological and nutritional status of animals are not present in in vitro systems, they cannot affect in vitro digestibility [2].

Various in vitro solubility techniques used for estimating mineral bioaccessibility indicated either a good or poor correlation between in vitro solubility and in vivo bioavailability of Zn and Fe, depending on used in vitro conditions and chemical species of dietary TEs [3,52,65]. As the good indicator of Zn bioavailability from different Zn sources seems to be their solubility in pH 5 and 2 buffers for poultry; however, an inverse relationship was found [14]. On the other hand, the positive relationship was found between Cu bioavailability and Cu solubility in pH 2 buffer [66]. Therefore, in vitro studies for measuring bioaccessibility and/or bioavailability of TEs are not suitable for fully substituted in vivo studies, and should be regarded as screening methods to help us identify dietary factors affecting the absorption of TEs and to study their interactions. There is a need for more validation studies in which the in vivo results are compared to in vitro results.

5. Conclusions

We conclude that feed supplementation with potato fibre can enhance the apparent digestibility of Zn and Cu from the gut of piglets, but it significantly decreased apparent total tract digestibility of Fe and Mn, and Fe apparent digestibility in the ileum. On the other hand, it seems that Zn glycinate is less soluble in the gut than Zn sulphate, resulting in decreased Zn absorption and digestibility in the lower small intestine. Moreover, ZnGly can affect in vitro solubility of other TEs in the different phases of simulated digestion, which could lead to the changed absorbability of TEs. Apparent digestibility of Zn and Cu

was not affected by Zn or fibre source in the separate small intestinal segments, while Mn digestibility in the duodenum increased by ZnGly supplementation.

Although in vitro solubility of TEs did not provide a good prediction of mineral bioaccessibility because of rapidly decreased solubility of Zn, Fe, and Mn in the simulated small intestinal phase, using a combination of the results from in vitro and in vivo experiments might be used for predicting the relative bioavailability of TEs and might help to improve understanding of important factors involved in digestion and absorption processes involving TEs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12040517/s1, Figure S1: In vitro solubility of Zn from both Zn sources ($ZnSO_4$, ZnGly); Table S1: Individual significant differences between parameters, in vitro solubility of Zn from both Zn sources ($ZnSO_4$, ZnGly).

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Review

Sustainable Livestock Production in Nepal: A Focus on Animal Nutrition Strategies

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Abstract: In many developing countries, the livestock sector plays a vital role in the national economy, providing a source of food, income, and employment. With changes in demographical, socio-economic, and environmental status, the livestock sector in the developing world is facing challenges of low productivity and has become both a culprit and a victim of undesirable climate change impacts globally. In this paper, we will review the status of Nepalese livestock production systems and evaluate possible livestock species-specific strategies to promote a more productive and sustainable livestock sector in the future. In Nepal, the livestock sector is deemed essential to alleviate poverty and improve the nutritional status of the population, as in many other developing countries. However, there is a need for substantial improvements in livestock productivity, in particular improvement of feeding strategies to exploit the genetic potential of livestock. For ruminants, the important issue is to improve nutritional value and hence utilization of existing feedstuffs. Use of, e.g., urea, molasses, and enzymes to improve feed digestibility and implementation of technologies to effectively preserve biomass from forages that are only seasonally available are necessary strategic measures. Identification and use of novel anti-methanogenic feed ingredients will be crucial to develop a ruminant livestock sector that is not only productive, but also environmentally sustainable. For monogastric animals, the development and use of novel protein feed ingredients, such as insects raised on indigestible (for monogastrics) plant residues, should become part of future feeding strategies in support of a circular bioeconomy and improved productivity, not least in small scale poultry production. Future policies should also include a strong focus on capacity building and development of research infrastructure, and promotion of collaborative activities among research and industry sectors to establish a productive yet sustainable livestock sector in Nepal.

Keywords: environment; feed resources; livestock productivity; policy; sustainability

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1. Background

Globally, the livestock sector is a principal source of food and employment. It is one of the fastest-growing agricultural sub-sectors, which provides livelihoods for ~1.3 billion people and accounts for ~40% of the global agricultural gross domestic product (AGDP) [1]. The livestock sector is expected to become even more crucial in the future, particularly in light of a rising human population, which is estimated to become ~9.7 billion in 2050 [2]. In this respect, the livestock sector will play a vital role in fulfilling a growing demand for animal-derived foods [3], while ensuring future global food security [4].

The livestock sector and the production of animal-derived foods are, however, also significant contributors to climate change. The livestock sector alone represents about ~18% of total anthropogenic greenhouse gas (GHG) emissions in the world [5]. There is an unfortunate association between the fermentation of feeds in the forestomach of ruminant animals and the enteric formation of the potent GHG, methane [6]. Growing concerns on the impacts of the livestock sector on environmental health have posed severe challenges to this sector, and there is increasing pressure for GHG mitigation from environmentalists, consumers, and policymakers, both locally and globally [7]. Thus, it is a primary issue for the livestock sector today to develop sustainable production systems, where animal-derived food can be produced with increased efficiency to accommodate growing demands from an increased human population, at the same time as impacts on the environment need to be decreased [8].

In the developing world, including Nepal and other Asian regions, livestock is a primary source of major dietary nutrients and a critical component of small-scale crop–livestock mixed farming systems [9]. The demand for livestock products is projected to increase in developing regions due to a rise in urbanization and income levels [10]. In addition, developing countries are predicted to contribute a significant part (up to 97%) of the global population rise [11], and the population of the developing world alone will reach 7.6 billion by 2050 [12]. Today, the livestock sectors in developing nations are facing two major challenges. Firstly, low animal productivity remains a significant problem, although some tendencies are seen towards scaling up production levels, mainly for monogastrics [13,14]. Secondly, livestock in developing nations accounts for 50–65% of the global GHG emissions from the livestock sector [10], thus contributing to climate change and global warming. However, there are a relatively limited number of studies evaluating the status of animal production in the developing world, particularly in very low-income countries, which limits our insight into the necessary measures to expand productivity at a lower environmental cost in the future. In this context, this paper aims to evaluate the current status of livestock production in Nepal to identify potential strategies that could be employed in the future to establish a more sustainable development of the livestock production systems, whilst considering the prevailing socio-economic conditions. We believe that the issues put forward here would also be relevant for other low-income countries with similar agro-climatic situations, which face a similar challenge with respect to the sustainable development of livestock production in the future.

2. Methodology

To evaluate the current status, identify key constraints, and elaborate suggestions for future policies to promote the development of the Nepalese livestock sector, we reviewed the limited body of available scientific publications related to Nepalese livestock production, animal nutrition, and livestock policies. To search peer-reviewed articles, databases such as Google Scholar, Web of Science, Agricola, Agricultural and Environmental Science Database, Agris, CAB, and Abstracts were used. Different keywords and search terms including "Feed resources" and "Livestock" and "Productivity" and "Sustainability," and "Nepal" were included as search terms. In addition, various reports and databases published by the Ministry of Agriculture and Livestock Development, Nepal (https://www.moald.gov.np/publication/Agriculture%20Statistics assessed on 6 December 2021); Central Bureau of Statistics, Nepal (https://cbs.gov.np/metadata/; assessed on 6 December 2021); National Population and Housing Census, Nepal (https://censusnepal.cbs.gov.np/Home/Index/EN; assessed on 6 December 2021); and the statistical database of FAOSTAT (https://www.fao.org/faostat/en/#data; assessed on 6 December 2021) were also used. All graphs were generated using GraphPad Prism software 7.0 (San Diego, CA, USA).

2.1. Data and Calculations Underlying Projections for Milk Production and Demands

We analyzed the trend of annual milk production compared to demand using data from the Nepalese ministry [15,16]. We calculated the current milk demand (ton/day)

using the FAO recommendations of 320 mL/day of milk or dairy products for adults (>18 years of age) for Nepal [17]. The values for daily milk demand would be even higher if they were corrected for the needs of non-breast-fed infants and children and teenagers below 18 years of age, who have a daily recommendation of 500 mL/person/day [17]. In addition, we made future projections of daily milk demands (ton/day) in Nepal for the period 2020–2030. The calculations for the projection of future demands were performed based on the projected increase in the total population of Nepal during that period, as presented by CBS [18], and the expected minimum demand for milk based on the FAO recommendations for daily milk consumption (320 mL/adult/day) for adults (>18 years of age) from developing countries [17].

2.2. Data and Calculations Underlying Projections for Meat Production and Demand

Projecting the future trends in annual meat production compared to demand was conducted in a similar way, using data from the Nepalese ministry [15,16]. The total annual meat production was calculated based on the amount of meat from major meat-producing animals, and also included the amount of duck meat which is negligible (217–281 tons/year) compared with other livestock species. The meat demand was calculated based on the FAO data for average meat consumption per capita (27.9 kg/person/year or 76.4 gm/person/day) in the developing countries [4]. We further made pessimistic projections for future meat demands in Nepal based on the FAO data for average annual meat consumption per capita for developing countries. Three separate projections were made based on the average annual meat consumption per capita for developing countries, worldwide population, and developed countries: 27.9 kg (76.4 gm/person/day), 38.7 kg (106 gm/person/day), and 80 kg (219.3 gm/person/day), respectively [4].

3. Nepalese Livestock Production

Nepal is an agricultural land-locked country situated in South Asia between India and China [19], where the agricultural sector is the prime source of national income, and it contributes about 32% to national gross domestic production (GDP), involving ~65% of the economically active population [20]. Nepal consists of high mountains, the Himalayan range (35% of total area), mid-hilly regions (42% of total area), and the lower plain of the Terai region (23% of total area) [20], each characterized by very distinct agro-climatic conditions. Thus, the Nepalese agricultural sector has regional and very distinct resource-use patterns from the lower plain areas bordering India (Terai region) to the Himalayan area at around 5000 m altitude near the Tibetan region.

Livestock is an important sub-sector within the Nepalese agricultural sector and accounts for ~25% of the national agricultural GDP [21]. The domestic livestock production in Nepal is based on 17 different species [22], which encompasses buffalo, cattle, goats, sheep, pigs, and poultry as the main farm animals (Figure 1), accounting for a total annual milk and meat production of 1,911,239 and 332,544 tons, respectively [20]. One of the critical features of Nepalese livestock is the subsistence nature of farming, where typically, a single household manages only a small number of animals. Accordingly, the Nepalese agriculture census defines the economic unit of an agricultural holding in terms of livestock production as at least one big head (cow, buffalo, etc.) or at least five small heads (goat, sheep, etc.) or poultry consisting of at least 20 birds (chicken, duck, etc.) managed under a single management system [23]. Farmers generally raise their few animals alongside other agricultural components, such as agronomical crops, fruits, or vegetables, and the animals are either kept in a shed or allowed to graze or scavenge freely during some hours of the day. In recent years, the number of commercial dairies (cow, buffalo), pig and poultry farms has been growing in Nepal; however, overcoming low livestock productivity is one of the significant challenges [24], as described in the following.

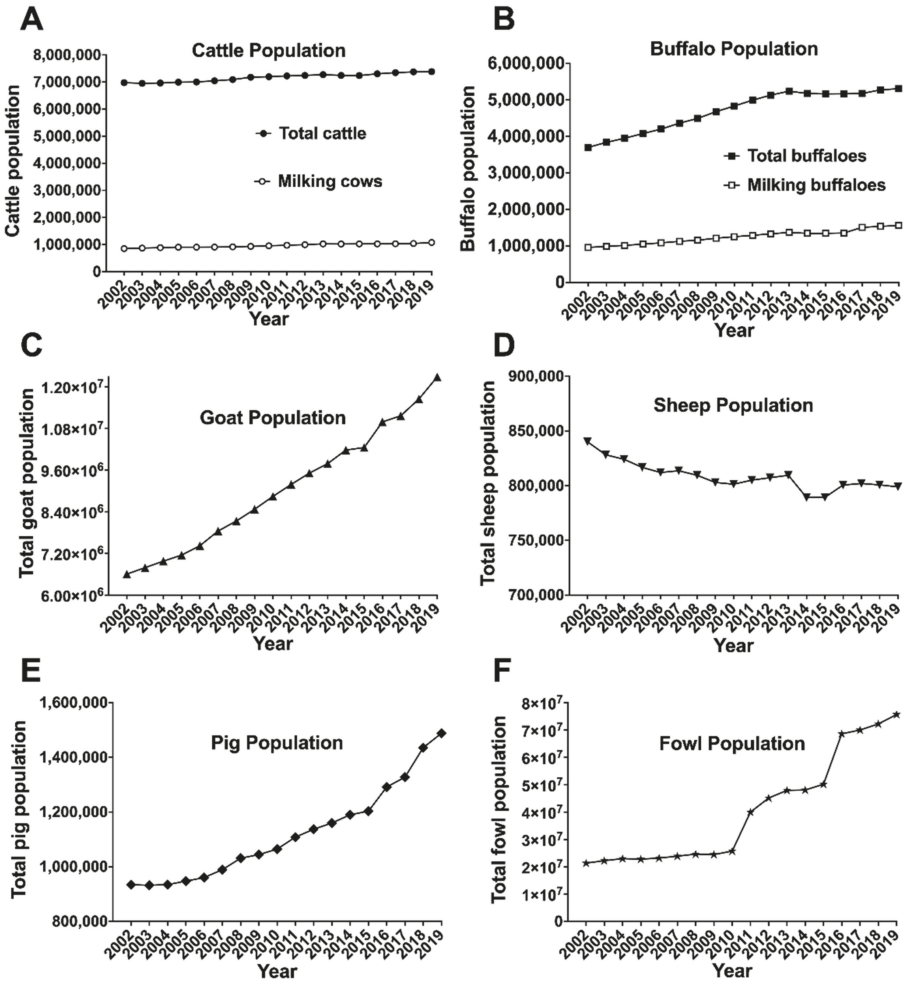

Figure 1. Population trend of major livestock species in Nepal from 2002 to 2019 (MOALD, 2017; MOALD, 2019) [15,16]. (**A**): Population trend of cattle (solid line, ●: total number of cattle; solid line ○: number of milking cows), (**B**): Population trend of buffalo (solid line, ■: total number of buffalo; solid line, □: number of milking buffalo), (**C**): Population trend of goats, (**D**): Population trend of sheep, (**E**): Population trend of pigs, and (**F**): Population trend of fowl.

