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Introducing novel trends in the nutrition of monogastric farm animals for the production of high-quality livestock products

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Introducing novel trends in the nutrition of monogastric farm animals for the production of high-quality livestock products

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Editorial: Introducing novel trends in the nutrition of monogastric farm animals for the production of high-quality livestock products

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Editorial on the Research Topic
Introducing novel trends in the nutrition of monogastric farm animals for the production of high-quality livestock products

The rearing of monogastric farm animals, especially pigs and poultry, is a significant animal husbandry activity worldwide, contributing ~75% to global meat production, fully meeting the demand for eggs, and providing animal protein sources of high nutritional and biological value (1). Pig and poultry production has shown adaptability to changing conditions and consumer concerns, through increased productivity, diverse products, and alternative production systems. However, challenges remain, particularly regarding climate change, welfare concerns, and production sustainability, especially given the ongoing energy and economic crises and food security threats. This Research Topic presents 10 papers covering novel aspects of these issues.

One of the key challenges in farm animal production in Europe is climate change adaptation. It affects animals through severe heat stress and feedstuff production. Corn, crucial in many countries, faces severe losses from heat and drought. Using alternative, stress-resistant cereal grains may be a solution. Barley, rye, and oats are less sensitive but contain fibers that can have depressive effects beyond certain inclusion rates. Lingens et al. investigated broken rye as a substitute for whole wheat with turkeys, finding that rye up to 10% can replace wheat and increase production sustainability. This aligns with earlier findings (2, 3), showing that feeding whole or broken cereal grains saves grinding costs, stimulates gizzard function, and positively affects digestion.

Another important issue in Europe is increasing protein self-sufficiency in animal nutrition. Importing soybean meal creates insecurity and a high carbon footprint. Other feedstuffs are available (legume seeds, extracted meals, dried distilled grains with solubles - DDGS, etc.), but their maximum inclusion rates are not always clear. Such et al. investigated the use of extracted sunflower meal (SFM) to replace soybean in the diets of pullets and laying hens. Results showed that SFM, even at a 30% inclusion rate, did not reduce ileal amino acid absorption and could fully substitute soybean.

A paper also explored low-protein diets for broiler chickens. While many results are available, practical implementation is constrained by special requirements and ratios of some essential and non-essential amino acids, such as threonine or glycine (4). Since crystalline glycine is not allowed in Europe, feedstuffs with higher glycine content can improve the efficiency of low-protein diets. Strifler et al. showed that increasing the threonine-to-lysine ratio or using meat meal as a glycine source improved weight gain and feed conversion of broiler chickens in the grower and finisher phases. However, changes in the starch-to-protein ratio in low-protein diets increased abdominal fat in birds.

Research has also focused on balancing growth performance and intestinal health in weaned piglets. Correia et al. examined different crude protein (CP) levels and supplementation with non-essential amino acids (NEAAs) like arginine, glutamine, and glutamate. Lowering CP in diets is often necessary to reduce nitrogen excretion but may compromise growth. The study found that while higher CP diet (24%) improved feed conversion, lower CP diet (18%) with NEAAs supported intestinal health by increasing villus height and goblet cells, and reducing inflammatory markers. This suggests that NEAA supplementation can mitigate the negative effects of low CP diets on gut health.

Wang et al. explored alternative feed ingredients, using storage japonica brown rice (SJBR) as a corn substitute in growing-finishing Min pigs. The aim was to improve feed efficiency while reducing costs. Bile acids were added to enhance fat digestion. Results indicated SJBR improved feed conversion ratios and increased beneficial gut bacteria. Adding bile acids further reduced back fat thickness and improved lipid metabolism, demonstrating the potential of these strategies for improving production efficiency while maintaining meat quality.

Yin et al. focused on enzyme supplementation to improve nutrient utilization and bone health in pigs. They evaluated phytase and a multi-carbohydrase-phytase complex (MCPC) on phosphorus digestibility and bone mineral content in growing pigs fed corn- or wheat-based diets. Enzyme supplementation, particularly MCPC, enhanced phosphorus absorption and bone strength while positively affecting gut microbial diversity. These findings emphasize the role of enzymes in boosting growth performance and nutrient utilization.

In livestock farming, nutrition is often the deciding factor for operational efficiency, both in terms of cost and productivity. A considerable amount of research is directed toward using alternative feedstuffs and feed additives to promote animal production in the face of modern challenges like climate change and rising raw material prices. Izuddin et al. evaluated the effects of different oil sources (crude palm oil, red palm oil, refined palm oil, palm kernel oil, or soybean oil) on blood lipid profiles, fatty acid deposition, and hepatic lipid and lipoprotein gene expression in laying hens. Their findings showed that all oils were suitable for use, but soybean oil increased omega-3 and omega-6 fatty acids in tissues. The choice of oil should reflect the producer's target, such as enhancing specific fatty acids in eggs and meat or reducing costs by using cheaper oils like crude palm oil.

Regarding feed additives, Almeldin et al. and Suliman et al. worked with broilers, using iron nanoparticles and algae, as well as nano-emulsified plant oil and probiotics, to promote meat production and quality. Almeldin et al. showed that green Nano-Fe up to 40 mg/kg, using 1 g/kg *Halimeda opuntia* as a carrier, or alone, enhanced broiler performance, carcass traits, and meat quality. Suliman et al. found that supplementing male broilers with essential oils and probiotics improved meat chewiness by reducing cohesiveness and hardness, while increasing springiness. Moniruzzaman et al. examined the effects of curcumin nanospheres in finishing pigs, finding promising results for enhanced growth, immunity, and gastrointestinal health.

In conclusion, the papers presented in this Research Topic offer valuable insights into innovative approaches in monogastric nutrition. The research emphasizes optimizing feed ingredients, alternative proteins, and dietary supplements to address climate change, economic pressures, and shifting consumer demands. By improving growth performance, nutrient utilization, and animal health, sustainable livestock farming becomes attainable. Despite the progress made, ongoing research is vital to further refine these strategies, ensuring animal production evolves in line with global sustainability goals. Together, these studies highlight the crucial role of nutrition in shaping the future of animal production.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of adding nano-emulsified plant oil and probiotics to drinking water during different periods besides sex on processing characteristics, physicochemical properties, and meat quality traits of broiler chickens

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Introduction: High-quality meat is one of the consumer demands. Therefore, several studies have concluded that supplementing broilers with natural additives can improve meat quality. This study was carried out to evaluate the effects of nano-emulsified plant oil (Magic oil®) and probiotic (Albovit®) as water additives (at the rate of 1 ml/L and 0.1 g/L, respectively) during different growing periods on processing characteristics, physicochemical properties, and meat quality traits of broilers chickens.

Methods: A total number of 432-day-old Ross broiler chicks were randomly assigned to one of six treatment groups according to the growing periods in which magic oil and probiotics were added to drinking water, each with nine replicates and eight birds per replicate (4♂ and 4♀). On day 35, birds' processing characteristics, physicochemical properties, and meat quality traits were examined.

Results and discussion: The results showed that treatments had a significant ($P < 0.001$) impact on cooking loss, cohesiveness, and chewiness. The male broiler chickens had higher ($P \leq 0.05$) initial lightness, initial whiteness index, water holding capacity, shear force, live weight, hot and chilled carcass weights, as well as lower gizzard and neck percentages than females. The interactions between treatments and sex showed a significant ($P < 0.001$) impact on cooking loss, shear force, hardness, springiness, and chewiness. In conclusion, supplementing male broiler chickens with Magic oil and probiotic, particularly from 0–30 days of age had favorable meat chewiness as a result of lower cohesiveness and hardness higher springiness, and the most convenient cooking loss value. Magic oil and probiotic, especially in males, is advisable to be supplemented in water of growing broilers chicken programs from 0 to 30 days of age. Moreover, further studies under commercial conditions are recommended to locate the most favorable combination of Magic oil/probiotic supplements for the best processing characteristics and meat quality attributes outcomes.

KEYWORDS

broiler sex, processing performance, physicochemical properties, breast meat quality, nano-emulsified plant-oil, probiotics

1. Introduction

Poultry production is presumably the most rapidly growing, adaptable, and profitable of all livestock sectors expanding in both developed and developing countries (1, 2). Poultry meat is widely accepted and consumed by people from all walks of life due to its high nutritional quality, delicious taste, low cost, and importance as an animal protein source in human growth and development (3–5). Continuous genetic selection has produced fast-growing broilers with a short production cycle that reach market weight at 6 weeks of age and have a high meat yield, accounting for 74.81% (1, 5–7). The selection has had a negative impact on meat quality by increasing fiber diameters and the ratio of glycolytic fibers (8). Meat quality is a multifaceted character that is influenced by breed, strain, age, genetic, environmental, sex, and nutritional factors (2, 9, 10). Several studies have been conducted to assess the impact of nutrient supplementation on meat quality in chickens (11–14).

Antimicrobial growth promoters (AGPs) have been used in the poultry production industry for decades, resulting in a high risk of antibiotic-resistant bacteria being transferred to humans. It is difficult to raise broiler chickens in an antibiotic-free production system, and finding an effective nutritional alternative to support growth performance, gut health, and functionality without using AGPs is critical (15). Several non-antibiotic growth promoters are commercially available to improve bird growth, control pathogens, and reduce the risks of antibiotic resistance and misuse (16, 17). Examples include herbal essential oils, extracts, nano-emulsions and secondary metabolites (18, 19). Additionally, exogenous enzymes (20), organic acids (21), probiotics (15, 22), prebiotics (23), amino acids (24, 25), and green nanoparticles (26).

Essential oils (EOs), a type of phytogenic, are viable substitutes for increasing meat broiler production efficiency (27). EOs can be used therapeutically in a variety of broiler production situations due to their antibacterial, antiviral, antifungal, and antiparasitic properties (18). A combination of canola oil nano-emulsion and 2% Satureja bachtiarica essential oil is an effective natural preservative for chilled chicken breast (28). Rabbits, monogastric, fed a diet containing nano-emulsified essential oil had a higher final live weight, a higher carcass weight, a higher meat protein content, a lower fat content, and higher monounsaturated and polyunsaturated acids (29). Probiotics in animal feed is projected to attain massive global growth, reaching USD 6.24 billion by 2026 (15). High dietary energy content increases carcass weight (CW), carcass yield, and abdominal fat (30). The quality of chicken meat is becoming a significant issue when viewed from the perspectives of consumers and industries (31). The criteria for meat quality include pH, color, water holding capacity, cook loss, myofibril fragmentation index, and shear force (2). Texture profile analysis is a constructive technique that uses a popular double compression test to mimic the bite action of the mouth to determine the textural properties of poultry meat (32).