4. Current Status

In the following sections, the status of livestock production from various species in Nepal will be highlighted in relation to their number, management, and overall production status, and strategic concerns regarding improvements in productivity of the different livestock species will be addressed.

4.1. Major Milk-Producing Animals in Nepal

In Nepal, buffalo and cattle are the major milk-producing livestock species. In this section, we will review the status and identify constraints for the improvement of productivity of the various livestock production systems.

4.1.1. Cattle

Cow milk (665,285 tons annually) is the second primary source of liquid milk consumption after buffalo (1,245,954 tons annually) in Nepal, accounting for about one-third of the total milk production [20]. Despite the significant contribution of cattle to the Nepalese dairy sector, cow milk productivity is very low compared with the global average. Additionally, milk production trends are not promising as annual dairy cattle milk productivity in 2001 was 412 kg/milking cow, and it had only increased to 627 kg/milking cow by 2015 [20]. One crucial aspect in this respect is that non-milking cows share a substantial proportion of the total cattle number since milking cows represent only ~14% of the entire cattle population [20]. Due to religious issues, it is not possible to cull non-productive cattle in Nepal.

When formulating future policies to improve the national self-sufficiency for milk, it is, therefore, recommended to focus on other milk-producing animals, such as buffalo, goats, and in mountainous regions, even yak. In contrast to cattle, these different species are associated with fewer religious constraints, allowing for efficient breeding and culling policies to be implemented. Details of the milk production status of goats, sheep, and yak are not available, and thus, in the following, the milk production status of only buffalo will be presented. However, goats are particularly very suited to Nepal's mountainous agricultural zones and can contribute to the national milk production and the development of milk-based industries also for small-holders, provided suitable breeding and proper livestock management approaches are applied. More recently, goat milk has been recognized as a particularly efficient tool to fight against malnutrition in the ultra-poor population, and the government has introduced various programs to promote dairy goat farming among poor farmers.

4.1.2. Buffalo

Indigenous buffalo *(Bubalus bubalis bubalis)* are distributed from sub-tropical to alpine zones of Nepal. Buffaloes are the greatest source of milk and meat production (Figures 2 and 3), contributing significantly to the agricultural gross domestic product (AGDP). They are exploited mainly for milk and meat, and to some extent, for draft power and manure. A recent report suggests that buffalo alone contribute to >50% of total meat and >60% of total milk production in Nepal, as shown in Figures 2 and 3 [20].

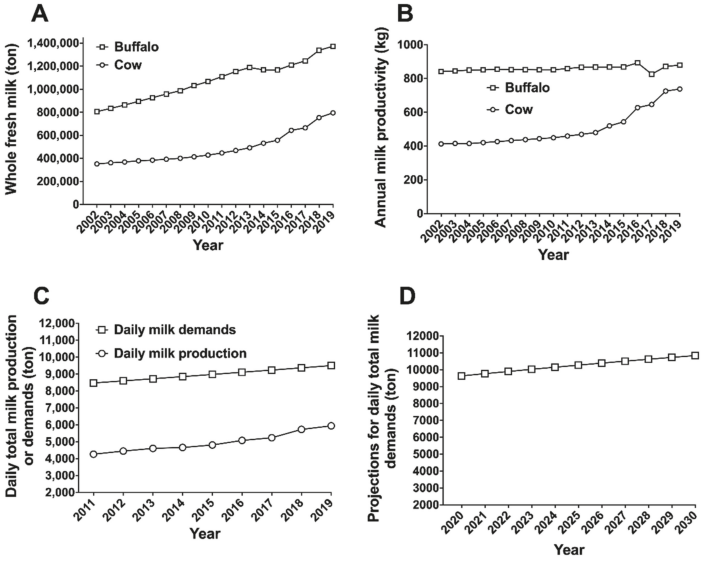

Figure 2. Status of milk production in Nepal (MOALD, 2017; MOALD, 2019) [15,16]. (**A**): Annual whole fresh milk production in ton for 2002–2016 (solid line, □: buffalo milk; solid line; ○: cow milk),

(**B**)**:** Annual milk productivity (kg/milking animal/year) of cow and buffalo for 2002–2016 (solid line, □: buffalo; solid line; ○: cow), (**C**): Status of annual milk production and demand (ton/day) in Nepal for 2011–2016 (solid line, □: daily milk demand; solid line; ○: daily milk production). The daily milk demand for Nepal was calculated using FAO recommendations of 320 mL/adult/day for adults (>18 years of age) [17]. The values for the daily milk demand would be even higher if they were corrected for the needs for non-breast-fed infants and children and teenagers below 18 years of age who have the daily demand of 500 mL/person/day [17]. (**D**): Projection of daily milk demand (ton/day) in Nepal for the period 2020–2030. The calculations for the projection were performed based on the projected total population of Nepal during that period presented by CBS [18] and the FAO recommendations for daily milk demand (320 mL/adult/day) for adults (>18 years of age) from developing countries [17]. The values for projected milk demands would be even higher if they were corrected as described in (**C**).

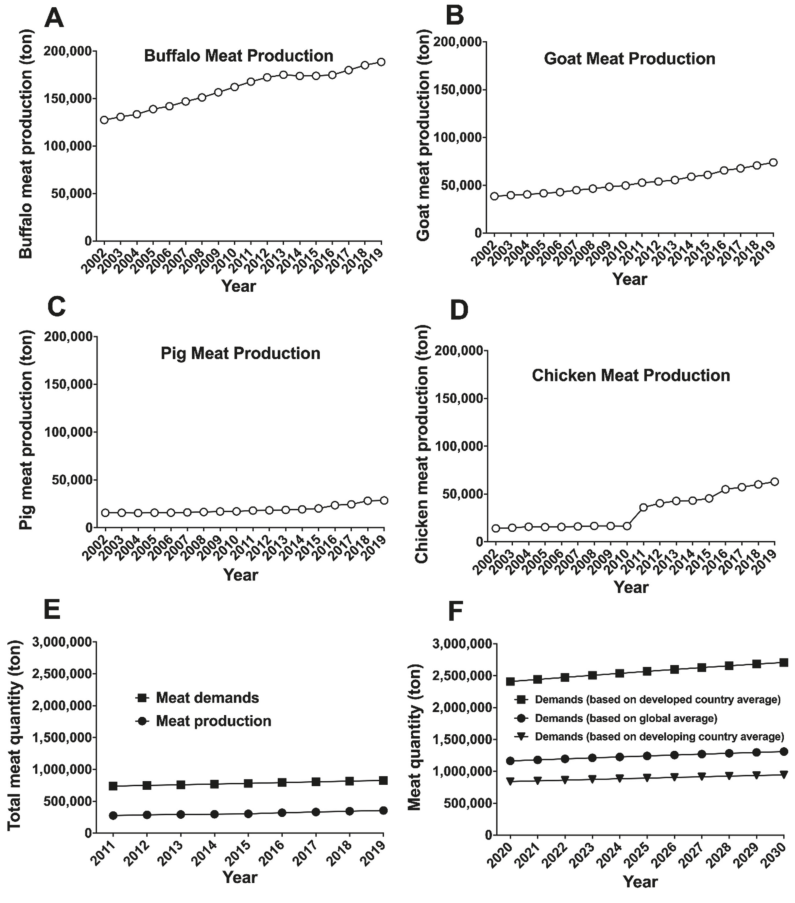

Figure 3. Status of meat production in Nepal (MOALD, 2017; MOALD, 2019) [15,16]. (**A**): The trend of total annual buffalo meat production (in ton) during the period 2002–2016, (**B**): The trend of yearly total goat meat production (in ton) for the period 2002–2016, (**C**): The trend of total annual pig meat production (in ton) during the period 2002–2016, (**D**): The trend of yearly total chicken meat production (in ton) during the period 2002–2016. (**E**): The trend of total annual meat production compared to demands for

2011–2016 in Nepal (solid line, ●: meat production; solid line, ■: meat demands). The total annual meat production was calculated based on the amounts of meat from major meat-producing animals and also included the amount of duck meat, which is negligible (217—281 ton/year) compared with other livestock species. The meat demands were calculated based on the FAO data for average meat consumption per capita (27.9 kg/person/year or 76.4 gm/person/day) in the developing countries [4]. (**F**): Projection for meat demands (in ton) during the period 2020–2030 based on the FAO data for average annual meat consumption per capita (solid line; ■: developed country and solid line; ●: global average, solid line; ▼: Nepal, developing country). The average annual meat consumptions per capita for developing countries, worldwide population, and developed countries, developing countries were used as 27.9 kg (76.4 gm/person/day), 38.7 kg (106 gm/person/day), and 80 kg (219.3 gm/person/day), respectively [4].

There are mainly three indigenous breeds of buffalo in Nepal: Lime, Parkote, and Gaddi, distributed predominantly in low hills, mid-hills, and far western hills, respectively [25]. However, imported breeds are becoming increasingly popular, with Murra now dominating current buffalo production among them. Low milk production (893 kg/milking buffalo) is a significant challenge in the buffalo production system, and realizing this, the Nepalese government launched the 20-year Agriculture Perspective Plan (APP) in 1995, where the priority was given to milk production. However, Nepal continues to import considerable amounts of whole milk and live buffalo from a neighboring country. To become self-sufficient in milk production, buffalo is the primary species to be targeted, and the focus should mainly be on buffalo milk productivity.

4.2. Major Meat-Producing Animals in Nepal

In Nepal, cattle do not contribute at all to national meat production as the consumption of meat from cattle and the use of cattle for meat production are prohibited for religious reasons. Therefore, buffalo, goat, sheep, pig, and poultry are the key sources of meat. In the following sections, the major constraints and possibilities for meat production from different livestock species will be presented.

4.2.1. Buffalo

In addition to a significant contribution to total milk production, buffalo are the major contributors to meat production in Nepal. Buffalo meat (cara or buff) accounts for nearly 60% of the national total meat consumption [20]. In general, male and unproductive female buffalo are slaughtered for meat consumption, whereas reproductive females are used for milk production. The female buffalo are mostly culled due to infertility, repeated breeding, and disease problems such as mastitis. With cultural transformation (relaxation of traditional norms and values for eating behaviors), so-called higher caste people like Brahmin have also started consuming cara meat in Nepal, thus increasing the overall demand for buffalo meat in recent decades. Additionally, cara meat is cheaper than other meat sources such as chevon, mutton, and chicken. Thus, in the future, it can be anticipated that the demand for buffalo meat will continue to increase, along with growing purchasing power among the lower strata in society. Therefore, it is recommended considerable strategic emphasis be placed on buffalo production to increase total meat production in the future.

4.2.2. Goat and Sheep

Small ruminants (goats and sheep) are vital components of farming systems in the developing world, and Asian countries account for about 57% and 23% of the world populations of goats and sheep, respectively [26]. In Nepal, goats are a crucial element of agricultural systems. Many farmers raise goats primarily for meat purposes (animal protein), and goats serve as an important source of income and savings in many rural families. Historically, goats have been well integrated into a typical crop–livestock mixed farming system, particularly in the hilly regions, due to their ability to capitalize available

plant biomass resources as feeds in mountain terrains. However, goats are also raised in the lower plain (Terai) regions of the country.

The total production of goat meat continues to grow in Nepal. Such an increase in production appears to be driven primarily by the rise in the population of goats, as recent trends indicate (Figures 1 and 3) [20], and to some extent also by improved feeding. It is estimated that goats contribute about 20% of the total Nepalese meat production. A large number of goats (~15% of the total population) is imported from India to fulfill the national demand for goat meat [27]. In Nepal, there are mainly four indigenous goat breeds: Chyangra, Sinhal, Khari, and Terai, where Khari is the predominant breed accounting for ~50% of the total goat population [28]. Although breed-specific production traits exist, all goat breeds generally have low productivity in Nepal, which is associated mainly with poor management practices. Chyangra and Sinhal are primarily found in the high hills and mountainous regions. They are managed under a migratory system, where animals graze alpine pasture areas during the summer months but are allowed to graze on the lower fallow crop fields and forests during periods of extreme winter conditions. Khari and Terai breeds are found primarily in the lower hills and Terai regions. Both of these breeds are generally managed under a sedentary system, where animals are allowed to graze for a few hours during the day in the nearby forest, fallow land, and pasture areas [28], complemented by feed brought to them in a cut-and-carry system. Several imported goat breeds are also being raised throughout the country, particularly Boer goats, which have become popular in recent times. In this context, the improvement of local indigenous breeds, the establishment of commercial farms with a higher stock density, and the introduction of proper nutritional strategies for sustained availability of feeding materials are the three key areas the Nepalese goat sector should focus on to improve the productivity of goats and fulfill the national demand for meat and milk from this species.

Sheep also constitute an important component of the Nepalese agricultural system, particularly in the Himalayan regions, where they serve as a source of meat, wool, and cash income. Most sheep in Nepal are reported to be of region-specific native breeds: Bhaynglung in the Transhimalaya, Baruwal in the mountains and Kage in the hills, and Lampuchhre in the Terai region. Nevertheless, sheep production is primarily limited to the upper Himalayan areas and adapted to the local climatic conditions, where sheep (like goats) are managed under a migratory or transhumant system. Sheep are well adapted to the local climatic conditions of the mountainous region with a diet primarily based on alpine pasture [29]. The sheep population and total sheep meat production have sharply declined in the past in Nepal (Figure 1 and Figure S1A) [20] which could, in part, be associated with the poor performance of local breeds, seasonal deficit of feed resources, and a lack of efficient market channels for sheep meat and wool [30]. Increased urbanization and migration of youth from villages to cities and abroad in search of employment may also have played a role. Since sheep are a crucial component of mountain agriculture, it is critical to develop efficient management and marketing strategies to establish a more profitable and sustainable livestock production, which can sustain a livelihood for people and encourage young people to reside in the high mountain regions, poorly suited for other agricultural activities.

4.2.3. Pigs

Pigs are one of the major livestock species in Nepal and are ranked as the 5th most important livestock species after fowl, cattle, buffalo, and goat in terms of population numbers. The trend for population size of pigs is increasing (Figure 1E) [15], and indigenous pig breeds contribute significantly (~50%) to the total pig population [31]. Due to certain cultural and religious taboos, pork production and consumption were limited to specific ethnic communities in the past. However, along with the transformation in cultural beliefs and values, pork has become a popular choice among the Nepalese, particularly in the urban areas, as evidenced by a sharp increase in pork production and consumption (Figure 3C) [15].

In the future, pork is expected to contribute substantially to the country's total meat production. With changing demands for pork, it is vital to conserve indigenous pig resources for future pig improvement programs [31]. Since the traditional small-scale subsistence piggery is mostly maintained under quite low planes of nutrition, particularly utilizing kitchen waste and by-products, it is essential to direct future efforts toward the commercialization of the pig industry. This should include a focus on improvements of farm structures, animal breeding, and feeding practices to improve not only pig performance but also to break routes of infectious diseases, particularly the zoonotic parasitic diseases taeniasis and cysticercosis. This will help to integrate a traditionally not well accepted or not recognized pig sector into the national livestock farming system with a massive potential for future meat production in the country. Recently, there has been an increasing number of piggery operations with a larger number of animals and a more commercialized approach. Piggery has been one of the popular agricultural business choices among youth who have returned from overseas.

4.2.4. Poultry

The poultry sector is one of the fastest-growing and probably the most commercialized livestock sub-sector in Nepal, and this sector alone contributes to the national GDP by around 3.5% [32]. Poultry production in Nepal consists of commercial broiler and layer farms, as well as village or backyard poultry production, where birds are raised in a small number in the scavenging or backyard system, while exploiting all sorts of inputs from the farmhouse itself [32]. Poultry farming in Nepal involves both commercial and native stocks, and their production shows an increasing trend [33]. A significant portion of the total poultry population consists of native breeds. Some of the documented indigenous chicken breeds of Nepal are Sakini (normal feather), Ghatikhuile (naked neck), and Pwakhulte (frizzled feather) [33]. Although the poultry sub-sector includes chickens, ducks, pigeons, and other kinds of birds kept under various production systems, the chicken population dominates by far [32]. The national population of fowl has gradually increased (~68 million in 2015 compared with ~21 million in 2001) along with a significant increase in the total chicken meat production (~55,000 tons in 2015 compared with ~14,000 tons in 2001) [20]. Chicken meat constitutes roughly 17% of total meat production, representing more than 99% of the total poultry meat production [20].

Regarding egg production, with increasing farm sizes, a higher mass of egg production and lower feed conversion rate have been observed, indicating a higher profit margin [34]. Sakini is a major native breed contributing to egg production in different regions of Nepal [35]. Support for a continued rise in commercial poultry production, improvement of the health of poultry species, and establishment of efficient marketing channels are some of the key issues the Nepalese government should prioritize in the future [36].

4.3. Wool Production in Nepal

In Nepal, no formal marketing channels exist for wool, unlike other livestock products, and farmers in different agro-ecological zones produce and utilize locally produced wool at a low scale. Although wool is recognized as a potentially valuable animal product, it has not been a major focus in the Nepalese livestock or agricultural policies, and Nepali pashmina or carpet industries rely on Tibetan wool producers due to insufficient local wool production or low wool quality [37]. Nepalese native sheep breeds have low wool productivity [38]. The production trends for wool in Nepal have, in fact, been stagnant during the past decades (60,9102 vs. 59,4312 kg in 2002 and 2017, respectively; Figure S1B) [15]. Thus, the Nepalese carpet industries rely on imported raw wool for wool-based products. Sheep are important livestock species for both meat and wool, particularly in the mountainous regions. Thus, focusing on improvements in wool, in addition to meat production from sheep, would be a sound priority when formulating regional livestock policies [39], to improve living conditions and avoid depopulation of the mountainous regions in Nepal. To

overcome the challenge of low wool production and quality in the sheep industry, strategic efforts should target a range of management practices, including breeding, harvesting, pre-processing of wool, and animal nutrition for general improvements in sheep (re-) productive performance.

5. Future Perspectives

In the following, the future perspectives for the Nepalese livestock sector will be discussed, and the role and importance of the livestock sector to ensure future food security in the country, and economic development, will be highlighted. Suggestions will be put forward for critical areas to include in Nepalese livestock development strategies to improve and sustain livestock production (Figure 4).

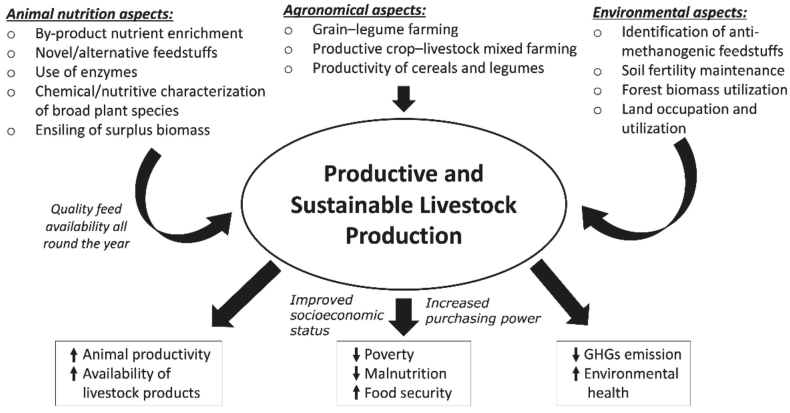

Figure 4. Future considerations to establish a productive and sustainable livestock production system in Nepal. Issues associated with animal nutrition, agronomy, and the environment are important to develop a productive livestock sector in Nepal, which could fulfill increasing demands for livestock production in changing demographic and socio-economic situations. (arrow; ↑: improvement, arrow; ↓: reduction); GHGs, greenhouse gases.

5.1. Productive and Sustainable Livestock Systems to Alleviate Poverty

Increasing population growth is a worldwide concern today. The most recent national population census of Nepal (2011) estimated the population to be 26.5 million, with a projection of a population increase to 30.4 and 33.6 million by 2021 and 2031, respectively [40]. Besides, Nepal is currently one of the least developed countries, where a significant proportion of the population lives below the poverty line (21.6%) [24]. Moreover, there is generally a poor nutritional status among people. Thus, 42%, 31%, and 14% of children were characterized as stunted, underweight, or suffering from wasting according to a Nepal Living Standards Survey (NLSS) in 2010/2011 [41]. In this respect, livestock could be a necessary tool to improve food and nutrition security and alleviate poverty, since (a) the majority of the poorer part of the rural population in the developing world (including Nepal) keep some kind of livestock, which represents a source of both income and valuable food protein [42], and (b) in a mountainous country like Nepal, conditions in vast areas for commercial crop production are poor, whereas livestock can be managed and valuable livestock-derived products can be produced in the higher hilly regions and mountains.

Previous reports have indicated that the poverty trend in Nepal is declining, particularly associated with the dynamics of both domestic migration (from rural to urban areas) and international migration (work-related migration abroad) [43], where foreign remittances have been particularly associated with poverty reduction [44]. Such migration had the following consequences for livestock production in the country: (a) the utilization of fertile land for the production of food and animal feed has declined, (b) availability of

local labor for livestock production has diminished, and (c) with increased income and better socio-economic status, per capita meat consumption and demands for livestock-derived products are growing, expanding the gap between demand and domestic supply, consequently leading to increased demand for imported meat and milk products.

As pointed out previously, the Nepalese livestock system is generally characterized by low productivity. In the following sections, the specific strategies required to develop more productive livestock systems will be discussed, since it is a priority in Nepal to become more self-sufficient in terms of feeding a growing population to alleviate (rural) poverty and improve the overall nutritional status of the population.

5.2. Alternative Livestock Feeding Strategies

In Nepal, both ruminants and monogastrics should be considered important production animals in the future. Since ruminant and non-ruminant animals have different digestive functions, nutritional requirements, and abilities to utilize various feed resources, species-specific feeding strategies need to be formulated.

Species-Specific Feeding Strategies

Ruminants: Buffalo, cattle, sheep, and goats are the major ruminant animals raised for meat or milk production in Nepal. One of the major challenges for improvement of productivity in the Nepalese ruminant livestock sector is to provide quality feed to the animals all year round, which is an important issue, since feed costs can amount to ~60% of total production costs in a typical livestock production system [45]. Due to land occupation and degradation, land availability for grazing is diminishing so the livestock sector relies more heavily on crop residues, by-products, and (novel) fodder and forage plants. For example, hempseed meal, a by-product of hemp oil production, was shown to be an alternative feeding resource for small ruminants [46]. Thus, strategies to supply sufficient amounts of nutritious feeding material all year-round should be a major strategic focus for the development of ruminant production in Nepal.

Crop–animal interaction is an integral component of mixed farming systems in Asia, including Nepal, where both crop and livestock complement resources to establish and sustain such farming systems [9]. A typical rice–wheat–maize cropping system in Nepal generates a substantial amount of straw and other by-products, which can be utilized for livestock feed. However, the nutritional quality of straw is low and methods to enrich such by-product nutrient content and use it efficiently have not been commonly practiced. It has been shown that urea treatment (up to 4% on a weight basis) enhances the voluntary intake of by-products like straw, and urea treatment can increase milk production in lactating buffalo [47]; this is due to an improved ability of microorganisms in the rumen to utilize fiber and synthesize protein for the animal, when the non-protein–nitrogen source, urea, is added to the diet. However, urea is not readily available to farmers. Alternatively, such by-products can also be pre-treated with sugar molasses [48] to increase feed intake and animal productivity. The nutritionally deficient crop by-products, such as rice straw (with high lignin and silica contents) could be enriched with the addition of alternative nutrient sources to stimulate microbial fermentation in the forestomach, such as urea, sugar molasses, ligninolytic enzymes, or microbial treatments [49].