There are very few studies in the literature that look at the effects of sex and water supplementation of nano-emulsified plant-oil on meat attributes *viz* processing characteristics, physicochemical properties, and meat quality traits of broiler chicken. On the other hand, the addition of nanoparticles such as zinc nanoparticles and curcumin nanoparticles to broiler feed and *Bacillus licheniformis* improved the weight, carcass characteristics, and meat quality of chickens (18). Thus, the objective of this study was to determine the effects of water supplements of Magic oil plus probiotics and sex on processing characteristics, physicochemical properties, and meat quality traits of broiler chickens.

2. Materials and methods

This study was approved by the Ethics Committee of Scientific Research, King Saud University (KSU), Saudi Arabia (Approval No: KSU-SE-21-02).

2.1. Nano-emulsified plant-oil and probiotics composition

2.1.1. Magic oil ™ Atcopharma

Each liter contains 98.5% nano-emulsified crude oil including 26% monounsaturated fat, 59% polyunsaturated fat (50% linoleic acid; omega-6 & 7% linolenic acid; omega-3;), 14% saturated fats only and vitamin E.

2.1.2. Albovit® Albafarma

Each kg contains *Enterococcus faecium* (3.3X1012 CFU), Galacto-oligosaccharides (136,000 mg), Vitamin D3 (200,000 IU), and Vitamin C (200,000 mg).

2.2. Experimental design and bird's management

On arrival from Alkhumasia commercial hatchery, Riyadh, Saudi Arabia, 432-day-old broiler chicks (Ross 308) were sexed and individually weighed before being divided into six treatments based on body weights. Each group was further redistributed to nine replicates with eight birds per replicate (4♂ and 4♀). The study was carried out in an environmentally controlled poultry unit at temperatures 22–24°C. All stages of growing period were performed in the Animal Production Department, College of Food and Agriculture Sciences, King Saud University (24°43′28.8″N 46°37′07.9″E). Broiler chicks were raised in flour cage pens under similar managerial and hygienic conditions. A standard starter (0–15) and finisher diets (16–35 days) as shown in Table 1, isocaloric and isonitrogenous contents were offered in mash form based on corn-SBM and were formulated to meet or exceed the recommendations in commercial practice in Saudi Arabia. The two additives were supplemented in drinking water and were not included in the nutrient matrix. Upon arrival, the chicks were randomly distributed to one of six treatments according to the periods in which Magic oil and probiotics were added to the drinking water: Control, no additive (A), Magic oil and probiotics from days 1 to 35 (slaughter day) (B), Magic oil and probiotics from days 1 to 4 then from days 17 to 21, and from day 25 to slaughter (C), Magic oil and probiotics from days 1 to 4 then from day 7 to slaughter (D), Magic oil and probiotics from days 1 to 4 and from day 21 to slaughter (E) and probiotic from days 1 to 4 and from days 16 to 18 (P). Magic oil and probiotic were added to water at the rate of 1 ml/L and 0.1 g/L, respectively. Water (with

TABLE 1 Dietary composition during starter and finisher periods.

Ingredient %	Treatment period (0–49) days	
	Starter (0–21)	Finisher (22–49)
Yellow corn	57.39	61.33
Soybean meal	27.00	22.80
Palm oil	2.20	2.80
Corn gluten meal	8.80	6.0
Wheat bran	0.00	3.0
DCP	2.30	2.09
Ground limestone	0.70	0.62
Choline chloride	0.05	0.05
DL-methionine	0.105	0.075
L-lysine	0.39	0.36
Salt	0.40	0.20
Threonine	0.17	0.17
V-M premix[a]	0.50	0.50
Total	100	100
Analysis		
ME, kcal/kg	3,000	3,050
Crude protein, %	23.0	20.5
Non phytate P, %	0.48	0.44
Calcium, %	0.96	0.88
Digestible lysine, %	1.28	1.15
Digestible methionine, %	0.60	0.54
Digestible sulfur amino acids, %	0.95	0.86
Digestible threonine, %	0.86	0.77

[a]Vitamin-mineral premix contains in the following per kg: vitamin A, 2,400,000 IU; vitamin D, 1,000,000 IU; vitamin E, 16,000 IU; vitamin K, 800 mg; vitamin B1, 600 mg; vitamin B2, 1,600 mg; vitamin B6, 1,000 mg; vitamin B12, 6 mg; niacin, 8,000 mg; folic acid, 400 mg; pantothenic acid, 3,000 mg; biotin 40 mg; antioxidant, 3,000 mg; cobalt, 80 mg; copper, 2,000 mg; iodine, 400; iron, 1,200 mg; manganese, 18,000 mg; selenium, 60 mg, and zinc, 14,000 mg.

or without Magic oil and probiotics) was available *ad libitum* for all birds in all groups.

2.3. Meat quality characteristics

2.3.1. pH and temperature

The initial pH and temperature of the breast muscle were measured directly after slaughter (~15 min) and then 24 h later (ultimate) using a microprocessor pH- Meter (Model PH 211, Hanna Instruments). Two readings were taken, and the mean value was calculated for each carcass.

2.3.2. Meat color

The color values of CIELAB Color System (1976), L* (lightness) a* (redness), and b* (yellowness), were determined on the breast muscles 15 min after slaughter using a Chroma meter (Konica Minolta, CR-400-Japan). As described by Valizadeh et al. (33) and Qaid et al. (34), values for L*, a*, and b* were converted to estimate the saturation index, total color change (ΔE), hue angle, browning index (BI), and whiteness index (WI). These parameters could provide more accurate assessment of how consumers perceive meat color.

2.3.3. Water holding capacity

It was determined based on the technique described by Wilhelm et al. (35). Two replicates of around about 2 g were collected from the breast muscle of each sample and were cut into cubes. Then, the samples were placed between two filter papers and two Plexiglas and were left under a 10-kg weight for 5 min. Afterward, the samples were weighed and WHC was determined as the difference between the initial and final weights.

2.3.4. Cooking loss

The frozen breast muscle (~100 g) was thawed overnight at 4°C. Then, they were placed in a commercial indoor countertop grill and were cooked to an internal temperature of 70°C. The temperature was monitored by inserting a thermocouple thermometer probe (Eco scan Temp JKT, Eutech Instruments) into the geometric center of the muscle. The muscles were weighed before and after cooking to determine cooking loss (CL) as the difference between the initial and final weights.

2.3.5. Myofibril fragmentation index

As an indirect measure of calpain activity, myofibril fragmentation (MFI) in muscle samples was assessed. A total of 4 g of minced muscles, free of visible connective tissue and external fat, were homogenized for 30 s in a blender (Ultra Turrax; IKA-Werke, Staufen, Germany) with 40 ml of cold MFI buffer at 2°C. Following several washes, suspension aliquots were diluted in MFI buffer to a final protein concentration of 0.5 mg/ml and poured into a cuvette for immediate absorbance measurement at 540 nm with a spectrophotometer (HACH DR/3000 Spectrophotometer, USA). Each sample's MFI was multiplied by 200.

2.3.6. Shear force

The cooked samples used in the cooking loss were reused for determining the shear force and were used to evaluate shear force according to Wheeler et al. (36). They were cooled to room temperature (21°C), then five 1.27 cm in diameter round cores were removed from each muscle sample parallel to the longitudinal orientation of the muscle fibers. Cores were obtained using a handheld coring device. Shear force was determined as the maximum force (*N*) perpendicular to the fibers using a Texture Analyzer (TA-HD-Stable MicroSystems, England) equipped with a Warner-Bratzler attachment. The crosshead speed was set at 200 mm/min.

2.3.7. Texture profile analysis

The TPA parameters of hardness, chewiness, springiness, and cohesiveness were conducted and measured in the same manner as described in Qaid et al. (34) and Novaković and Tomašević (37).

2.4. Carcass measurements

At day 35 of age, 18 birds per treatment (nine males and nine females) were selected randomly. After slaughtering, feathers, heads, and shanks were removed and the remaining carcass was dissected to separate breast and thigh. Similarly, fat, liver, heart, gizzard, wings, and drumstick were also separated and weighed. The percentage of the yield of each part was calculated based on dressing weight (38).

2.5. Statistical analysis

The data were subjected two-way analysis of variance (ANOVA) in a general linear model (GLM) using Statistical Analysis System package (SAS) version 9.4 software (SAS Institute Inc., Cary, NC, USA) (39).

The model equation is described as follows:

$$\gamma_{ij} = \mu + T_i + G_j + TG_{ij} + e_{ijk}$$

Where:

Yij = the individual observation;
μ = the general experimental mean;
Ti = the effect of ith treatment;
Gj = the effect of *j*th sex;
TGij = the effect of treatment by sex interaction;
eijk = a random error.

Means for measurements showing significant differences in the analysis of variance were tested using the PDIFF option. The overall level of statistical significance was set at $P \leq 0.05$. All values were expressed as statistical means ± standard error of the mean (SEM).

3. Results

The results of the physicochemical properties are shown in Tables 2–4. The results of the main effects: treatments and sex of broilers, and their interactions on the initial and ultimate color components (lightness, redness, and yellowness) and their derivatives of breast meat at 35 days of age are summarized in Tables 2, 3, respectively. While the results of the main effects; treatments and sex and their interactions on the initial and final values of pH and temperature of breast meat at 35 days of age are shown in Table 4. The results of meat quality characteristics of both male and female broiler chickens supplemented with Magic oil and probiotic are shown in Table 5. The data in Table 6 show the main effects of treatments and sex on carcass measurements at 35 days of age, as well as the effects of interaction between treatments and sex of broilers. The effects of treatments, sex, and their interaction on processing performance (weight of chilled carcass, yield of cooked carcass, breast, legs, wings, back, and neck in percent) at 35 days of age are shown in Table 7.

3.1. Effects of treatments

Data analysis revealed that water supplementation of Magic oil and probiotic had no significant effect on the initial and final color components of breast flesh (Tables 2, 3). The supplementation also had no significant effect on the initial and final values of pH and temperature of breast meat at 35 days of age, as shown in Table 4. Table 5 shows that treatments had no effect on WHC, MFI, SF, hardness, and springiness but had an effect on CL, cohesiveness, and chewiness. Compared to the other groups, Magic oil supplementation had the lowest (most convenient) value (18.04) for cooking loss at 0–30 days of age. The most convenient values for cohesiveness and chewiness were obtained by the control group. In contrast, probiotic supplementation at 0–4 and 16–18 days of age resulted in the highest values for cohesiveness and chewiness. In Magic oil groups, the values were intermediate. The experimental treatments had no effect ($P \geq 0.05$) on carcass measurements and processing performance at 35 days of age (Tables 6, 7).