In Nepal, precipitation during the rainy monsoon season (June–August) allows different grasses, pasture, and other fodder plants to grow, which represent substantial natural plant biomass of good nutritional quality and which can be used to feed ruminant livestock. However, in other seasons of the year (such as in winter or spring conditions), natural biomass availability is low, and particularly larger ruminants hardly get anything to eat other than the dried crop by-products of low nutritional quality. This seasonal deficit of feed materials is probably the major constraint for the improvement of ruminant production [30] and must be overcome to improve the productivity of ruminant livestock production systems. Preservation/conservation of plant biomass during the season, when it is available, is an important strategy to ensure more consistent availability of feed for

ruminants throughout their production cycle. The substantial amount of biomass available during the rainy season could be stored as hay or silage, thus preserving the available nutrients for use during the dry season, with a positive impact on overall annual animal productivity. It is noteworthy that it is not feasible to operate ensiling on a larger scale in all regions of Nepal due to topographical constraints, space limitations, and the lack of proper technologies to harvest and chop the harvested plant biomass. However, the application of low-scale ensiling techniques, such as using synthetic plastic bags or pits, could be an economical option also for small-scale farmers [50]. However, the implementation of such technologies is presently hindered by ineffective extension services and hence knowledge transfer.

In Nepal, more than 170 different species of trees, shrubs, and vine are used to provide fodder for ruminants [51]. However, there is limited information on their nutritional contents and potential biomass yields; nevertheless, three different commonly used fodder species had a large annual biomass yield of ~26–39 kg dry matter (DM)/tree [52]. Chemical analyses of ~30 different types of fodder-tree leaves revealed a relatively high crude protein content of ~10–22%, but also high contents of neutral detergent fiber (~27–74%) and acid detergent lignin (~6–30%) on a DM basis; these have strong negative impacts on DM digestibility [53]. In the hilly regions, the fodder species available for ruminant feeding as green feed will only support moderate levels of growth and milk production [54] due to the high content of indigestible components, such as lignin, which also limit the availability of protein and other nutrients for the animal [53]. Detailed chemical and nutritional characterization of various forage and fodder species potentially available as ruminant feeds are yet to be performed, and this knowledge is needed for estimations of possible improvements in productivity in the ruminant livestock sector.

Non-ruminants: Poultry and pigs are the major land-based non-ruminant species being raised in Nepal. The poultry sector is, as mentioned before, the most commercialized animal production sector, although people, particularly in rural areas, still keep a small number of local poultry breeds as a source of quality food protein, as well as for additional income from sales of eggs and meat [55]. Like in many other countries, poultry feed can contain up to 65% of corn [56]; corn is a significant energy source used for poultry production in Nepal. Corn is also one of the staple foods for human consumption in Nepal, and corn productivity is low despite some efforts in the past to try to increase productivity [57]. Due to the competition for corn for human food versus animal feed, the total annual demand for corn is growing, and thus ~45% of total corn used for animal feed is imported from India [57]. There have been some research efforts made by the National Maize Research Program, under the Nepal Agricultural Research Council, Government of Nepal, to increase the productivity of corn by developing more stress-tolerant corn varieties [58]. However, they are yet to be tested and commercialized under farm conditions in different agro-ecological zones. Pig production is still at an infant stage, although recent trends show that pork consumption is rising, particularly in urban areas, and this will call for even more efforts to improve the production of suitable feeds for monogastric animals, without competing for arable land needed for the production of food for human consumption.

In Nepal, various grain legumes are available in the winter (such as lentil, chickpea, etc.) and summer (such as soybean, black gram, etc.) seasons and they are important components of the cropping systems but are mostly used for human consumption, and their productivity is remarkably low (<1 ton/ha) [59]. Hence, overall availability of soybean for animal feeding in Asian regions is generally low [60], but soybean is, nevertheless, the major and probably most expensive feed resource used in Nepal for poultry and swine to fulfill their protein requirements. Grain legumes, including soybean, are mostly cultivated under an intercropping system, but they also have the potential to be grown as sole crops, particularly in the lower plain areas of the Terai region. Diseases and pests, soil moisture deficits in winter crops, and the unavailability of high-yielding varieties appear to be the major constraints for the upscaling of grain legume production [61]. To increase the productivity of Nepalese poultry and swine production systems, it is, therefore,

imperative to find alternative sources of feed protein to partially replace the expensive soybean meal as a protein source. Such alternative feed items could be of both animal or plant origin. One alternative protein source for non-ruminant animals, which presently is attracting interest in more developed regions of the world, is insects. Nepal hosts a diverse range of insect species [62,63], and they could become alternative sources of protein in diets for non-ruminants in the future [64]. This requires that insects depending on their growth substrate can be safely added to livestock diets without compromising the quality, safety, or palatability of meat and milk [65]. Insects can be grown on different cheap organic substrates, such as household wastes. This is an important feature in relation to the development of a more circular bioeconomy, where unutilized waste is converted into valuable protein food sources and the pollution load is reduced.

Other alternative sources of protein could be plant-based feed materials. Sunflower or mustard meals also contain high levels of protein. In one study, sunflower meal (with the addition of microzyme) could substitute up to 35% of soybean meal in the feed without compromising the growth performance of broilers [66]. Additionally, the water plant Azolla, like terrestrial legumes, can fix atmospheric nitrogen and convert it into protein. Azolla has been identified as a possible economical substitute for soybean-based poultry feed and can replace up to 10% of soybean meal in the overall diet [67].

The availability of quality feeds all year round is important to sustain animal production and exploit the genetic potential of livestock. Specific research and extension services should be prioritized in Nepal to effectively utilize current feed resources and formulate implementable, alternative, and efficient new feeding strategies. Particular attention should be paid to enrich the nutritional values of various plant-based by-products. Also, cost-effective small-scale technologies should be developed to preserve the chemical and nutritional properties of seasonally available surplus plant biomass. Additionally, a detailed evaluation of biomass yields, chemical and nutritional properties, and digestibility of a broader range of available forage and fodder species is vital to optimize future feed formulations. Future strategies should also be directed towards identifying alternative nutritious feed resources, such as insects, and assessing them for palatability, utilization, and impacts on animal health, growth, and performance. While formulating future livestock strategies, it is noteworthy that ruminants have advantages over non-ruminants, since they are not necessarily in direct competition with humans for food resources, which is critical for a developing country like Nepal, but feed resources for ruminant animals should nevertheless have a reasonably high digestibility to achieve significant improvements in animal production.

5.3. Feeding Strategies for Reduced Climate Impact

The livestock sector's contribution to climate change has become a global issue, as the livestock sector alone accounts for about one-fifth of total GHG emissions [68]. In Nepal, it has been estimated that agriculture and forestry, and other land-use sectors account for >80% of national GHG emissions [69]. Enteric emissions from ruminant livestock and emissions from agricultural soils are the major contributors [70]. The climate mitigation policies directed at GHG emission from the livestock sector and particularly enteric methane emission from ruminant livestock should be implemented with extreme caution. Assessing the negative impact of livestock-derived GHGs should be balanced against the positive benefits relating to food security and poverty alleviation since livestock is a vital source of nutrition and livelihood for many low-income people in countries like Nepal [71,72]. The global average meat consumption is ~100 g per person per day with an about ten-fold variation between the highest and lowest consuming parts of the population [68]. In Nepal, with a transition in socio-economic status and income rise, per capita meat consumption will increase, which is expected to lead to beneficial trade-offs in terms of improvements in nutritional status. However, this also calls for a transition of livestock production systems from the traditional extensive production system to more intensified and resource-efficient systems to ensure increments in productivity are achieved without associated increments

in GHG emissions. Improving livestock production efficiency through better breeding techniques and feed utilization strategies is essential in this respect since the most important determinant for methane emission per kg produce is animal productivity [73].

Other possibilities to reduce emissions from the ruminant livestock sector could be to exploit feed additives with anti-methanogenic properties in diets for ruminants. This is becoming a topic of interest in the European Union, where climate policies dictate a 40% reduction in GHG emissions by the year 2030 compared with 2014, which is likely to be backed by political instruments, such as taxation on livestock production [74]. Various types of plants have been shown to contain bioactive, anti-methanogenic compounds, but to the best of our knowledge, no research has been conducted in this field on indigenous plants from Nepal.

To sum up, long-term strategies to minimize GHG emissions from the livestock sector in a developing country like Nepal should involve (1) the transition of particularly the existing ruminant livestock sector towards more productive systems, and (2) identification of potential anti-methanogenic plants/compounds suitable to use as feed additives for ruminant livestock.

The livestock sector is not only a culprit, but also itself becoming a victim, of climate change; it is vulnerable to the adverse impacts of climate change on the environment and feed availability for livestock in the developing world [75]. In Nepal, the livestock sector is, as already mentioned, an integral component of mixed farming systems. The increased frequency of drought, extreme rain, hailstorms, floods, erratic rainfall patterns, etc., are some of the observed effects of climate change with negative impacts on the existing farming system [76,77]. Thermal stress has direct adverse effects on animal health, reproductive, and productive performances of livestock, whereas adverse abiotic conditions, such as droughts and heat stress, lead to undesirable impacts on forage and crop production, thus limiting plant biomass supply for feeding of production animals [78,79]. Although there are some early indications of adverse climate change impacts, it is yet too soon to fully understand the extent of climate change impacts on the livestock sector in different regions of Nepal with its diverse agro-climatic conditions and agro-livestock systems. Climate impacts on livestock production may, therefore, be very different in different areas [80]. In Nepal, limited research has been conducted concerning livestock adaptation to climate change, and most research efforts have taken only ruminant species into account. Thus, future studies are needed to acquire a better understanding of the impacts of climate change on different livestock production systems, taking both ruminants and non-ruminants into account, and considering key issues, such as forage and pasture availability, animal performance characteristics (meat, milk, and egg production and feed conversion efficiency). Such information is essential to perform a holistic economic analysis of livestock systems under climate change and develop the most appropriate region-specific future strategies for adaptation and reduction of GHG emissions.

6. Future Livestock Policies

In Nepal, the Ministry of Agriculture and Livestock Development (MOALD) (http://moald.gov.np/, accessed on 8 December 2021) is responsible for the formulation, implementation, monitoring, and evaluation of livestock-related policies, plans, and programs, and the Department of Livestock Services (DLS) is mainly liable for livestock-related extension activities. Moreover, the Nepal Agricultural Research Council (NARC) (www.narc.gov.np, accessed on 8 December 2021) carries out major agricultural research activities at the National Animal Science Research Institute (NASRI), with various disciplinary divisions targeting different livestock sectors. Agricultural universities, mainly the public Agriculture and Forestry University (AFU) and Tribhuvan University (TU), and some private institutions carry out teaching activities to fulfill academic requirements for various study programs in animal and veterinary sciences. In Nepal, collaboration within and across governmental livestock bodies and industry sectors could generate needed

synergies to improve livestock productivity. Unfortunately, such coordinated efforts have been missing so far.

Detailed analysis of the national agricultural policies of Nepal have been reported elsewhere, suggesting a need for a separate livestock policy rather than the previously formulated common agricultural policies (see review by [37]) and agriculture perspective plans (1995/96–2014/2015) [81]. In this paper, we propose that a future separate livestock policy should be formulated, which (a) acknowledges animal feed as a pivotal component to improve livestock productivity, (b) prioritizes the capacity building of research institutions to uplift livestock research, and (c) recognizes the industry sector as a key player in Research–Academia–Industry (RAI) partnerships to promote future research and sector development activities (Figure 5).

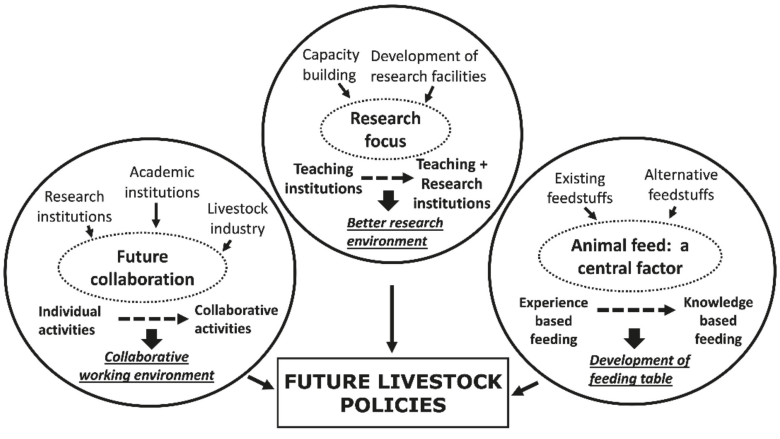

Figure 5. Formulation of future livestock policies in Nepal. Future livestock policies should focus on creating a better research environment and collaborative networks among research, academic, and industry sectors where animal nutrition should be recognized as a central player to improve existing production systems and exploit the genetic potentials of future livestock production systems.

6.1. Animal Feed—A Pivotal Component of the Future Livestock Policies

Poor animal productivity is recognized as a major issue in Nepal, like in many other developing countries [82]. We highlight that providing quality feed for livestock is probably the first and most important factor in improving animal productivity in Nepal as this strategy is also helpful in reducing methane production by increasing the production per unit of animal [83]. This is particularly important because feed costs account for ~60% of total livestock production costs [45], and the provision of quality feeds is necessary to fully exploit the genetic potential of production animals. Thus, any breeding program, particularly for ruminants, would not succeed without ensuring the availability of quality feed year-round. In this context, Nepal's future national livestock policies should identify production, supply and optimized use of quality animal feeds as the central focal point.

Compiling information about feed quality in the form of feeding tables is a critical step in ensuring that proper feeding plans can be formulated to fulfill the nutritional requirements of specific animals. Such tables should integrate nutrition characteristics of (a) existing commonly used feedstuffs and the impact of post-harvest processing, (b) identify alternative feedstuffs, and (c) encompass seasonal and regional/geographical variations and other potential factors that could influence the nutritional values of the feedstuffs. Presently, planning of animal feeding in Nepal is rarely based on scientific knowledge about the feed, but rather on the livestock farmers' experiences, which may not always ensure the most resource-efficient outcomes.

To improve the preservation, quality, and utilization of existing feedstuffs for ruminant species, preservation, and storage of seasonally available forage and fodder biomass should be a top priority. Farmers must have easier access to nutrient enrichment technologies to improve the nutritional values of crop residues and by-products. The use of marginal or underutilized lands, where virtually no crop production is possible, for growing grasses, forages, and fodder trees should be prioritized, and potentials for the utilization of forest biomass and new plant species under agroforestry models should be evaluated.

As an immediate action, the livestock sector should collaborate with crop development companies and research divisions to increase maize productivity and expand legume cultivation to ensure a greater future reliance on home-grown energy and protein resources. Expanded productivity of these crops is imperative to prevent increased competition from livestock for these crops that are primarily utilized as foods for humans. The utilization of natural pastures and meadows in hilly areas can also improve the export potential of organic livestock products in the future. The long-term policy strategy should focus on the potential for producing novel protein sources that can be less competitive with humans for food, and insects and Azolla are fascinating in this regard. Proper capacity and infrastructure building of research institutions are crucial to identify and evaluate the suitability of existing and novel feed resources, as discussed in the following sections.

6.2. Capacity and Infrastructure Building of Research Institutions

Like in many other developing countries, an insufficient research capacity, not least in the livestock field, is a significant concern in Nepal. Future livestock policies should be directed towards developing better research environments to address upcoming vital issues associated with livestock production and management, particularly efficient utilization of existing and alternative feedstuffs and the development of new feed resources. Traditionally, the NARC has been the only institution devoted to livestock research. Its available laboratory facilities and human resources should be upgraded to perform, for example, large-scale in vitro analyses to characterize feed digestibility, which is the major determinant of energy and protein value of feeds. Besides, animal experimental facilities should be equipped to enable in vivo studies that characterize the impacts of existing and novel feeding resources on animal performance. Facilities to assess potential anti-methanogenic properties of existing or novel feedstuffs are also highly relevant in this context, so as to be able to design the most productive, as well as climate-friendly, strategies for livestock sector development.

Historically, Nepalese academic institutions are dedicated to fulfilling only the academic requirements of various study programs, including animal and veterinary sciences. Future livestock education policies should aim to transform the existing universities into more research-based institutions, thus prioritizing both research and teaching as their major activities. This will help to uplift the research capability and contribute to establishing research-based university education training and teaching programs. This will also strengthen positive collaborations between universities and research institutions to develop joint project proposals and establish a research-based, potentially international, learning environment in animal science.

6.3. Research–Academia–Industry (RAI) Collaborations

In Nepal, research and academia are separate pillars, and neither appear to have strong traditions for research collaboration with the industry sector. Livestock industries are currently in a growing phase, and many feed industries of different sizes are already in existence, mainly focusing on poultry feed formulations. Research efforts in Nepal are poorly connected with business sectors, which makes it difficult to commercialize research outputs to promote industrial growth in the livestock sector. Industry sectors can provide important tools for livestock research, such as animals and animal facilities, as well as resources to carry out different research activities. Both research and the academic level in Nepal could benefit from such collaborations with the industry sector, as international

experiences have shown in other parts of the world. Thus, integrated future livestock policies should include instruments (e.g., National research funding) to promote research–academia–industry (RAI) collaborations to develop a sustainable livestock industry sector.

7. Conclusions

The livestock sector is an integral component of Nepalese mixed farming systems, providing high-quality food, income, and livelihood to millions of people residing in a poor socio-economic setting. The livestock sector is currently facing the principal challenge of low productivity and cannot fulfill the national demands for livestock products. This situation will be exacerbated in the future in light of population growth and increased per capita meat and milk consumption associated with improved socio-economic status. Thus, to ensure that the livestock sector will be able to feed future generations, specific livestock-associated future policies must be developed to promote the sustainable development of the livestock sector and livestock management systems. In future livestock policies, the availability of quality feed for livestock should be acknowledged as one of the primary elements to exploit the existing or to-be-improved genetic potentials of production animals, and efforts should be directed towards developing an easy-to-use feeding table for farmers by evaluating nutrient and digestibility characteristics of existing, as well as potential future, feed resources. Additionally, capacity building and infrastructure development at public universities and research institutions relating to animal nutrition, feeding, health, and performance are important to formulate needed region-specific strategies to ensure the development of a productive, as well as climate-friendly, livestock sector, adapted to the prevailing agro-ecological conditions in the future. Industry sectors should be identified and acknowledged as important players in this respect. Future livestock policies should include instruments that will encourage RAI partnerships within research collaboration and promote the implementation of future strategic measures targeting the Nepalese livestock sector.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12050679/s1, Figure S1: Status of sheep meat and wool production.

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Article

Growth Performance, Meat Quality, and Fecal Microbial Population in Limousin Bulls Supplemented with Hydrolyzable Tannins

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Abstract: The objective of this study was to investigate the effects of supplementation of hydrolyzable tannins (HT) from sweet chestnut wood extract (*Castanea sativa* Mill.) to the diet of Limousin bulls on growth rate, carcass and meat quality traits, and fecal *Clostridia* strain levels in a 7-month feeding trial. Thirty-two bulls were randomly assigned to four treatment groups (CON (without addition of HT); TAN 1 (1 g HT kg^{-1} DM); TAN 2 (1.5 g HT kg^{-1} DM); and TAN 3 (1.5 g HT kg^{-1} DM with a nominally lower dose of concentrate). Compared with the CON group, supplementation with HT significantly ($p < 0.050$) increased bull growth rate during 4–7 months, whereas carcass and meat quality traits were unaffected during the last three months of fattening. Supplementation of HT significantly reduced meat drip loss ($p = 0.000$) compared with the CON group. No effects were observed on the total number of fecal *Clostridia* strains; however, the concentration of *Clostridium perfringens* was significantly lower ($p = 0.004$) in TAN 1 than that in the CON group. The results obtained in fattening bulls indicate that the addition of HT is justified in practice to improve growth performance and feed efficiency without adverse effects on the carcass and meat quality.

Keywords: carcass characteristic; chestnut tannins; fecal *Clostridia*; growth performance; Limousin bulls; meat quality

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1. Introduction

The use of plant extracts has increased significantly in recent years, especially after the ban on the use of nutritive antibiotics in animal production in Europe in 2006 [1]. The emergence of resistance in pathogenic microorganisms mainly originating from the digestive tract of animals has caused public concern and led breeders to reflect on the necessary changes and immediate measures in the practical conditions of breeding. The relatively positive effects of various feed supplements of plant origin, such as essential oils, tannins, and others, was quickly discovered in non-ruminant breeding, which is not identical to the effect in ruminants [2]. A digestive tract adapted to the voluminous nature of the ration, with foregut-specific microbiota, is an additional obstacle to the rapid and effortless transfer of the norms of nutritional supplements of another animal groups.

Because of the voluminous nature of the diet, cattle can already absorb many of the above-mentioned amounts of active ingredients in a varied ration. Nevertheless, in intensive beef production, it is essential to control the microbial population in the foregut and reduce opportunistic pathogenic microbes of the normal intestinal microflora. Such properties are also possessed by plant secondary metabolites and are referred to as phytochemicals, phytogenes, or phytobiotics and could be a very suitable substitute for

antibiotics in animal nutrition [3–6]. Tannins from higher plant species are proving to be very promising [7].

Tannins are found in different plant species and different plant parts, including bark, fruits, leaves, and roots, resulting in different physical and chemical properties [8,9]. Therefore, the bioactive properties of tannins depend on their chemical structure, which is even more important than their concentration [10,11]. Plants produce them as secondary metabolites for their own protection against consumption by herbivores [11]. The group of tannins is very diverse and can be divided into four groups based on their chemical structure: (i) condensed tannins or proanthocyanidins; (ii) HT; (iii) florotanins from brown algae; and (iv) complex tannins conjugated with metals or proteins [12]. In the literature, they are usually classified into two broad groups: HT gallic acid and glucose and condensed tannins consisting of flavonoids [13,14], both of which are found in small amounts in forage ingredients < 450 μg g^{-1} DM, [15]. HT is divided into two subgroups, gallotannins and ellagitannins [16]. Hydrolyzable tannins are a group of water-soluble polyphenolic compounds that possess antimicrobial, anti-inflammatory, antiviral, antioxidant, and antiparasitic activities [17,18].

Feed additives according to (EC) No. 1831/2003 [19] are divided into four categories: technological additives, sensory additives, nutritional additives, and zootechnical additives. According to current knowledge, HT from sweet chestnut is classified in the second category, sensory additives, and in the subcategory (b) flavoring agents with the active substance level [20]. Therefore, our research aims to elucidate and expand the knowledge of the activity of HT beyond the bureaucratically limited framework that describes only the definition of potential effects. In the scientific and professional literature, we often find a general use of the term food supplement, which does not necessarily correspond to the definition and categorization of the EU standards for plant extracts. Some studies on tannins intended for transfer to ruminants have been performed in vitro [21], on cannulated or fistulated animals, on small ruminants, and in individual small laboratory tests. Mostly, these tests examine the effective concentration (fattening lambs—20.8 g HT kg^{-1} DM and sheep—34.0 g HT kg^{-1} [22], higher concentrations (generally >50 g kg^{-1} DM [23]) on rumen microflora, mechanism and efficiency of bypass proteins [24–26], growth of animals, meat quality [18,27,28], health problems caused (kidney and liver damage [8]), and antimicrobial activity (mechanism [29] on microbes in feces (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Candida albicans* [30], *Clostridium perfringens* [31], and *Clostridia* [30,31]). However, a variety of plant materials with unclear composition and varying tannin content have been used in research [21,23,25,32,33].

In the available literature, we found no study in which bulls were fattened under commercial fattening conditions at modest feed rations with a small amount HT of known composition. Therefore, the objective of this study was to determine the effects of adding chestnut (*Castanea sativa* Mill.) tannin extracts with a high HT content at low concentrations of 1.0 to 1.5 g kg^{-1} DM to the diets of fattening bulls. We investigated their effect on growth and fattening traits, carcass quality and meat quality of bulls. In addition, the effect of HT on the microbiological condition (*Clostridia*) of bull feces was estimated.

2. Materials and Methods

The animal procedure was conducted at an experimental farm located in NE Slovenia (46°5′ N, 15°8′ E), followed the Slovenian Law on Animal Protection, and approved feed additives were used (European Union Register of Feed Additives, 2013) [34]. The ethics committee's approval was not required.

2.1. Animals, Housing and Diets

Thirty-two Limousin bulls were included in the 7-month trial to evaluate the effect of various levels of supplementation of hydrolyzable tannin (chestnut tannin: *C. sativa* Mill.) on growth, carcass and meat quality traits, and fecal *Clostridia* strains.

At an average body weight (BW) of 432 ± 43 kg, thirty-two bulls were randomly assigned to one of four treatment groups (a completely randomized design). The bulls were housed in collective pens (eight animals per pen), where they were kept for the duration of the trial. All identical pens located in the same barn consisted of a standard slatted concrete floor area 6 × 4 m, equipped with identical water troughs. Equal feeding conditions were ensured for all bulls and individual bulls served as experimental units.

Animal BWs were equalized at the beginning as much as possible, both within and between individual groups. Highly homogeneous groups facilitated a viable experiment and comparison of groups during the trial.

Animals were fed a total mixed ration (TMR). The composition of the basal diet is presented in Table 1. Diets were prepared daily, and rations were formulated to meet the requirements of medium-frame finishing bulls [35]. During the feeding trial, the feed ratios for bulls were optimized twice according to BW to meet their nutritional requirements.

Table 1. Ingredients and chemical composition of total mixed ration (g kg^{-1} DM).