3.2. Effects of sex

Statistical analysis of the data revealed that sex had no significant effect ($P > 0.05$) on the initial and final color components of breast meat (Tables 2, 3) and on the initial and final pH and temperature values of breast meat at 35 days of age (Table 4). However, the initial lightness index ($P < 0.001$) and initial whiteness index ($P = 0.001$) were influenced by sex, with male chickens having a higher value than female chickens. As shown in Table 5, the results of CL%, MFI%, and texture profile analysis were not significantly different in male and female broiler chickens ($P > 0.05$). However, the results showed that sex had a significant effect ($P = 0.02$) on SF and tended to be significant on the WHC ratio ($P = 0.05$), with male chickens having a higher value than female chickens (Table 5).

The effects of sex on carcass measurements of birds at 35 days of age are shown in Table 6. Male chickens had significantly ($P \leq 0.05$) higher LW (2,591.9) and CW (1,904.2) at marketing age than females, which had LW and CW of 2,195.5 and 1,605.4, respectively. On the other hand, females had significantly higher gizzard weight (1.6%) than males (1.5%). The percentage of carcass yield, abdominal fat, liver, and heart did not differ significantly ($P > 0.05$) between male and female birds. At 35 days of age, sex had a significant effect on chilled carcass weight, wings, and neck percentage (Table 7). Males had a higher chilled carcass weight, lower relative wing, and neck weight than females. On the other hand, there were no significant ($P > 0.05$) differences between males and females in other processing performances (carcass yield, breast, legs, back, and neck percentages), as shown in Table 7.

3.3. Interaction of treatment and sex

Statistical analysis of the data revealed that the interaction of supplements and sex had no significant ($P > 0.05$) effect on the initial and final color components of breast flesh (Tables 2, 3). On the other hand, the interaction of supplements and sex shown in Table 4 had no significant effects ($P > 0.05$) on the initial and final values of pH and temperature of breast meat at 35 days of age.

In Table 5, the results show that the interaction of treatments and sex had a significant effect ($P \leq 0.05$) on WHC, CL, SF, and texture profile analysis, except for cohesiveness. Males in the treatment group

TABLE 2 Effects of adding nano-emulsified plant oil and probiotics to drinking water during different periods besides sex on initial color components and its derivative of breast meat of broiler chickens at 35 days of age.

Sex	Treatment[a]	*n*[b]	Initial color components			Initial color derivatives				
			Li∗	ai∗	bi∗	ΔE	Chroma	HA (○)	BI	WI
Interaction effects:										
Males	A	9	48.29	3.73	4.24	46.12	5.73	46.85	14.74	47.95
	B	9	47.1	3.68	4.88	47.28	6.13	52.73	16.45	46.74
	C	9	46.3	3.9	4.29	48.10	5.92	47.31	15.66	45.97
	D	9	46.62	3.78	4.33	47.78	5.85	48.57	15.52	46.29
	E	9	47.48	4.19	4.98	46.97	6.60	49.42	17.26	47.05
	P	9	46.89	4.82	4.51	47.75	7.29	43.24	18.50	46.33
Females	A	9	43.67	4.98	4.47	50.86	6.92	42.27	18.98	43.22
	B	9	45.27	3.35	5.14	49.09	6.22	56.28	17.26	44.90
	C	9	45.87	4.34	5.03	48.60	6.81	49.07	18.48	45.43
	D	9	45.28	4.07	4.8	49.14	6.35	49.53	17.67	44.89
	E	9	44.52	3.94	4.87	49.89	6.38	49.01	17.81	44.13
	P	9	44.85	4.77	5.03	49.68	7.07	46.84	19.57	44.36
SEM			1.06	0.45	0.48	0.764	0.400	2.70	1.11	0.757
Main effects:										
Sex	Males	54	47.11[a]	4.02	4.89	47.33[b]	6.25	48.02	16.35	46.72[a]
	Females	54	44.91[b]	4.24	4.54	49.54[a]	6.62	48.83	18.29	44.49[b]
SEM			0.43	0.19	0.2	0.441	0.231	1.56	0.642	0.437
Treatment	A	18	45.98	4.35	4.36	48.49	6.32	44.56	16.86	45.59
	B	18	46.18	3.52	5.01	48.18	6.17	54.51	16.85	45.82
	C	18	46.09	4.12	4.66	48.35	6.36	48.19	17.07	45.70
	D	18	45.95	3.92	4.56	48.46	6.10	49.05	16.60	45.59
	E	18	46	4.07	4.92	48.43	6.49	49.22	17.54	45.59
	P	18	45.87	4.8	4.77	48.71	7.18	45.04	19.03	45.35
SEM			0.75	0.32	0.34	0.764	0.400	2.70	1.11	0.757
Source of variation:						*P*-values				
Sex			<0.001	0.39	0.21	0.001	0.26	0.71	0.04	0.001
Treatment			0.99	0.13	0.79	1.00	0.45	0.13	0.67	1.00
Sex × treatment			0.47	0.53	0.97	0.47	0.74	0.90	0.84	0.47

[a]A, unsupplemented control; B, Magic oil supplementation from 0 to 30 days of age; C, Magic oil supplementation from 1 to 4, 17 to 21, and 25 to 35 days of age; D, Magic oil supplementation from 1 to 4 and 17 to 35 days of age; E, Magic oil supplementation from 1 to 4 and 21 to 35 days of age; P, probiotic supplementation from 1 to 4 and 16 to 18 days of age.

[b]Number of replicate pens.

L* lightness, a* redness, and b* yellowness; ΔE, total color change; Chroma, saturation index; HA, hue angle (○); BI, browning index; WI, whiteness index. Means within a column with no common superscript differ significantly ($P \leq 0.05$). SEM, standard error of the mean.

fed Magic oil from 0 to 4, 17 to 21, and 25 to 35 days of age, achieved the best WHC ratio (36.18), followed by females in the control group (35.25), and males in the treatment group (34.12) fed Magic oil from 0 to 30 days of age. In contrast, females in the treatment group fed probiotic at 1–4 and 16–18 days of age had the lowest WHC ratio values (29.62). Male and female birds in the treatment group receiving Magic oil supplementation from 0 to 30 days of age had the best CL ratios (18.96 and 17.12, respectively), whereas male birds in the control group had the worst (25.69). The female fed the Magic oil from 1 to 4 and 17 to 35 days of age had the lowest shear forces (4.87 *N*), indicating the greatest tenderness, and males fed the Magic oil from 0 to 30 days of age had the highest tenderness (5.24 *N*). The highest value of shear force in the males of the control group (6.57 *N*) indicated tough meat. The males fed Magic oil from 0 to 4, 17 to 21, and 25 to 35 days of age had the highest hardness (11.01 *N*), while the females fed Magic oil from 0 to 30 days of age had the lowest hardness (7.38 *N*). Compared to the other groups, female broilers supplemented with Magic oil at 1–4 and 21–35 days of age and male broilers supplemented with Magic oil at 0–30 days of age had the highest springiness values (0.87 and 0.86, respectively). Male broilers fed Magic oil at 0–30 days of age had the lowest values for chewiness

TABLE 3 Effects of adding nano-emulsified plant oil and probiotics to drinking water during different periods besides sex on ultimate color components and its derivatives of breast meat of broiler chickens at 35 days of age.

Sex	Treatment[a]	*n*[b]	Ultimate color components			Ultimate color derivatives				
			Lu*	au*	bu*	ΔE	Chroma	HA (○)	BI	WI
Interaction effects:										
Males	A	9	50.02	5.54	9.13	44.90	10.71	58.83	28.19	48.85
	B	9	50.27	5.86	9.3	44.75	11.07	57.75	29.00	49.00
	C	9	48.9	5.38	8.6	45.95	10.23	58.60	27.11	47.85
	D	9	47.27	5.48	8.7	47.64	10.51	57.49	28.59	46.15
	E	9	51.64	5.97	9.99	43.54	11.78	59.17	29.58	50.16
	P	9	48.62	6.87	9.09	46.56	11.78	54.42	31.00	47.25
Females	A	9	48.64	5.97	9.34	46.39	11.36	57.15	30.04	47.37
	B	9	48.59	5.15	10.73	46.50	12.00	64.05	32.60	47.16
	C	9	47.74	5.87	9.57	47.25	11.29	58.50	31.49	46.50
	D	9	48.99	6.16	9.11	46.04	11.15	56.42	29.46	47.74
	E	9	48	5.7	9.16	46.91	10.84	57.56	29.74	46.86
	P	9	50.19	5.26	10.14	44.84	11.59	62.60	29.96	48.83
SEM			1.20	0.70	0.64	0.85	0.49	2.24	1.46	0.84
Main effects:										
Sex	Males	54	49.45	5.85	9.14	45.56	11.01	57.71	28.91	48.21
	Females	54	48.69	5.69	9.67	46.32	11.37	59.38	30.55	47.41
SEM			0.49	0.29	0.26	0.49	0.28	1.29	0.84	0.48
Treatments	A	18	49.33	5.75	9.24	45.65	11.03	57.99	29.12	48.11
	B	18	49.43	5.5	10.01	45.63	11.54	60.90	30.80	48.08
	C	18	48.32	5.63	9.09	46.60	10.76	58.55	29.30	47.17
	D	18	48.13	5.82	8.9	46.84	10.83	56.96	29.03	46.95
	E	18	49.82	5.84	9.57	45.23	11.31	58.36	29.66	48.51
	P	18	49.41	6.07	9.62	45.70	11.69	58.51	30.48	48.04
SEM			0.85	0.5	0.45	0.85	0.49	2.24	1.46	0.84
Source of variation:						*P*-values				
Sex			0.27	0.69	0.15	0.27	0.37	0.36	0.17	0.24
Treatments			0.66	0.98	0.55	0.74	0.70	0.89	0.93	0.75
Sex × treatment			0.2	0.55	0.56	0.23	0.69	0.44	0.77	0.23

[a]A, unsupplemented control; B, Magic oil supplementation from 0 to 30 days of age; C, Magic oil supplementation from 1 to 4, 17 to 21, and 25 to 35 days of age; D, Magic oil supplementation from 1 to 4 and 17 to 35 days of age; E, Magic oil supplementation from 1 to 4 and 21 to 35 days of age; P, probiotic supplementation from 1 to 4 and 16 to 18 days of age.

[b]Number of replicate pens.

Lu* ultimate lightness, au* ultimate redness, and bu* ultimate yellowness; ΔE, total color change; Chroma, saturation index; HA, Hue angle (○); BI, browning index; WI, whiteness index. Means within a column with no common superscript differ significantly ($P \leq 0.05$). SEM, standard error of the mean.

(2.38), whereas female broilers fed probiotic at 1–4 and 16–18 days of age had the highest values for chewiness (3.95). Taken together, male broilers supplemented with Magic oil at 0–30 days of age had the best options in terms of chewiness (2.38) because they had lower cohesiveness (0.39) and hardness (7.38) and higher springiness (86), as well as the most favorable cooking loss value and water holding capacity.

The interaction of supplements and sex had no effect ($P > 0.05$) on carcass measurements and processing performance at 35 days of age (Tables 6, 7).

4. Discussion

The efficacy of Magic oil, a natural nano-emulsified plant-oil, was compared to probiotic on carcass traits and breast quality in broiler chickens in this study. Nano emulsions are used in the food industry to encapsulate, protect, deliver, and transport hydrophobic (low water solubility) bioactive components such as nutrients, nutraceuticals, antimicrobials, and antioxidants (40, 41). They are composed of tiny oil droplets suspended in water and act as a vehicle for essential oils to be bioavailable

TABLE 4 Effects of adding nano-emulsified plant oil and probiotics to drinking water during different periods besides sex on breast meat pH and temperature of broiler chickens at 35 days of age.

Sex	Treatment[a]	n[b]	pH		Temperature (°C)	
			Initial	Ultimate	Initial	Ultimate
Interaction effects:						
Males	A	9	6.06	5.93	27.78	13.41
	B	9	6.14	5.96	27.44	12.68
	C	9	6.10	5.99	27.32	12.82
	D	9	6.03	5.98	27.48	12.43
	E	9	6.14	5.87	27.30	12.81
	P	9	6.19	5.99	26.51	12.71
Females	A	9	6.14	5.97	27.68	12.46
	B	9	6.10	5.99	27.27	12.48
	C	9	6.06	5.95	27.37	12.30
	D	9	6.05	5.99	27.58	12.69
	E	9	6.07	5.99	27.26	12.32
	P	9	6.21	5.97	27.01	12.80
SEM			0.06	0.04	0.31	0.30
Main effects:						
Sex	Males	54	6.11	5.95	27.31	12.81
	Females	54	6.10	5.98	27.36	12.51
SEM			0.02	0.02	0.13	0.12
Treatment	A	18	6.10	5.95	27.73	12.94
	B	18	6.12	5.98	27.36	12.58
	C	18	6.08	5.97	27.24	12.56
	D	18	6.04	5.98	27.53	12.56
	E	18	6.10	5.93	27.28	12.57
	P	18	6.20	5.98	26.76	12.76
SEM			0.04	0.03	0.22	0.22
Source of variation:				*P*-values		
Sex			0.85	0.35	0.77	0.09
Treatment			0.22	0.78	0.07	0.76
Sex × treatment			0.84	0.43	0.92	0.39

[a]A, unsupplemented control; B, Magic oil supplementation from 0 to 30 days of age; C, Magic oil supplementation from 1 to 4, 17 to 21, and 25 to 35 days of age; D, Magic oil supplementation from 1 to 4 and 17 to 35 days of age; E, Magic oil supplementation from 1 to 4 and 21 to 35 days of age; P, probiotic supplementation from 1 to 4 and 16 to 18 days of age.
[b]Number of replicate pens. SEM, standard error of the mean.

(18). Recent studies on the quality of meat or carcass traits of birds supplemented with powder, essential oils, or extracts of phytogenic in diets have been conducted (42–46). Unfortunately, little or no research has been conducted to examine the effect of water supplementation of Magic oil as nano-emulsified plant-oil and probiotic on the meat attributes of birds. At a probability level of $\alpha \leq 0.05$, the null hypothesis states that the effects of Magic oil and probiotic, sex, and their interactions on processing characteristics, physicochemical properties, and meat quality traits of broiler chickens are equal to the effects of the control group. Treatments, sex, and their interactions, according to the alternative hypothesis, improved some or all of the selected parameters.

Meat color is influenced by many factors, such as pre-slaughter factors, heme pigments, stunning methods, moisture content, cooling regimes, sex, strain, stress, and protein physical status (47). In agreement with Yetişir et al. (48), who noted that a higher L* value would be preferable in terms of consumer acceptance. In our study, male birds had significantly higher initial L* and whiteness index values (47.11 and 46.72, respectively) than females (44.91 and 44.49, respectively). Identifying color is an easy way to determine the pH of meat. If the meat is very dark, the pH is high, and if it is very light, the pH is low. Female birds in the control group, for example, were very light (Li* = 48.29) and had a low pH (initial pH = 6.06). Birds given Magic oil between the ages of 0 and 30 days had a numerically higher initial Li* = 46.18, resulting in a numerically higher initial WI

TABLE 5 Effects of adding nano-emulsified plant oil and probiotics to drinking water during different periods besides sex on meat quality of broiler chickens at 35 days of age.

Sex	Treatment[a]	*n*[b]	WHC%	CL%	MFI	SF (*N*)	Texture profile analysis			
							Hardness (*N*)	Springiness	Cohesiveness	Chewiness
Interaction effects:										
Males	A	9	31.31^{bc}	25.69^{a}	85.28	6.57^{a}	8.72^{abc}	0.78^{b}	0.41	2.90^{abc}
	B	9	34.12^{abc}	18.96^{e}	80.47	5.24^{c}	7.38^{c}	0.86^{a}	0.39	2.38^{c}
	C	9	36.18^{a}	22.80^{b}	88.86	5.35^{bc}	11.01^{a}	0.77^{b}	0.42	3.66^{ab}
	D	9	33.03^{ab}c	23.63^{b}	85.48	5.49^{bc}	9.29^{abc}	0.79^{b}	0.40	3.12^{abc}
	E	9	32.96^{ab}c	20.14^{e}	72.72	5.66^{bc}	9.56^{abc}	0.77^{b}	0.43	3.66^{ab}
	P	9	34.49^{ab}	20.78^{cde}	84.62	5.68^{bc}	10.58^{a}	0.78^{b}	0.45	3.67^{ab}
Females	A	9	35.25^{ab}	19.95^{e}	75.35	4.97^{c}	9.14^{abc}	0.78^{b}	0.37	2.54^{bc}
	B	9	31.68^{abc}	17.12^{f}	88.49	5.88^{ab}	10.52^{a}	0.77^{b}	0.40	3.74^{ab}
	C	9	32.00^{ab}c	21.88^{bcd}	85.72	4.95^{c}	8.65^{bc}	0.77^{b}	0.41	2.71^{abc}
	D	9	31.25^{bc}	22.52^{bc}	82.45	4.87^{c}	8.85^{abc}	0.81^{ab}	0.42	3.19^{abc}
	E	9	32.45^{abc}	22.75^{b}	90.54	5.42^{bc}	9.23^{abc}	0.87^{a}	0.44	3.54^{ab}
	P	9	29.62^{c}	22.72^{bc}	89.60	5.54^{bc}	10.72^{a}	0.77^{b}	0.45	3.95^{a}
SEM			1.44	0.77	5.48	0.28	0.74	0.02	0.01	0.25
Main effects:										
Sex	Males	54	33.68	21.99	82.90	5.66^{a}	9.42	0.79	0.42	3.23
	Females	54	32.04	21.16	88.59	5.27^{b}	9.52	0.80	0.42	3.28
SEM			0.59	0.31	2.24	0.12	0.30	0.01	0.01	0.10
Treatment	A	18	33.28	22.82^{a}	80.32	5.77	8.93	0.78	0.39^{d}	2.72^{c}
	B	18	32.90	18.04^{b}	84.48	5.56	8.95	0.82	0.40^{cd}	3.06^{bc}
	C	18	34.09	22.34^{a}	87.29	5.15	9.83	0.77	0.42^{bc}	3.19^{bc}
	D	18	32.14	23.07^{a}	93.65	5.18	9.07	0.80	0.41^{bcd}	3.16^{bc}
	E	18	32.70	21.45^{a}	81.63	5.54	9.40	0.82	0.43^{ab}	3.60^{ab}
	P	18	32.06	21.75^{a}	87.11	5.61	10.05	0.77	0.45^{a}	3.81^{a}
SEM			1.02	0.54	3.88	0.20	0.52	0.02	0.01	0.18
Source of variation			*P*-values							
Sex			0.05	0.06	0.08	0.02	0.82	0.72	0.91	0.76
Treatment			0.74	<0.001	0.19	0.18	0.16	0.07	<0.001	<0.001
Treatment × sex			0.04	<0.001	0.09	0.01	0.02	0.01	0.09	<0.001

[a]A, un-supplemented control; B, Magic oil supplementation from 0 to 30 days of age; C, Magic oil supplementation from 1 to 4, 17 to 21, and 25 to 35 days of age; D, Magic oil supplementation from 1 to 4 and 17 to 35 days of age; E, Magic oil supplementation from 1 to 4 and 21 to 35 days of age; P, probiotic supplementation from 1 to 4 and 16 to 18 days of age. Means within a column with no common superscript differ significantly ($P \leq 0.05$).
[b]Number of replicate samples.

= 45.82. Treatments, sex, or their interactions had no effect on the other color components and derivatives in breast meat. According to Abudabos et al. (49), no treatment effect of nano-emulsified plant oil or betaine was observed in L*, a*, b*, color saturation, hue angle (H°), and a* to b* ratio.

The pH is defined as the negative log of the concentration of hydrogen ions and the pH parameter was a good predictor of meat characters (9). After slaughter, oxygen deprivation raises hydrogen ion concentrations due to lactic acid dissociation *via* the anaerobic glycolysis pathway, resulting in a pH drop. The pH declines directly affect the protein solubility, protein denaturation, protein's capacity bind water, and shelf life (50–52). Thus, broiler breast meat with a high pH has a higher water-binding capacity than meat with a lower pH. Lower pH in bird meat groups with essential oil or phytogenic nano-emulsions essential oil may be responsible for inhibiting the integration of the deterioration of microorganism growth (53, 54). High pH (over 6.2) and low pH values (below 5.8) can negatively influence meat quality of broiler breast meat [dark, firm, and dry (DFD) vs. pale, soft, exudative (PSE), respectively] (46, 50, 55). As a result, our data fell within the normal meat quality pH values (5.9–6.2), with initial pH ranging from 6.03 to 6.21 and final pH ranging from 5.87 to 5.99. Protein denaturation, a protein's ability to bind water, tenderness, and springiness were not impacted by treatment because the concentration of hydrogen ions in the muscle of broiler

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TABLE 6 Effects of adding nano-emulsified plant oil and probiotics to drinking water during different periods besides sex on carcass measurements of broiler chickens at 35 days of age.