Ingredients	Treatment Rations			
	CON	**TAN 1**	**TAN 2**	**TAN 3**
Corn maize silage	126.8	126.6	126.6	132.7
Wheat straw	158.4	158.3	158.2	165.9
Sodium bicarbonate	10.5	10.5	10.5	11.0
Concentrate	568.5	567.9	567.6	546.5
Corn maize	135.8	135.7	135.6	142.0
Tanin additive	/	1.0	1.5	1.5
Nutrient content				
DM	737.9	737.7	738.2	732.8
Crude ash	48.5	47.1	47.4	47.2
Crude protein	158.1	156.6	159.7	149.2
Crude fibre	54.4	46.1	50.4	56.2
Ether extract	42.2	40.0	42.1	41.7
Sugar	33.5	30.5	35.6	30.7
Starch	510.2	529.4	537.8	534.9
Calcium	8.5	6.8	8.7	6.3
Phosphorus	5.6	5.8	5.8	5.5
Sodium	4.0	3.3	4.3	4.5
Magnesium	2.1	1.5	1.9	2.1
Potassium	7.7	6.3	8.0	7.7
ADF	94.4	95.4	86.3	91.2
NDF	198.5	186.0	180.6	188.4
ADL	18.2	15.1	15.1	15.2
ME (MJ kg^{-1})	12.6	12.6	12.6	12.6

No tannin additive in the diet (CON); 10 g of mixture (HT + soy protein additive) per animal—1.0 g kg^{-1} DM (TAN 1); 15 g of HT additive per animal—1.5 g kg^{-1} DM (TAN 2); and 15 g of HT additive per animal added to the TMR diet with reduced quantity of concentrate in nominal value—1.5 g kg^{-1} DM (TAN 3). Dry matter (DM); acid detergent fiber (ADF); neutral detergent fiber (NDF); acid detergent lignin (ADL); metabolizable energy (ME).

Commercially available Farmatan-D®, wood extract rich in HT, was obtained from Tanin d.d. Sevnica (Sevnica, Slovenia); the supplement originating from sweet chestnut wood (*Castanea sativa* Mill.). All wood extracts were in powder form before use. Suitable quantities of wood extracts were mixed into feed rations on the DM bases. The chemical compositions of the wood extracts rich with hydrolyzable tannins: major components (%) of Farmatan-D (hydrolyzable tannins 74.3, vescalin 0.9, castalin 1.7, roburin A 0.2, gallic acid 2.4, roburin B/C 2.1, grandinin 0.9, roburin D 1.0, vescalagin 4.7, roburin E 1.5, castalagin 4.1, ellagic acid 0.8.). The supplement was used in the three groups and compared with the control group.

The four trial groups were comprised of thirty-two animals, eight per group:

The control group (CON)—no tannin additive in the diet. The first treatment group (TAN 1)—10 g of mixture (HT + soy protein additive) per animal added to the TMR diet (1.0 g kg^{-1} DM). The second treatment group (TAN 2)—15 g of HT additive per animal added to the TMR diet (1.5 g kg^{-1} DM).The third treatment group (TAN 3)—15 g of HT additive per animal added to the TMR diet (1.5 g kg^{-1} DM) with reduced quantity of concentrate in nominal value of the HT additive (Table 1).

Feed rations were prepared daily for all treatment groups. The bulls were fed their respective diets once a day (07:00 h), following a 20 d diet adaptation period. During the adaptation period, the diets were not supplemented with tannin extract. Access to feed and water was provided ad libitum. Ration components for complete feed rations, and refusal samples were collected monthly at the beginning and during fattening period, and were sent to the laboratory for chemical analyses (LKS-Landwirtschaftliche Kommunikations-, und Servicegesellschaft mbH, Germany). The mill was washed after preparation of each diet to prevent cross-contamination with tannins. The ratio was adjusted on a weekly basis. During the feeding trial, the feed rations and feed refusals were recorded on a daily basis for each treatment group. In the 7-month feeding trial, a total of 852 offered rations and 852 residual feeds were precisely measured. From the data, we calculated the dry matter intake (DMI).

2.2. Recording of Dry Matter Intake

Feed and feed refusals were collected and recorded daily for DM analysis and calculation of DMI. To calculate the monthly average DMI, the average of all daily feed and feed refusals for each pen (each group) were calculated. The results are presented as monthly average DMI per pen, as descriptive statistics.

2.3. Recording of Body Weight

Following a 20 d diet adaptation period, bulls were weighed and started the 213-day experimental period. During the experimental period, bulls were weighed at the beginning and at 30-day intervals thereafter. At the end of the experiment, the bulls were weighed 24 h before slaughter and on the day of slaughter. Body mass data were recorded using a digital walk-through scale (EC 2000 Tru-test). The average daily gain (ADG) was calculated between the monthly recordings and throughout the experiment. At each weighing, body parameters were also recorded: height at withers (measured from the highest point of the shoulder blade to the ground) and hip height (measured from the highest point of the hip bones to the ground).

2.4. Carcass Classification

At the end of the experiment, the bulls were transported by truck to a local commercial abattoir. At the end of the slaughter line, warm carcass weight (CW) was recorded, and carcass classification, i.e., evaluation of conformation and fatness, was performed by an accredited classification body using the European Union beef carcass classification system (EUROP). For the statistical analysis, scores of the conformation (E, U, R, O, and P) and fatness (1–5) scale were transformed into numerical classification units on a 15-point scale. Age at slaughter (AS) was calculated as the difference between the date of birth and the date of slaughter. The dressing percentage (DP) was calculated as the ratio of CW to its live weight at slaughter (LWS). A measuring tape was used to record the carcass length and chest depth as described by Campion, Keane, Kenny and Berry [36].

2.5. Measurements of Meat Quality Traits

A day after slaughter, samples of *Longissimus dorsi* (LD) were taken from the left carcass at the level of the last rib to measure meat quality traits. The muscle was cut into two 5 cm-wide large sections. One part was used for analysis of fresh meat, whereas the other was weighed, vacuum-packed, and subjected to vacuum aging for 14 days at 4 °C (matured sample). Fresh samples were used to measure pH value; color parameters (L*, a*, and b*) chemical composition by NIR; marbling; water-holding capacity (WHC); and tenderness.

The pH value was measured at the center of the LD muscles at 24 and 48 h post-mortem using an MP120 Mettler Toledo pH meter fitted with a combined glass electrode InLab427 (Mettler-Toledo, GmbH; 8603 Schwarzenbach, Switzerland). The samples were evaluated in two replicates at two different sites [37].

To evaluate meat color, a Minolta L*a*b* colorimeter was used. Analysis was performed on a freshly cut surface of LD exposed for 60 min to bloom [38]. Measurements were taken in triplicate using a Minolta Chroma Meter CR-300 (Minolta Co., Ltd., Osaka, Japan). Color stability measurements were performed 24 h after slaughter.

Determination of moisture, intramuscular fat (IMF), and protein content (chemical composition) were determined by near-infrared spectroscopy (NIR Systems 6500 Monochromator; Foss NIR System, Silver Spring, MD, USA), as described previously by Prevolnik et al. [39].

Water-holding capacity (WHC) was determined by three different methods: drip loss, cooking loss, and thawing loss. Drip loss was determined using the EZ-DripLoss method as described by Christensen [40]. Two cylindrical pieces (2.5 cm) were cut from the central area of the LD. The samples were weighed and sealed in plastic sealable cups (Sarstedt AG & Co., Nümbrecht, Deutschland meat extract collector), and weight loss was recorded after 24 and 168 h of storage [41].

Samples for determining cooking loss were first weighed and then cooked in a thermostatic water bath (ONE 7-45), until the interior of the sample reached 72 °C. After cooking, the samples were dried with paper towels and reweighed to obtain data on water loss during cooking.

To determine thawing loss, samples were weighed, vacuum packed, and frozen at −20 °C. Samples were then thawed overnight at 4 °C, gently dried with a paper towel, and reweighed [42].

After cooking, the samples were left to cool and objective determination of tenderness was performed by determining the shear force (N) with a TA Plus texture analyzer (Ametek Lloyd Instruments Ltd., Fareham, UK). The same procedure was repeated for the aged samples.

To assess marbling, cross-sections of LD were visually compared using a reference standard scale ranging from 1 (devoid of marbling) to 10 (abundantly marbled). Two operators independently evaluated each sample, and an average of the two scores was obtained [43].

2.6. Fecal Clostridia Analysis

The fecal samples were collected (using individual plastic sleeves) from each animal on the day before the start of the trial and on the day of slaughter. The first fecal samples were obtained on a scale by catching feces of individual animals into a clean plastic bucket. At the end of the fattening trial, colon fecal samples were collected from the slaughterhouse. The fecal samples were stored at 4 °C until freezing (–72 °C). Samples were then sent by express mail in controlled conditions to the laboratory for further analysis (Miprolab GmbH, Göttingen, Germany).

Two methods were applied for the analysis of *Clostridia*. With the "Most Probable Number" (MPN), the total number of sulfite-reducing *Clostridia* was determined (pathogenic and non-pathogenic species) to get an overall count of all viable *Clostridia*. Selective *Clostridia* enrichment and counting analyses allowed for the detection, identification, and semi-quantitative counting of the relevant pathogenic *Clostridia* species.

2.6.1. Total Number of *Clostridia*

The total number of viable sulfite-reducing *Clostridia* was determined using the MPN adhering to the principles of EN 26461-1 in a miniaturized design. Briefly, 50 g of each sample was added to 100 mL of differential reinforced *Clostridia*l medium (DRCM, Heipha, Germany) and homogenized using two cycles of 30 s in a paddle blender (Stomacher 400, Seward, UK). From each homogenized sample, 1 mL was pipetted into 9 mL of DRCM and thoroughly mixed. Further dilutions were performed in 96-well plates with a transfer volume of 25 µL

and a total volume of 250 µL per well. Each sample was tested in five replicates. The plates were covered and incubated at 37 °C for 14 days. The incubation atmosphere consisted of 85% N_2, 10% CO_2, and 5% H_2 generated with an Anoxomat (Mart, The Netherlands). All black wells were counted as positive for the growth of sulfite-reducing *Clostridia*, and the total number was calculated using the MPN table taking into account the initial dilution. *Clostridia* counts are given as bacteria per gram of sample.

2.6.2. Selective *Clostridia* Enrichment and Counting Analysis

To selectively identify and count relevant pathogenic bacteria (*C. perfringens* and *Clostridium sporogenes*), samples were spread on egg yolk lactose agar (Heipha, Germany). Aliquots of the homogenized fecal samples were heated at 80 °C for 10 min to activate the spores and to reduce the interfering accompanying flora. Plates were incubated for 48 h at 37 °C in an anaerobic atmosphere as described above. The incubation time was extended to 72 h in the case of insufficient growth. Bacterial colonies were identified by colony properties, including lipase and lecithinase activity. Colonies of the same type, appearance, and macromorphology were randomly selected for confirmation by Remel RapID ANA (Thermo Fisher Scientific, Lenexa, KS, USA) and Gram strain. *C. perfringens* was additionally tested by PCR. Colonies of the various species were counted semi-quantitatively on the plates, and the results were grouped into four classes (Number 0 = no growth; Number 1 = 1–10 colonies; number 2 = 11–50 colonies; number 3 = >50 colonies) between treatments. A quantitative counting of the colonies was not performed, because plate cultures were obtained from pre-enriched cultures and were only able to provide a semi-quantitative assessment of the distribution of the individual bacterial species. However, the pre-enrichment process still reflected the initial concentration ranges. The range for the semi-quantitative data was calculated as follows: 0, no growth; range 1, 1–10 colonies; range 2, 11–50 colonies; range 3, >50 colonies. All four ranges reflect the initial concentrations in the samples.

2.7. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 25. Means and standard errors of the means were computed for all parameters separately for each treatment. To determine the differences in growth performance, carcass, and meat quality parameters for all treatments, we applied one-way analysis of variance (ANOVA). The statistical model used was as follows:

$$Y_{ijk} = \mu + T_{ij} + e_{ijk}$$

where Y_{ijk} is *k*- investigated the characteristics of *j*- bull within *i*-treatment, μ is total mean, T_{ij} is the *j*-bull of *i*-treatment (CON, TAN 1, TAN 2, TAN 3), and e_{ijk} is error component.

The assumptions of normality and homoscedasticity were verified using Shapiro–Wilk's and Levene's tests. In case of finding significant results in an ANOVA, post hoc analysis using the Duncan method was performed.

One-way ANOVA was applied to examine the differences in the total number of *Clostridia* (MPN) at the beginning and the end of the feeding trial. In the case of selected *Clostridium*, the non-parametric Kruskal–Wallis test was used and pairwise comparisons were performed using the Dunn–Bonferroni post hoc test. If the *p*-value was lower than 0.05, the results were considered significant.

3. Results

3.1. Growth Performance and Carcass Characteristics

The monthly average dry matter intake (DMI) data are presented in Table 2. In the CON group, we detected the smallest DMI in almost all months (with an exception between 4–5 months) of the feeding trial.

Table 2. Monthly average dry matter intake per pen.