Sex	Treatment[a]	n[b]	Live weight (g)	Hot carcass weight (g)	Carcass yield (%)	Abdominal fat (%)	Liver (%)	Heart (%)	Gizzard (%)
Interaction effects:									
Males	A	9	2,590.6	1,898.4	73.3	1.3	2.2	0.5	1.5
	B	9	2,683.3	1,977.8	73.7	1.3	2.2	0.5	1.4
	C	9	2,546.7	1,857.9	72.9	1.3	2.4	0.5	1.5
	D	9	2,507.8	1,859.9	74.2	1.3	2.2	0.6	1.5
	E	9	2,658.9	1,961.3	73.8	1.4	2.3	0.6	1.4
	P	9	2,564.4	1,870.0	73.0	1.3	2.3	0.5	1.5
Females	A	9	2,103.3	1,529.3	72.7	1.2	2.3	0.5	1.6
	B	9	2,235.0	1,642.1	73.4	1.5	2.3	0.6	1.5
	C	9	2,150.0	1,570.0	73.0	1.4	2.4	0.5	1.6
	D	9	2,269.4	1,643.1	72.4	1.3	2.4	0.6	1.5
	E	9	2,169.4	1,601.1	73.8	1.3	2.3	0.5	1.6
	P	9	2,245.6	1,646.6	73.3	1.5	2.3	0.6	1.5
SEM			61.4	48.2	0.5	0.1	0.1	0.0	0.1
Main effects:									
Sex	Males	54	2,591.9[a]	1,904.2[a]	73.5	1.3	2.3	0.5	1.5[b]
	Females	54	2,195.5[b]	1,605.4[b]	73.1	1.4	2.3	0.5	1.6[a]
SEM			25.1	19.7	0.2	0.1	0.0	0.0	0.0
Treatment	A	18	2,346.9	1,713.9	73.0	1.3	2.2	0.5	1.5
	B	18	2,459.2	1,809.3	73.5	1.4	2.3	0.5	1.5
	C	18	2,348.3	1,713.9	73.0	1.4	2.4	0.5	1.5
	D	18	2,388.6	1,751.5	73.3	1.3	2.3	0.6	1.5
	E	18	2,414.2	1,781.2	73.8	1.4	2.3	0.5	1.5
	P	18	2,405.0	1,758.3	73.1	1.4	2.3	0.5	1.5
SEM			43.4	34.1	0.4	0.1	0.1	0.0	0.1
Source of variation:					*P*-values				
Sex			<0.001	<0.001	0.24	0.47	0.39	0.49	0.02
Treatment			0.44	0.30	0.60	0.91	0.66	0.49	0.96
Sex × treatment			0.25	0.44	0.37	0.54	0.89	0.20	0.93

[a]A, unsupplemented control; B, Magic oil supplementation from 0 to 30 days of age; C, Magic oil supplementation from 1 to 4, 17 to 21, and 25 to 35 days of age; D, Magic oil supplementation from 1 to 4 and 17 to 35 days of age; E, gic oil supplementation from 1 to 4 and 21 to 35 days of age; P, probiotic supplementation from 1 to 4 and 16 to 18 days of age.

[b]Number of replicate samples. The relative weights of abdominal fat, liver, heart, and gizzard were calculated relative to live weight. Means within a column with no common superscript differ significantly ($P \leq 0.05$). SEM, standard error of the mean.

breasts was unaffected by treatment. This contradicts Abudabos et al. (49), who stated that diet only affected breast filets pH at 24 h post-mortem; the control had lower breast pH than betaine, while nano-emulsified plant oil had an intermediate temperature.

The term "water holding capacity" describes a muscle's capacity to bind water under specific circumstances. Usually, a sharp pH drop in meat can denaturize proteins, leaving behind pale meat with low WHC. According to Hughes et al. (56), decreasing water retention is linked to a decrease in the nutritional value of the meat due to the loss of some nutrients, which makes the breast meat less tender. It also tends to result in less reflective surface light, which lowers L* values. Cooking loss is the proportion of water lost during cooking due to shrinkage, which is related to the loss of juiciness to the palate. Usually, an increase in WHC accompanied by a decrease in the percentage CL (57, 58). Although cooking loss was significantly lower when broilers were supplemented with Magic oil from 0 to 30 days of age in both males and females, WHC in this study did not show any significant differences in treated groups compared with control.

Myofibrillar fragmentation is the extent of myofibrillar destruction caused by homogenization. The treatments had no effect on the MFI of the breast muscle. Myofibrillar fragmentation index values, according to Olson and Stromer (59), are strongly correlated with other muscle measurements such as tenderness. Magic oil supplementation, on the other hand, did not differ from the control

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TABLE 7 Effects of adding nano-emulsified plant oil and probiotics to drinking water during different periods besides sex on processing performance of broiler chickens at 35 days of age.

Sex	Treatment[a]	n[b]	Chilled carcass weight (g)	Carcass yield (%)	Breast (%)	Legs (%)	Wings (%)	Back (%)	Neck (%)
Interaction effects:									
Males	A	9	1,871.6	72.3	26.4	19.7	6.6	13.1	4.9
	B	9	1,944.4	72.4	28.1	19.3	6.4	12.9	4.2
	C	9	1,832.2	71.9	26.9	19.6	6.5	13.3	3.9
	D	9	1,759.1	70.5	26.9	19.2	6.5	12.6	3.7
	E	9	1,932.7	72.7	27.0	20.2	6.5	13.2	4.2
	P	9	1,834.4	71.5	27.1	19.6	6.4	12.9	4.1
Females	A	9	1,504.5	71.5	27.0	18.9	6.8	12.8	4.5
	B	9	1,606.4	71.8	26.4	19.0	6.7	13.2	4.7
	C	9	1,617.8	75.6	29.3	19.4	6.8	13.4	5.2
	D	9	1,618.9	71.3	26.5	19.1	6.6	13.2	4.4
	E	9	1,573.6	72.5	27.8	17.2	6.9	13.6	5.0
	P	9	1,627.3	72.5	26.9	19.4	6.6	13.2	5.0
SEM			50.4	1.4	0.8	0.7	0.2	0.4	0.3
Main effects:									
Sex	Males	54	1,862.4[a]	71.9	27.1	19.6	6.5[b]	13.0	4.2[b]
	Females	54	1,591.3[b]	72.5	27.3	18.8	6.7[a]	13.3	4.8[a]
SEM			20.6	0.6	0.3	0.3	0.1	0.2	0.1
Treatment	A	18	1,687.8	71.9	26.7	19.3	6.7	12.9	4.7
	B	18	1,775.4	72.1	27.3	19.2	6.6	13.1	4.5
	C	18	1,725.0	73.8	28.1	19.5	6.6	13.3	4.5
	D	18	1,689.0	70.9	26.7	19.1	6.6	12.9	4.1
	E	18	1,753.1	72.6	27.4	18.7	6.7	13.4	4.6
	P	18	1,730.9	72.0	27.0	19.5	6.5	13.1	4.5
SEM			35.6	1.0	0.6	0.5	0.1	0.3	0.2
Source of variation:					P-values				
Sex			<0.001	0.43	0.59	0.07	0.008	0.27	0.002
Treatment			0.45	0.50	0.57	0.86	0.80	0.72	0.52
Sex × treatment			0.12	0.64	0.26	0.28	0.94	0.92	0.21

[a] A, unsupplemented control; B, Magic oil supplementation from 0 to 30 days of age; C, Magic oil supplementation from 1 to 4, 17 to 21, and 25 to 35 days of age; D, Magic oil supplementation from 1 to 4 and 17 to 35 days of age; E, Magic oil supplementation from 1 to 4 and 21 to 35 days of age; P, probiotic supplementation from 1 to 4 and 16 to 18 days of age.
[b] Number of replicate pens. Means within a column with no common superscript differ significantly ($P \leq 0.05$). SEM, standard error of the mean.

in terms of myofibril fragmentation. The SF of the birds' breast muscle, on the other hand, ranged from 5.5 to 5.8 kgf/g (60) and from 2.71 to 3.31 kgf/g (61). As the SF values in this trial ranged between 5.15 and 5.77 *N*, the Magic oil or probiotic supplementation groups had no effect on meat tenderness. These findings are consistent with those of Pokoo-Aikins et al. (46), who found that different levels of dietary DL-Methionine supplementation had no effect on the meat toughness value of broilers. The dietary methionine level, the sex of the bird, and their interactions had no effect on the textural properties of cooked meat (46). According to Hussein et al. (10), females had more tender pectoral muscles and more myofibrillar fragmentation than males.

The texture profiles (cohesiveness and chewiness) of the treatments differed significantly, with the Magic oil groups having lower levels of cohesiveness and chewiness than the probiotic group. In general, the effect of treatment and sex interaction on meat tenderness resulted in variation between sexes rather than between treatment groups; thus, female meat was tenderer than male meat in each treatment group except in the group supplemented with Magic oil from 0 to 30 days of age, where male meat was tenderer than female. Furthermore, male meat in the control group was the highest SF value compared to other groups. Thus, meat quality could be enhanced by adding natural antioxidant compounds, and Magic oil has the highest antioxidant capacity due to its high phenolic content.

Foods fortified with micro/nano encapsulated vegetable-essential oils can improve their functional properties such as antioxidant and antimicrobial activity, as well as having more healthy unsaturated fatty acids (62).

The current study found that Magic oil additives improve meat quality, especially when supplemented from 0 to 30 days of age, with favorable chewiness in female broiler chickens (2.38) that resulted in lower cohesiveness (0.39) and hardness (7.38) and higher springiness (86). Female cage-reared broilers had higher meat quality in the breast muscle (63). Due to chewiness equal cohesiveness*hardness*springiness, which indicated chewiness was influenced by one or more of these parameters and has a direct relationship. In addition, the group that supplemented with Magic oil from 0 to 30 days of age were the most convenient cooking loss value and holding water.