Month	Treatment Groups				*p*-Value
	CON	TAN 1	TAN 2	TAN 3	
1	8.1 ± 0.2	8.3 ± 0.2	8.2 ± 0.1	8.3 ± 0.3	0.072
2	8.1 ± 0.1	8.3 ± 0.1	8.2 ± 0.2	8.2 ± 0.3	0.087
3	8.0 ± 0.2	8.2 ± 0.2	8.2 ± 0.1	8.3 ± 0.1	0.079
4	8.5 ± 0.1	8.6 ± 0.2	8.4 ± 0.2	8.7 ± 0.2	0.120
5	8.6 ± 0.2	9.0 ± 0.4	8.6 ± 0.2	8.8 ± 0.2	0.089
6	8.8 ± 0.2	9.0 ± 0.2	8.9 ± 0.1	9.1 ± 0.3	0.101
7	9.3 ± 0.1	9.4 ± 0.1	9.3 ± 0.2	9.4 ± 0.2	0.077

No tannin additive in the diet (CON); 10 g of mixture (HT + soy protein additive) per animal—1.0 g kg^{-1} DM (TAN 1); 15 g of HT additive per animal—1.5 g kg^{-1} DM (TAN 2); and 15 g of HT additive per animal added to the TMR diet with reduced quantity of concentrate in nominal value—1.5 g kg^{-1} DM (TAN 3). *p*-value lower than 0.05, the result is considered significant.

The growth performance results are listed in Table 3. The final BW was not affected (p = 0.273) by treatment, and there were no statistical differences between the groups, nevertheless, we found a 10–40 kg difference. Significant differences in average daily gain (ADG) (measured monthly) were observed in the last months of the trial in ADG 4–5 (p = 0.012), ADG 5–6 (p = 0.049), and ADG 6–7 (p = 0.041), with the lowest growth rate noted in the control group. However, the ADG calculated for the entire experimental period was not significantly different between the treatment groups, indicating no effect of dietary tannins (p = 0.142).

Table 3. Effect of supplemented hydrolyzable tannins on growth performance of Limousin bulls.

Items	Treatment Groups				*p*-Value
	CON	TAN 1	TAN 2	TAN 3	
Feeding trial	Body weight, kg				
Initial	411 ± 11.3	429 ± 18.9	416 ± 14.0	426 ± 11.8	0.092
Final	659 ± 13.2	695 ± 22.8	669 ± 13.2	684 ± 19.2	0.273
Month	Average daily gain, g/day				
1	1361 ± 73.9	1073 ± 143.4	1261 ± 131.3	1193 ± 45.2	0.440
2	1176 ± 54.5	1440 ± 106.4	1336 ± 128.1	1214 ± 44.4	0.201
3	1352 ± 53.8	1368 ± 45.1	1412 ± 85.9	1282 ± 58.0	0.532
4	1402 ± 38.1	1330 ± 16.0	1322 ± 39.5	1456 ± 52.1	0.074
5	1124 ± 58.1 ^{a}	1429 ± 74.5 ^{b}	1217 ± 53.8 ^{b}	1290 ± 50.2 ^{b}	0.012
6	979 ± 52.3 ^{a}	1198 ± 26.0 ^{b}	1031 ± 76.2 ^{b}	1123 ± 46.3 ^{b}	0.049
7	857 ± 37.5 ^{a}	985 ± 58.4 ^{b}	916 ± 97.7 ^{b}	964 ± 73.1 ^{b}	0.041
1–7	1172 ± 22.2	1260 ± 30.2	1214 ± 56.2	1217 ± 27.1	0.142

No tannin additive in the diet (CON); 10 g of mixture (HT + soy protein additive) per animal—1.0 g kg^{-1} DM (TAN 1); 15 g of HT additive per animal—1.5 g kg^{-1} DM (TAN 2); and 15 g of HT additive per animal added to the TMR diet with reduced quantity of concentrate in nominal value—1.5 g kg^{-1} DM (TAN 3). ^{a,b} Mean values in the same row with different letters are significantly different (Duncan, $p < 0.05$), ± standard error of the mean.

There were no significant differences in carcass weight (p = 0.289) (Table 4), conformation score (p = 0.086), fatness score (p = 0.130), dressing percentage (p = 0.090), and additional body measurements (depth of chest and length of carcass).

3.2. Meat Quality

A statistically significant difference was obtained for drip loss measured after 48 h of storage (p = 0.000) (Table 5), which was higher in the CON group than in the tannin-supplemented group. In the two-week old samples, all tannin-supplemented groups showed significantly higher thawing loss than the CON group (p = 0.008). Additionally, the lowest cooking loss of aged meat was observed in the CON group.

Table 4. Effect of supplemented hydrolyzable tannins on carcass quality of Limousin bulls during a 213-day trial.

Items	Treatment Groups				*p*-Value
	CON	TAN 1	TAN 2	TAN 3	
Carcass weight (kg)	418.0 ± 10.0	434.0± 8.6	422.0 ± 18.6	429.0 ± 16.7	0.289
Conformation score *	11.8 ± 0.5	12.5 ± 0.6	12.1 ± 0.7	10.8 ± 0.7	0.086
Fatness score **	7.7 ± 0.6	8.1 ± 0.3	6.5 ± 0.6	7.0 ± 0.4	0.130
Dressing percentage, %	60.4 ± 0.5	60.1 ± 0.8	62.1 ± 0.7	60.5 ± 0.7	0.090
Depth of chest, cm	42.3 ± 0.6	44.3 ± 0.5	43.1 ± 0.6	43.8 ± 0.5	0.070
Length of carcass, cm	137.0 ± 1.4	139.0 ± 1.7	139.0 ± 1.3	141.0 ± 0.9	0.084
LD muscle area, cm^2	117.0 ± 4.3	118.0 ± 4.0	122.0 ± 4.3	112.0 ± 2.5	0.321
LD fat area, cm^2	15.2 ± 1.9	15.1 ± 1.5	11.8 ± 1.7	12.2 ± 1.2	0.295

No tannin additive in the diet (CON); 10 g of mixture (HT + soy protein additive) per animal—1.0 g kg^{-1} DM (TAN 1); 15 g of HT additive per animal—1.5 g kg^{-1} DM (TAN 2); and 15 g of HT additive per animal added to the TMR diet with reduced quantity of concentrate in nominal value—1.5 g kg^{-1} DM (TAN 3). * Carcass classification system (EUROP) evaluating the conformation—scale 1 (poorest) to 15 (best). ** Carcass classification system (EUROP) evaluating the fatness-scale 1 (least) to 15 (fattest). *p*-value lower than 0.05, the result is considered significant.

Table 5. Effect of supplemented hydrolyzable tannins on meat quality parameters of Limosin bull muscles.

Items	Treatment Groups				*p*-Value
	CON	TAN 1	TAN 2	TAN 3	
		Fresh meat samples			
pH 24 h	5.7 ± 0.1	5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.1	0.266
pH 48 h	5.8 ± 0.0	5.5 ± 0.1	5.5 ± 0.1	5.6 ± 0.0	0.254
L* (lightness), 24 h	35.4 ± 1.2	36.2 ± 1.1	37.6 ± 1.0	34.9 ± 1.1	0.324
a* (redness), 24 h	17.4 ± 0.7	19.9 ± 1.3	19.8 ± 1.0	18.4 ± 1.1	0.303
b* (yellowness), 24 h	5.6 ± 0.4	7.1 ± 0.8	7.2 ± 0.5	6.3 ± 0.6	0.171
L* 48 h	36.4 ± 1.3	38.0 ± 1.1	37.5 ± 1.1	36.7 ± 1.0	0.750
a* 48 h	19.1 ± 0.9	21.7 ± 1.4	19.3 ± 0.7	20.2 ± 0.9	0.265
b* 48 h	5.7 ± 0.5	7.6 ± 0.6	6.1 ± 0.5	6.4 ± 0.5	0.143
Drip loss h, %	1.4 ± 0.3 [b]	0.8 ± 0.1 [a]	0.6 ± 0.0 [a]	0.8 ± 0.1 [a]	0.000
Thawing loss, %	5.3 ± 0.7	6.8 ± 0.4	7.4 ± 0.6	6.3 ± 0.5	0.067
Cooking loss, %	25.1 ± 2.2	29.7 ± 1.0	27.1 ± 1.5	26.9 ± 1.0	0.207
Marbling[1]	2.8 ± 0.6	2.2 ± 0.4	1.4 ± 0.1	2.0 ± 0.3	0.088
IMF, %	2.8 ± 0.3	2.9 ± 0.4	2.1 ± 0.2	2.5 ± 0.3	0.201
Protein, %	22.2 ± 0.2	22.3 ± 0.2	22.5 ± 0.2	22.4 ± 0.2	0.617
Water, %	74.1 ± 0.5	73.7 ± 0.3	74.3 ± 0.2	74.1 ± 0.3	0.591
Protein to water ratio	3.4 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	0.818
WBSF fresh, N	77.5 ± 3.3	65.1 ± 3.6	64.5 ± 5.5	66.7 ± 3.4	0.126
WBSF cooked, N	221.4 ± 37.0	222.7 ± 34.0	287.1 ± 25.0	275.7 ± 26.0	0.297
		Aged meat samples—2 weeks			
L*	38.5 ± 1.5	38.2 ± 1.2	40.5 ± 1.0	38.4 ± 1.3	0.640
a*	22.1 ± 1.1	23.3 ± 1.3	24.3 ± 0.9	23.2 ± 1.1	0.476
b*	8.4 ± 0.9	9.5 ± 0.9	10.3 ± 0.7	9.8 ± 0.9	0.465
pH	5.7 ± 0.1	5.6 ± 0.1	5.5 ± 0.0	5.7 ± 0.1	0.491
Vacuum loss, %	2.1± 0.3	2.1 ± 0.5	2.4 ± 0.3	2.9 ± 0.5	0.414
Thawing loss, %	3.9 ± 0.4 [a]	4.8 ± 0.8 [b]	4.9 ± 0.3 [b]	5.2 ± 0.5 [b]	0.008
Cooking loss, %	19.9 ± 2.4	28.6 ± 1.1	24.3 ± 1.2	23.9 ± 1.4	0.080
WBSF fresh, N	72.9 ± 6.2	55.8 ± 8.0	62.2 ± 5.3	64.2 ± 4.9	0.310
WBSF cooked, N	147.3 ± 17.6	133.8 ± 4.9	111.6 ± 10.8	141.4 ± 11.6	0.183

No tannin additive in the diet (CON); 10 g of mixture (HT + soy protein additive) per animal—1.0 g kg^{-1} DM (TAN 1); 15 g of HT additive per animal—1.5 g kg^{-1} DM (TAN 2); and 15 g of HT additive per animal added to the TMR diet with reduced quantity of concentrate in nominal value—1.5 g kg^{-1} DM (TAN 3). [a,b] Mean values in the same row with a different letter are significantly different ($p < 0.05$) ± standard error of the mean. L* = lightness; a* = redness; b* = yellowness; marbling 1 = visually assessed on a freshly cut *Longissimus dorsi* (LD) using a scale from 1 (extremely lean) to 10 (extremely marbled sample); WBSF, Warner–Bratzler shear force; IMF, intramuscular fat.

3.3. Clostridia Counts

The total number of *Clostridia* was determined by the MPN method. No significant difference was found among groups in the total number of *Clostridia* before ($p = 0.173$) and after ($p = 0.582$) the feeding trial (Figure 1).

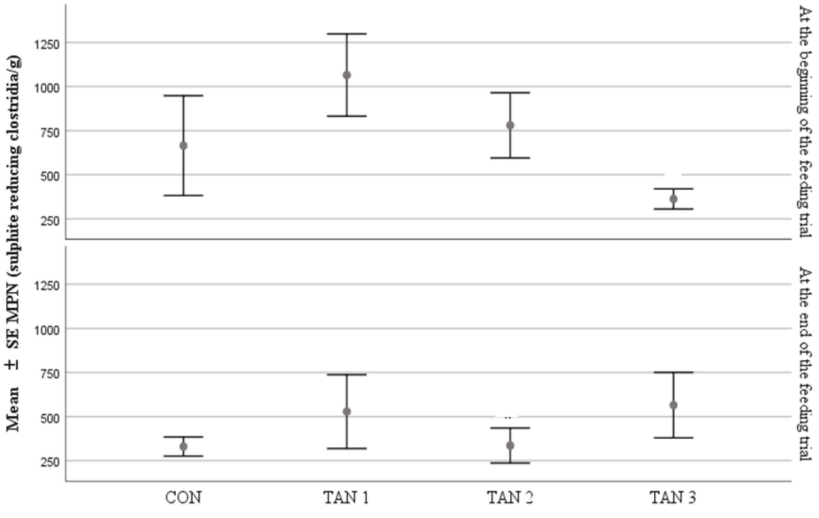

Figure 1. Total number of *Clostridia* in fecal samples before and after tannin supplementation determined by the most probable number method. No tannin additive in the diet (CON); 10 g of mixture (HT + soy protein additive) per animal—1.0 g kg^{-1} DM (TAN 1); 15 g of HT additive per animal —1.5 g kg^{-1} DM (TAN 2); and 15 g of HT additive per animal added to the TMR diet with reduced quantity of concentrate in nominal value—1.5 g kg^{-1} DM (TAN 3).

The concentrations of individual Clostridium species (*C. perfringens* and *C. sporogenes*) were analyzed with selective *Clostridia* enrichment and counting analysis. The results were counted and ranged within four concentration classes (Figure 2). All four ranges reflect the initial concentrations in the samples.

No significant differences between the treatments were observed at the beginning of the feeding trial for *C. perfringens* ($p = 0.764$) and *C. sporogenes* ($p = 0.979$). At the end of the feeding trial, there were no differences in the *C. sporogenes* concentrations between the groups. Differences in concentrations of *C. perfringens* were detected ($p = 0.004$) between treatments. The lowest concentration was determined for the TAN 1 group.

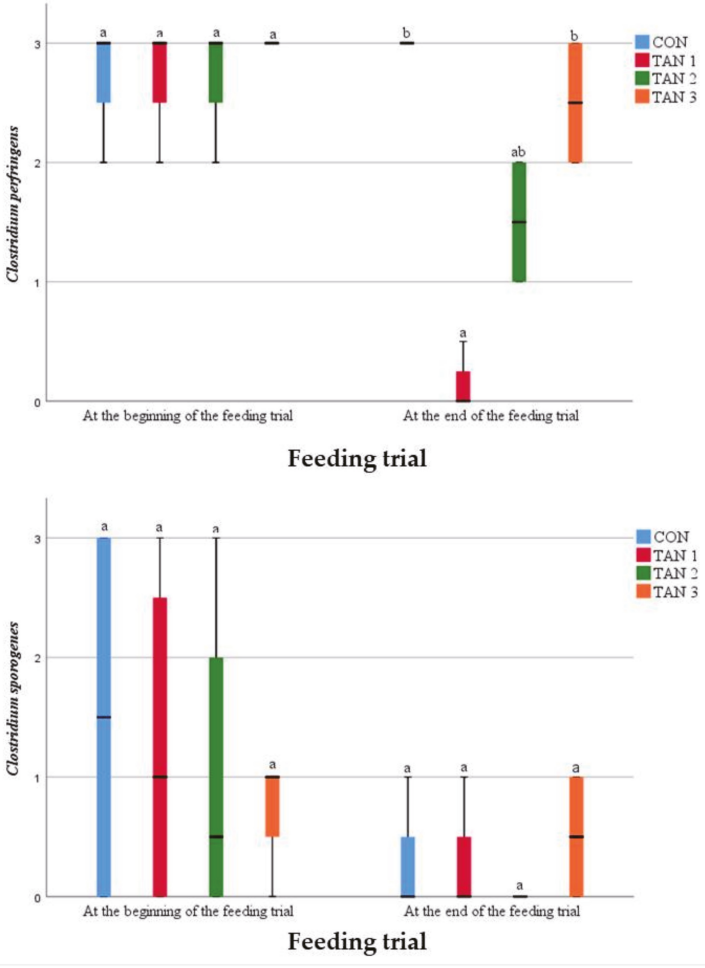

Figure 2. Concentration of selected *Clostridia* in 1 g of fecal samples before and after tannin supplementation. No tannin additive in the diet (CON); 10 g of mixture (HT + soy protein additive) per animal—1.0 g kg^{-1} DM (TAN 1); 15 g of HT additive per animal—1.5 g kg^{-1} DM (TAN 2); and 15 g of HT additive per animal added to the TMR diet with reduced quantity of concentrate in nominal value—1.5 g kg^{-1} DM (TAN 3). Number 0 = no growth; number 1 = 1–10 colonies; number 2 = 11–50 colonies; number 3 => 50 colonies; a, b different superscript letters indicate differences (Dunn–Bonferroni post hoc test; $p < 0.05$) between treatments.

4. Discussion

When discussing tannins, it must be noted that there are many published studies with different, even contradictory results, which report positive, negative or no effect of tannin supplementation on the growth performance of bulls. In this context, Patra and Saxena [10] reported that the growth performance was associated with various responses to tannin supplementation using different chemical structures and concentrations of tannins used in studies, different animals, and basal diet used in feeding trials. Different effects are often attributed to condensed tannins, compared with hydrolyzable tannins. Although research on these two types of tannins is important, and previous studies have reported similar effects [44–46], generalizations should be carefully considered [47]. In the present

study, we focused on hydrolyzable tannins and their effect on ruminants. However, some comparisons are discussed, as many more studies associated with the effects of tannins are conducted with condensed tannins [9].

4.1. Effects of Tannins on Growth Performance of Bulls

As previously mentioned, tannins can improve the ratio of bypass proteins, which can lead to improved intestinal absorption of amino acids and thus have a positive effect on daily gain. Our results show that supplementation of the diet with HT affected the growth of bulls, and significant differences were detected in the last three months of our trial when the bulls reached 538 ± 20.3 kg BW until the slaughter maturity (677 ± 19.2 kg). In the present study, there were no differences in final ADG and BW between the groups. Furthermore, no reduction in the daily DMI was detected. This is in agreement with the feeding study of Aboagye et al. [48]. They studied the effects of feeding chestnut (HT) extract and a combination (50:50) of HT and quebracho (CT) extracts in a powdered form at different concentrations of dietary DM for three months on steers. There were no effects of treatment on DMI, BW, ADG in both HT groups, or a combination of both tannins and both concentrations. In the current study, total ADG and BW were not affected, although we observed a higher ADG at the end of the trial. Tabke et al. [49] supplemented tannic acid (HT) into steam-flaked corn-based finishing diets of steers. Overall, no effects were observed for ADG, carcass characteristics, hot carcass weight, *longissimus* muscle area, fat thickness, and yield grade during the study in any treatment. The study by Krueger et al. [44] used commercially available mimosa (CT) and chestnut (HT) extracts and added them to a high-grain diet to fatten crossbred steers. Including tannins in the diet resulted in similar DMI for steers in the control and both tannin-treatment groups. Tannin supplementation had no effect on animal performance or carcass, except for HCW. We conclude that the absence of an effect of tannins on animal performance observed in this study could be due to the conservative dose of tannins.

We recorded differences in ADG during the last three months of the feeding trial. The positive impacts of tannin supplementation during the last feeding period were also observed by Brus et al. [50]. Commercial HT additives in powder form were used in the diet of Simmental bulls. After month 8 of the study, ADG was significantly higher in the group with tannin wood extract and lower in the control group. The higher measured ADG in the last period of the feeding trial is in agreement with our findings. Tannin supplementation in the finishing feeding phase of Holstein steers was studied by Rivera-Mendez et al. [46]. Supplementation with tannins increased average daily gain (ADG, 6.8%) and dry matter intake (DMI, 4.0%) in the finishing feeding phase. The authors concluded that the mechanism responsible for the higher ADG has not been fully understood and explained.

A possible explanation for the achievement of significantly higher ADGs in the final phase of bull fattening in the present study is the nature of nutrient metabolism of ruminants and the specific effect of hydrolyzable tannins. The extent and composition of daily gain in the final phase of fattening depends on the available energy and proteins in the animal's diet, which is above the maintenance requirement [51]. In this context, ruminants can take advantage of the symbiotic effect of digestion with microorganisms in the rumen, which decompose the nutrients by using their own digestive enzymes. Microbes can form ideal nutrients for ruminants from the decomposition products. Ruminants meet their metabolic protein needs from two sources: true microbial proteins from microbial synthesis and proteins from feed (bypass proteins) [52]. The addition of HT influenced the microbial metabolic process of nutrient synthesis in the rumen, which may lead to better feed efficiency and a higher growth rate of fattening bulls. The chemical composition of the tested feed mixtures is the same in terms of energy content, thus, differences in ADG size are not due to energy alone, as the authors claim [53]. The possible explanation for the differences in ADG can be attributed to the different amounts of crude protein in the TMR. In the present study, all groups with HT had significantly higher ADG than the control

group. Therefore, we hypothesized that HT influenced the ruminal digestion of rumen degradable and undegradable dietary proteins. Consequently, increased protein production in the rumen from both sources resulted in greater availability of enzymatically degradable proteins in the small intestine. It can be assume that the addition of HT increased the availability of amino acid absorption, which could pass into the amino acid pool.

4.2. Effect of Tannin Supplement on Meat Quality

In this study, meat quality traits such as meat color, IMF, protein content, marbling, and tenderness were not affected by HT plant extract. Similar results regarding meat quality traits were observed in a study by Joo et al. [54]. The objective of this study was to examine the effects of dietary fermented chestnuts on growth performance, carcass, and meat quality parameters (cold carcass weight, back fat thickness, *longissimus* muscle area, marbling score, and fat color) in the late fattening period of Hanwoo steers. No effects on growth performance or carcass traits were observed. Moreover, differences were observed in physicochemical characteristics (cooking loss, water-holding, shear force), except meat pH. Beef meat quality in relation to added tannins was also studied by Larraín et al. [55]. They observed an effect on meat color and lipid oxidation in beef *longissimus lumborum* (LL) and *gluteus medius* (GM) muscles. They found that supplementation with a diet of high-tannin sorghum increased the rate of color change during aerobic oxygenation and modulated lipid oxidation in two ways: it reduced oxidation before aerobic storage and accelerated oxidation during aerobic display of the tissue.

In other species, supplementation with tannins or feeding tannin-rich feeds had little or no effect on meat quality, as seen when supplementing a natural extract of chestnut wood to rabbits [56] or pigs [57]. Similarly, de Jesús et al. [58], who studied the effect of feeding dried chestnuts (15% and 25% of the formulation), found no effect on the physiochemical properties (color parameters, water holding capacity, and shear force) of *Longissimus dorsi* muscle.

Our research has shown that adding tannins to a bull's diet significantly reduces drip loss in fresh meat. However, after two weeks, meat from bulls that received tannin supplementation exhibited significantly higher meat thaw loss. Similar conclusions were also reported by Joo et al. [59]. They reported that adding chestnut meal at 30 g kg^{-1} resulted in lower drip loss in pigs. Drip loss is a method of evaluating water holding capacity, which is an essential quality parameter for both the industry and the consumer and is related to the status of proteins that bind the water that is mainly affected by post-mortem conversion of muscle to meat (pH decline) and the rate of carcass refrigeration (especially deep muscles) [60].

4.3. Effect of Supplement on Fecal Clostridia Concentration

In the present study a notable reduction was observed when the concentration of individual *Clostridium* species was compared. Differences in the concentration of *C. perfringens* ($p = 0.004$) were observed between groups at the end of the feeding trial. The results of our study are in good agreement with previous studies. Redondo et al. 2015 [61] found that *C. perfringens* isolated from bovine feces had difficulty forming resistance to hydrolyzable tannins. The study of Elizondo et al. [62] confirmed bacteriostatic and bactericidal activities in vitro experiments with *C. perfringens*. The bacteriostatic activity of quebracho and chestnut tannins was tested on selected *C. perfringens* strains of toxin types A, C, D, and E. The concentrations of quebracho required to inhibit the growth of *C. perfringens* were 7–85 times higher than those of chestnut tannin (0.6–1.2 mg/mL vs. 0.003–0.15 mg/mL). The bactericidal effect of quebracho occurs within 5 h of administration and, in the case of chestnut tannin, virtually immediately in 5 min. Our results are in agreement with the previous study on steers, in which an antimicrobial effect of hydrolyzable tannins on *E. coli* and coliform bacteria was observed [63]. The effect was limited to the rumen, whereas in present study to the entire digestive tract.

5. Conclusions

This study provides new information about the effect of HT supplements in the diet of bulls on growth pattern, carcass and meat quality, and reduction of pathogenic strains of *Clostridia* in feces. It demonstrated that tannins had a significant effect on increasing growth rate when bulls averaged 538 kg BW to slaughter maturity. In addition, tannin supplementation decreased meat drip losses and was very effective in reducing *C. perfringens* in the selective test. Therefore, given the same energy content in the diet of fattening bulls, the addition of HT can affect protein synthesis in the rumen, thereby increasing the availability of amino acids in the amino acid pool for body protein synthesis and consequently ADG. Our research on large ruminants under practical breeding conditions allowed us to approach a statistically balanced experiment. In practice, a cheaper meal with a lower protein content together with the addition of hydrolyzable tannins can increase nutrient utilization efficiency, improve growth intensity, and effectively reduce *Clostridia*l pathogens in bulls without adverse effects on carcass and meat quality. Our results suggest that the addition of hydrolyzable tannins is justified in practice because their activity is effective against pathogenic bacteria and could be very promising in controlling ruminant diseases, reducing antibiotic use, and improving overall welfare of domestic animals. Further research should elucidate the response of the microbiome and the methane reduction potential in bull fattening.

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Institutional Review Board Statement: Feed additives were used in this study (European Union Register of Feed Additives, 2013). No animal procedures were performed, which would require ethics approval according to Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available within the article.

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