Female broiler chickens in the supplemented probiotic group from 0 to 4 and 16 to 18 days of age had a rapid drop in meat pH (0.24) within 24 h postmortem compared to the other group, resulting in low WHC (29.62). Furthermore, the probiotic group had the highest cohesiveness (0.45), greater hardness (10.72), and the lowest springiness (0.77), which resulted in higher chewiness (3.95) and lower tenderness (5.54). These findings support the findings of Loddi et al. (64) who noticed that probiotics added to water and feed had no effect on the sensory characteristics of meat. On the other hand, Jensen and Jensen (65) found a positive impact of probiotics including *Bacillus licheniformis* and *Bacillus subtilis* spores on the flavor of broiler meat after cooling for 5 days. Several studies have suggested that nanoemulsion-based products can positively influence the physicochemical and sensory properties of breast muscles (66). Conversely, some authors have observed that natural antioxidants have little or no effect on the sensory characteristics of meat. For example, dietary supplementation with nano-emulsified vegetable-essential oils (49) or probiotic (16) had no effect on the quality of chicken meat in terms of myofibril fragmentation index, cooking loss, shear force, and texture profile analysis.

In the current study, male birds had 18.06% more live weight, 18.61% more hot carcass weight, and 17.04% more chilled carcass weight, resulting in 0.55% more carcass yield than female birds at marketing age. Female birds, on the other hand, weighed 6.67% more relative gizzards, 14.29% more relative neck weight, and 3.08% more relative wings than males. These results agree with those reported by others (10, 30, 67). The percentages of carcass yield, abdominal fat, liver, and heart were not significantly different between males and females. These findings partially contradict Majid et al. (68), who found a significant difference between males and females in most carcass cut weights.

In this experiment, the same level of Magic oil was used at different stages of bird development: from day one to slaughter, from 1 to 4 days, then 17 to 21 days and 25 days, and from 1 to 4 days, then 7 days to slaughter. However, the obtained results in this study varied depending on the treatment period. As a result, more researches are prompted to investigate different levels of Magic oil at different growth periods of birds in order to determine the best level of supplementation. These findings are in contrast with Abudabos et al. (49) who stated that dietary supplements of nano-emulsified plant oil/betaine had no effect on dressing, leg, fat, gizzard, spleen, or thymus, but had a significant effect on breast meat, liver, and bursa and in contrast to Nisar et al. (16), who claimed that treatments had no effect on carcass characteristics.

5. Conclusions

In conclusion, both water supplements of Magic oil and probiotic had no negative effects on the color, pH, temperature, or processing performance of breast meat. Male broiler chickens supplemented with Magic oil from 0 to 30 days of age had the best options in terms of chewiness as they had lower cohesiveness and hardness besides higher springiness, as well as the most convenient cooking loss value and water holding capacity. The results showed that male birds had higher initial lightness, water holding capacity, shear force, live weight, hot and chilled carcass weights, as well as a lower gizzard and neck percentage than female. These findings could also be used as a foundation for future studies of water supplementation of nanoemulsified plant oil and probiotics and their effects on performance, carcass quality and meat traits of broiler chickens.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of Scientific Research, King Saud University (KSU), Saudi Arabia (Approval No. KSU-SE-21-02).

Author contributions

GS and EH: conceptualization. AA, GS, and EH: methodology and investigation. HA-B: software. RA, GS, and EH: validation. RA: formal analysis. AA-O: resources and funding acquisition. HB-A: data curation. MQ, GS, and EH: writing—original draft preparation. AS: writing—review and editing and supervision. GS: visualization. GS and AA-O: project administration. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Evaluation of dietary curcumin nanospheres as phytobiotics on growth performance, serum biochemistry, nutritional composition, meat quality, gastrointestinal health, and fecal condition of finishing pigs

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Curcumin is a bioactive functional feeding stimulant that is widely used as an additive in cuisine and animal feeds. Owing to its hydrophobic nature and low bioavailability, the nanoformulation of curcumin has recently received special attention from researchers. In this study, we investigated the effects of curcumin nanospheres (CN) on the growth performance, serum biochemistry, meat quality, intestinal immunohistochemistry, fecal malodors and microbes in finishing pigs. A total of 90 crossbred pigs (Duroc × [Yorkshire × Landrace]) with an average initial body weight of 73.77 ± 0.08 kg were randomized into 3 dietary groups in triplicate pens (10 pigs in each pen): control (CON) without supplementation of CN and the pigs in the remaining two groups were supplemented with CN at 1.0 (CN1) and 2.0 (CN2) mL/kg diet for a 40-day long experiment. The results showed that pigs fed the higher CN supplemented diet (CN2) had significantly higher final weight (FW) and weight gain (WG) than those fed the CON diet, and no significant differences were observed in the feed conversion ratio (FCR) and average daily feed intake (ADFI) after 28 days. At the end of the experiment, pigs fed the CN supplemented diet showed no significant difference in WG, ADFI or FCR compared to those on the CON diet. Overall, at the termination of the 40-day feeding trial, dietary CN had a significant effect on FW and WG, except for ADFI and FCR, in finishing pigs. After 40 days of the feeding trial, serum biochemical parameters such as glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, triglycerides, and total cholesterol levels were significantly decreased in pigs fed the CN supplemented diet. However, high density lipoprotein levels were significantly increased in pigs fed the CN diets. Protein and lipid contents, as well as yellowness and lightness of the neck and longissimus dorsi muscles were not significantly affected by CN supplementation; however, there was a tendency to increase the redness of the longissimus dorsi muscle in pigs fed the CN2 supplemented diet compared to the CON diet. Meat grading and carcass weight significantly increased in pigs fed a higher CN supplemented diet. Fecal *Escherichia coli* and ammonia gas

were significantly depleted in pigs fed CN diets. Histomorphological parameters, such as villus height, crypt depth and goblet cells in the jejunum of the intestine were significantly increased in pigs fed CN diet. Immunohistochemical staining showed that pro-inflammatory cytokine like tumor necrosis factor-α expression was reduced in pigs fed CN supplemented diets compared to the CON diet; however, antibodies such as immunoglobulin A and tight junction proteins such as claudin 3 were highly expressed in the intestine of pigs fed the CN diets. Overall, the results demonstrate the potential of dietary curcumin nanospheres as a nanobiotechnology tool as well as an effective feed additive for improving the performance and health status of finishing pigs.

KEYWORDS

curcumin nanospheres, growth, serum biochemistry, immunohistochemistry, malodors, microbes, meat quality, finishing pigs

Introduction

Curcumin is a polyphenolic bioactive compound extracted from the turmeric plant, *Curcuma longa*, which has phytotherapeutic potential in terms of its antimicrobial, antioxidant, anti-inflammatory, and immunostimulatory properties. It can be used as a nutritional supplement or phytobiotic in the diet of animals because of its pharmacological properties, especially as a growth promoter, immunomodulator and gastro-protector (1, 2). The most important functional property of curcumin is its highly safe and non-toxic nature, making it sutable for biomedical applications (3). Interestingly, no studies have reported the toxic effects of curcumin in humans or animals at higher levels of supplementation (3). Despite the non-toxic and beneficial nature of curcumin, the drawbacks of native curcumin is its hydrophobic (insoluble in water) nature and its oral bioavailability in human or animals is very low. This is a major challenge for supplemention of curcumin in the diets as an additive or as a drug from a clinical perspective. It has been reported that bioaccumulation of native curcumin levels in blood and tissues were very poor in hepatic and intestinal metabolism, and dietary curcumin is rapidly eliminated from the body through excreta, which ultimately lowers the bioavailability of native curcumin in organisms (4, 5). In this regard, nanoformulation of native curcumin could be a better option for higher utilization of curcumin in humans or animals in terms of making native curcumin more soluble and bioavailable. Several dosage forms have been proposed for the nanoformulation of curcumin, such as surfactant micelles, microemulsions, nanoemulsions, emulsions, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), biopolymer nanoparticles, and microgels, which show higher water solubility, chemical strength, and bioavailability of curcumin (6). For instance, Tabatabaeain et al. (7) postulated that *Satureja khuzistanica* essential oil loaded SLNs modified with chitosan folate (as NLCs) can be useful to enhance the bioavailability and degradability as well as encapsulation efficiency of the hydrophobic essential oils. Shaikh et al. (3) reported that the bioavailability of nanoencapsulated curcumin on oral administration was 9-times higher compared to native curcumin. In addition, Jaguezeski et al. (8) found that the dietary incorporation of curcumin nanocapsules can enhance milk quality by increasing the antioxidant activity and reducing lipid peroxidation in milk, where curcumin nanocapsules were used at 10-fold lower doses than native curcumin in the diet of dairy sheep. Furthermore, Marchiori et al. (9) postulated that dietary nanoencapsulated curcumin (10 mg/kg of feed) can improve the egg quality of quail using 3 times lower concentration than that of normal curcumin (30 mg/kg of feed) under cold stress conditions.

Curcumin in nanoencapsulated form is effective for growth and immunity enhancement in poultry, livestock and aquatic animals (8–15). However, information on the application of nanocurcumin in animal health is scarce compared to that on human health (4). In our previous studies, we reported the physical and chemical characteristics of curcumin in nanoencapsulated form, hereafter, curcumin nanospheres (CN), as well as findings of *in vitro* and *in vivo* studies using cell lines and murine models, respectively (16, 17). Recently, we discovered the efficacy of dietary CN in weaned piglets, where we demonstrated that dietary supplementation with CN could improve growth performance, feed utilization, and immune functions, as well as deplete the fecal infectious bacteria and ammonia gas discharge in piglets (12). Based on our previous findings, in the present study, we hypothesized that dietary CN would have potential effects on the growth performance, serum biochemistry, meat composition and quality, pro-inflammatory cytokines, gut barrier junction protein, and antibody expression in the jejunum of the intestine, as well as fecal noxious gases and pathogenic bacterial contents in finishing pigs.

Materials and methods

Ethics statement

Experimental protocols describing the management and care of animals were reviewed and approved by the Animal Care and Use Committee of Jeju National University, Republic of Korea (approval no. 2021-0056). Samples from the animals were taken

carefully to minimize suffering and slaughtered humanely at the end of the experiment.

Chemicals and kits

Curcumin powder (99% purity) extracted from the turmeric plant, *Curcuma longa* Linn, and lecithin (L-α-phosphatidylcholine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Toluene and dichloromethane (DCM) as the organic solvents were collected from Sigma-Aldrich (St. Louis, MO, USA) and Acros Organics (Janssen Pharmaceuticals, Geel, Belgium), respectively. The rest of the kits used were: goat anti-porcine immunoglobulin A (IgA) secondary antibody (NB724, Novus Biological, Abingdon, UK), horse anti-goat immunoglobulin G (IgG) antibody (BA-9500, Vector Laboratories, Inc., Burlingame, CA, USA), and bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), the rest were purchased from Abcam (Abcam, Waltham, CA, USA) such as hematoxylin and eosin staining kit (ab245880), 3,3'-diaminobenzidine (DAB) detection immunohistochemistry (IHC) kit (ab64261), goat anti-rabbit IgG (ab6721), recombinant anti-TNF-α (tumor necrosis factor- alpha) antibody (ab270264), claudin 3 (CD3) monoclonal antibody (ab135372), and normal goat serum (ab7481). All other reagents used in the present study were laboratory grade.

Curcumin nanospheres

The production protocol for CN and its physico-chemical characteristics have been previously described by our research group (12, 16, 17). Briefly, 200 mg of commercial curcumin powder were mixed with 40 mL of toluene, and the mixture was poured into 2 L of distilled water. The mixture was then sonicated at 50 kHz for 4 h (Sonictopia, Rep. of Korea) with continuous stirring. After sonication, toluene was fully evaporated from the mixture using a rotary evaporator (Buchi AG, Meierseggstrasse, Flawil, Switzerland) at 40°C. The concentrated liquid without toluene was then freeze dried for 72 h to obtain curcumin powder in nanoform (hereafter, nanocurcumin). An aliquot of 40 mg of nanocurcumin was added to a mixture of 200 mg phosphatidylcholine (lecithin) and 40 mL dichloromethane. In this study, lecithin was used to coat the nanocurcumin in the mixture. The CN solution was produced continuously and maintained at −70°C until use in pig feeds.

Animals and diets

A total of 90 crossbred pigs (Duroc × [Landrace × Yorkshire]) with an average initial body weight of 73.77 ± 0.08 kg (average age 112 days) were reared in a regional commercial pig farm (Bada Pig Farm, Hanlim, Jeju, Korea) for 40 days. The finishing pigs were arbitrarily distributed into nine pens based on average body weight using a randomized block design (RBD) according to three dietary treatments. Each treatment consisted of three replicate pens, each of which contained 10 pigs (30 pigs per treatment). In the present study, we used commercial pig feed for the supplementation of CN in the experimental diets of pigs (Neopigg, Purina Korea). Three diets were designed as control (CON) without supplementing CN, one diet was supplemented with 1 mL CN/kg diet (CN1), and the diet of the remaining group was supplemented with 2 mL CN/kg (CN2). The CN solution was mixed with the commercial mash feed using a stainless steel mixer. The amount of CN supplemented in the diets and the preparation of the diets were based on previous studies (10, 12). The pigs were reared in concrete-floored pens with steel fencing, including the entrance. Each pen was provided with a self-feeding and drinking water system in a controlled environment with proper air supply and temperature (28°C). Pigs were allowed continuous access to feed and drinking water. All the pigs were ear tagged with a number to identify them individually for recording the sampling data.

Growth and feed utilization

Growth and feed utilization in finishing pigs were measured in two stages: (i) Days 1–28 and (ii) Days 29–40. Pig weights and feed consumption data were measured, growth and feed utilization were calculated to obtain the final weight (FW), weight gain (WG), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) after each stage. The overall growth performance and feed utilization were calculated/assessed after the end of the 40-day experiment.

Serum biochemistry

For serological analyses, an aliquot of 5 ml of whole blood sample was obtained from the jugular vein of each pig using a non-heparinized Vacutainer syringe (24 gauge) and stored in a tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Whole blood samples were collected early in the morning at day 28 and day 40 randomly based on the visual average size of the randomly selected pigs (three pigs from each pen) according to the three treatment groups (nine pigs per treatment). The obtained whole blood samples were left at room temperature (RT) for 10 min and centrifuged at 1,500 ×g at 4°C for 20 min. After centrifugation, blood cells were precipitated, and the transparent supernatant of the blood (serum) was obtained using a micropipette and stored in Eppendorf tubes at −20°C for further analyses. Serological parameters such as glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), bilirubin (BIL), triglycerides (TG), alkaline phosphatase (ALP), high-density lipoprotein (HDLP), cholesterol (CHOL), and blood urea were determined using Refloton kits compatible with a serolocial diagnostic machine (Refloton Plus, Hoffmann-La Roche, Rotkreuz, Switzerland).

Meat quality, nutritional composition, and carcass grading

At the termination of the 40-day experiment, finishing and marketable pigs were transported from the commercial pig farm

to a regional government approved pig slaughterhouse (Nonghyup Slaughterhouse, Hanlim, Jeju, Korea). Pigs were slaughtered by the stunning process and bleeding by cutting the head, and visceral parts were collected by evisceration. Intestinal parts (three samples from three pigs per pen of the treatments) were separated for further microbial and histological studies. Carcass weight and backfat thickness of the pigs were determined according to Kim et al. (18). A part of the neck and longissimus dorsi muscle at the 10th rib (∼250 g each) (three samples from three pigs for each pen of the treatments) was collected, and meat color reading was performed at least at three sites of the respective sample based on lightness (L*), redness (a*), and yellowness (b*) by placing the measuring head vertically above the muscle samples using a digital chroma meter (8 mm aperture size, diffuse illumination/0<° viewing angle, and illuminant type C) using the Minolta CR-410 instrument (Minolta CR-410, Konica Minolta Sensing Inc., Osaka, Japan). The neck and longissimus dorsi muscle samples were stored at −20°C for proximate composition analysis. The carcass quality of pork was graded as "Grade 1+," "Grade 1," or "Grade 2," based on marbling, lean color, and conditions of belly streaks of pork (19).

The proximate composition of pork meat in terms of protein, lipid, ash and moisture contents of neck and longissimus muscle samples based on the dietary treatments was analyzed using the conventional methods followed by AOAC (20). Briefly, representative test samples (1 g each) were dried at 135°C for 3 h in a dryer to measure the moisture content of the meat samples. The ash content of the meat samples was obtained by burning the samples at 550°C in a muffle furnace. The Kjeldahl method was used to obtain the nitrogen (N) content in the meat samples (0.1 g), and the crude protein was measured through acid digestion, distillation, and titration of the samples using the formula, N × 6.25. The crude lipid content of the neck and longissimus dorsi meat samples (1 g each) was obtained by the ethyl-ether extraction method using a lipid extraction unit (Soxhlet apparatus 1,046, Tacator AB, Hoganas, Sweden).

Intestinal and fecal microbial contents

From the collected intestinal parts of slaughtered pigs, approximately 5 cm sections were cut from the middle of the small intestine (jejunum), separated and stored in a plastic falcon tube under chilling conditions in an ice box for microbial analysis in the laboratory. For fecal microbial content, fresh fecal samples were obtained from every pen and collected in plastic zipper bags marked with diet numbers, and all zipper bags were stored in an ice box. Microbial analyses of the jejunum and feces were performed immediately after collection from the site. For this, 1 g of feces and 1 g of intestinal pieces (3 pigs from each pen; total 9 pigs for each treatment) were mixed with 9 ml of peptone water (CM0009; Oxoid Ltd, Basingstoke, Hampshire, UK), and homogenized by vortexing, and the supernatant of was collected by pipetting. The decimally (10 fold) diluted fecal and intestinal sample solutions were then poured onto MacConkey (CM0115, No.3; Oxoid Ltd, Basingstoke, Hampshire, UK) serial agar plates and incubated at 37°C for 24 h. The viable number of pathogenic bacteria, *Escherichia coli* in the fecal and intestinal samples were counted and calculated the number of bacterial colonies using a colony counter (Suntex automatic colony counter, Taiwan). Likewise, the *Lactobacillus* spp. in fecal and intestinal samples were identified on de Man-Rogosa-Sharpe (MRS) agar (CM0361; Oxoid Ltd., Basingstoke, Hampshire, UK) after incubation at 37°C for 48 h. Furthermore, the *Salmonella* spp. in fecal and intestinal samples were identified on Salmonella-Shigella (SS) agar (CM0099; Oxoid Ltd., Basingstoke, Hampshire, UK) after incubation at 37°C for 24 h.

Fecal gas contents

Fecal gas analysis was performed based on the method described by Moniruzzaman et al. (12), with slight modifications. Briefly, the fecal samples (250 g each) in plastic zipper bags were kept in airtight conditions at RT (25°C) for 24 h. Then, the bags were connected using a hose pipe to the probes of specific gas sampling kits for the measurement of noxious gases such as ammonia (NH_3) and hydrogen sulfide (H_2S) in the feces of finishing pigs. The NH_3 and H_2S gases in the feces of pigs were measured in parts per million (ppm) using colorimetric analysis kits with a portable Gastec instrument (model GV-100S; Gastec Corp., Tokyo, Japan).

Intestinal histomorphology

Histomorphological studies of the jejunum of finishing pigs fed with or without curcumin nanospheres were conducted using a standard protocol (21). Briefly, small sections (5 cm) of the middle parts of the small intestine (jejunum) from the eviscerated pigs of each treatment group (three pigs per pen) were cleaned with water and fixed in 4% paraformaldehyde for 24 h at 4°C. Gut samples were further cleaned, infiltrated, and dehydrated with different alcohols at different concentrations. The tissue sections along with the paraffin cubes were sliced (4 μm thickness) using a sharp cutter adjusted with a histological microtome machine (HistoCore, Leica Biosystems, Buffalo Grove, IL, USA), and hematoxylin and eosin (H&E) was used for staining (H&E, ab245880, Abcam, Cambridge, UK) of the tissue sections. The tissue sections were mounted with Canada balsam (mountant) and closely monitored using an Olympus light microscope (AX70 Olympus, Tokyo, Japan) fitted with a digital camera (DIXI Optics, Daejeon, Republic of Korea) to capture the tissue images. These images were then examined using the Image J operating system (Image J 1.32j, National Institute of Health, Bathesda, MD, USA). To measure the measurement of the villus height, crypt depth, villus height/crypt depth, intestinal muscular thickness and goblet cell number per villus, at least six images were obtained from each slide in pooled form with triplicate slides for each treatment.

Intestinal immunohistochemistry

Tissue sections from the jejunum were deparaffinized with xylene and graded alcohols. The tissue sections were then treated with 3% H_2O_2 in methanol and 0.3% Triton X-100 was added

for tissue permeabilization for 30 min. Tissue sections were further blocked in 20% normal goat serum (ab7481, Abcam, Cambridge, UK), and antigen retrieval was performed using trypsin antigen retrieval solution (ab970, Abcam, Cambridge, UK) at 37°C for 30 min. The tissue sections were incubated with antibodies, such as goat polyclonal anti-porcine IgA (NB724, Novus Biologicals, Centennial, CO, USA), recombinant anti-TNF alpha antibody (ab270264, Abcam, Cambridge, UK), and claudin 3 (CD3) monoclonal antibody (ab135372, Abcam, Cambridge, UK) overnight at 4°C according to the manufacturer's protocol. The tissue sections were washed with PBS buffer four times and incubated with secondary antibody, biotinylated goat anti rabbit IgG (H+L) (ab6721, Abcam, Cambridge, UK) for 10 min at RT. The sections were washed four times in PBS, and streptavidin peroxidase was applied and incubated for 10 min at RT. Finally, 1 ml of 3,3'-diaminobenzidine (DAB) substrate was added to 20 µl DAB chromogen (ab64261, Abcam, Cambridge, UK), mixed by swirling, and applied to the tissue sections. The tissue sections were further incubated for 10 min and counterstained with hematoxylin. The expression of IgA, TNF-α, and CD3 was quantified using the Image J program (Image J 1.32j, National Institute of Health, Bathesda, MD, USA). To obtain data on the expression of TNF-α, IgA, and CD3 in the jejunum of finishing pigs, at least six images from each histological slide were pooled for statistical analysis in triplicate.

Statistical analysis

For statistical analysis, the pig pen mean values were expressed in triplicates. Normality and homogeneity of variance were assessed for arcsine-transformed percentage data using the Shapiro–Wilk and O'Brien tests, respectively. The data were initially analyzed using two-way analysis of variance (ANOVA) to check the interaction effect between the treatments and replication pens. As no significant interaction effects were found among the treatments and replication pens, we conducted a one-way ANOVA to check the dietary effects of CN supplemented or control diets in finishing pigs. Tukey's honestly significant difference (HSD) *post-hoc* test was used to check the significance of the treatment means. The effects of treatment means were analyzed based on a significance level of $P < 0.05$. All statistical analyses were performed using SAS version 9.1 operating system (SAS Institute, Cary, NC, USA).

Results

Dietary effects of CN on performance and feed usage in finishing pigs

The effects of CN on the growth performance and feed utilization of finishing pigs are shown in Table 1. The growth performance data showed that pigs fed the CN2 diet had significantly greater FW, WG and ADG than those fed the CON diet at the end of the 28-day rearing period. However, there were no significant differences in FW, WG, and ADG of pigs fed the CON and CN1 diets or the CN1 and CN2 diet groups. At the end of remaining 10 days (from 29 to 40 days), the results showed that dietary CN had no significant effects on WG, ADG, ADFI, and FCR in finishing pigs. Collectively, at the end of the 40 days of feeding trial in finishing pigs, we found significant effects of dietary CN on FW, WG, and ADG in pigs; however, no significant effect was observed in ADFI and FCR of pigs fed CN supplemented diets compared to the CON diet.

Dietary effects of CN on serological indices in finishing pigs

Serum biochemical analyses of pigs fed the experimental diets are presented in Table 2. The results showed that pigs fed the CN supplemented diets had significantly higher HDLP levels than those fed the CON diet; however, other parameters such as GPT, GOT, BIL, TG, ALP, CHOL, and urea levels were unchanged at the end of 28 days of pigs. Furthermore, at the end of the 40 days, the serological data demonstrated that pigs fed the CN2 supplemented diets had significantly decreased levels of GPT, GOT, and CHOL compared to the CON diet. The TG level in pigs fed the CN1 and CN2 supplemented diets was significantly lower than the CON diet group. However, HDLP levels were significantly decreased in pigs fed CN supplemented diets, but BIL, ALP, and urea levels were unaltered in pigs fed experimental diets.

Dietary effects of CN on nutritional composition and meat color in finishing pigs

The neck and longissimus dorsi muscle chemical compositions in terms of protein, lipid, and ash contents were not significantly affected by supplementation of CN in the pig diets compared to the CON diet (Tables 3, 4). However, moisture content of neck muscle was significantly lower and it was significantly higher in case of longissimus dorsi muscle in pigs fed the CN1 diet compared to the CON group of pigs. Meat characteristics of the neck muscle in terms of lightness, redness, and yellowness as well as lightness and yellowness in the longissimus muscle were not significantly affected by CN supplementation compared to the CON diet group; however, there was a tendency of increasing the redness in the longissimus dorsi muscle of pigs on higher CN supplementation (2 ml/kg) than that in the CON diet group (Tables 3, 4).

Dietary effects of CN on meat carcass quality and grading of finishing pigs

The effects of CN on meat carcass quality and grading during finishing are presented in Table 5. The carcass weight and backfat thickness of pigs were significantly increased in pigs fed the high supplementation of CN diet; however, no significant differences in carcass weight and backfat thickness of pigs were observed between the low CN supplementation and CON diet groups. Furthermore, pigs fed the CN supplemented diets showed a gradual increase in the percentage of higher class meat grades (1+) as well as a

TABLE 1 Dietary curcumin nanospheres (CN) on performance and feed utilization in finishing pigs for 40 days[1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Days 1–28				
FW (kg)[2]	92.6 ± 0.2[b]	94.3 ± 0.7[a,b]	95.5 ± 1.6[a]	0.0345
WG (kg)[3]	18.8 ± 0.2[b]	20.5 ± 0.7[a,b]	21.7 ± 0.7[a]	0.0345
ADG (kg/d)[4]	0.67 ± 0.01[b]	0.73 ± 0.03[a,b]	0.78 ± 0.06[a]	0.0354
ADFI (kg/d/pig)[5]	1.9 ± 0.4[a]	1.9 ± 0.2[a]	1.7 ± 0.2[a]	0.5414
FCR[6]	2.8 ± 0.7[a]	2.6 ± 0.3[a]	2.1 ± 0.2[a]	0.2268
Days 29–40				
WG (kg/pig)	10.0 ± 0.9[a]	14.8 ± 3.5[a]	15.1 ± 1.4[a]	0.0536
ADG (kg/pig)	0.83 ± 0.1[a]	1.24 ± 0.3[a]	1.26 ± 0.1[a]	0.0554
ADFI (kg/d/pig)	2.0 ± 0.3[a]	2.2 ± 0.1[a]	2.1 ± 0.3[a]	0.5808
FCR	2.4 ± 0.4[a]	1.8± 0.4[a]	1.7± 0.2[a]	0.0886
Days 1–40				
FW (kg/pig)	102.6 ± 0.9[b]	109.1 ± 3.5[a]	110.6 ± 1.4[a]	0.0101
WG (kg/pig)	29.2 ± 0.2[b]	34.5 ± 4.7[a]	36.9 ± 1.9[a]	0.0106
ADG (kg/pig)	0.72±0.02[b]	0.88 ± 0.09[a]	0.92 ± 0.03[a]	0.0106
ADFI (kg/d/pig)	2.7 ± 0.6[a]	2.8 ± 0.3[a]	2.5 ± 0.4[a]	0.7237
FCR	2.7 ± 0.6[a]	2.2 ± 0.1[a]	1.9 ± 0.2[a]	0.1146

[1]Values are mean ± SD from three replicate groups of pigs ($n = 3$), where mean ± SD in each row with different superscripts (a,b) are statistically significant at the 5% level of significance ($P < 0.05$).
[2]Final weight; [3]Weight gain = (final weight -initial weight)/initial weight; [4]Average daily gain; [5]Average daily feed intake; [6]Feed conversion ratio = feed intake/weight gain.

gradual decline in the percentage of relatively lower class meat grades compared to the CON diets.

Dietary effects of CN on fecal and intestinal bacterial contents in finishing pigs

The intestinal (jejunum) bacteria, *Lactobacillus* spp., *Escherichia coli* and *Salmonella* spp. in finishing pigs were not significantly affected by CN supplemented diets compared to the CON diet group (Table 6). The results showed that pigs fed the CN1 and CN2 supplemented diets had significantly lower fecal *Escherichia coli* contents than in those of pigs fed the CON diet. Moreover, pigs fed the CN2 diets had significantly lower fecal *E. coli* content than in the CN1 diet group. On the other hand, fecal *Lactobacillus* spp. and *Salmonella* spp. levels were unaltered in pigs fed the experimental diets (Table 6).

Dietary effects of CN on fecal noxious gas emissions in finishing pigs

Emission of fecal noxious gases such as ammonia was significantly reduced by dietary administration of CN in pigs compared to the CON diet; however, emission of fecal hydrogen sulfide gas was not significantly different among the pigs fed the experimental diets (Table 7).

Dietary effects of CN on intestinal histomorphology of finishing pigs

Histological sections from the jejunal intestine of finishing pigs fed the control and CN supplemented diets are shown in Figure 1. The results showed that villus height (VH) and crypt depth (CD) were significantly increased in pigs fed the CN supplemented diets compared to those fed the CON diet; however, there were no significant differences in the VH and CD of pigs fed the CN1 and CN2 diets (Table 8). Moreover, there were no significant differences in the VH/CD ratio or muscular thickness in pigs fed the experimental diets. The number of goblet cells was significantly higher in pigs fed the CN1 and CN2 diets than in the CON diet group.

Dietary effects of CN on intestinal immunohistochemistry of finishing pigs

Immunohistochemistry of jejunum sections of the intestine of finishing pigs demonstrated that the expression of TNF-α was reduced in the CN supplemented diet groups compared to the CON diet group (Figure 2). On the other hand, IgA and CD3 were found to be highly expressed in the intestine of pigs fed the CN1 and CN2 diets compared to the CON diet (Figures 3, 4) at different magnifications on the immunohistochemical slides.

TABLE 2 Dietary curcumin nanospheres (CN) on serum biochemical parameters in finishing pigs for 40 days [1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Day 28				
GPT[2]	21.8 ± 2.6[a]	19.1 ± 4.9[a]	18.9 ± 0.8[a]	0.5203
GOT[3]	16.1 ± 2.9[a]	14.1 ± 2.2[a]	11.6 ± 1.4[a]	0.1202
BIL[4]	0.5 ± 0.1[a]	0.5 ± 0.1[a]	0.6 ± 0.2[a]	0.0000
TG[5]	73.7 ± 3.3[a]	71.9 ± 3.2[a]	71.2 ± 2.1[a]	0.5858
ALP[6]	20 ± 1.0[a]	21 ± 1.0[a]	20 ± 1.0[a]	0.0000
HDLP[7]	32.8 ± 2.1[b]	40.9 ± 2.3[a]	40.0 ± 2.9[a]	0.0120
CHOL[8]	109.0 ± 2.0[a]	110.7 ± 2.5[a]	109.7 ± 2.9[a]	0.7251
Urea	27.0 ± 2.3[a]	27.4 ± 1.6[a]	27.9 ± 2.7[a]	0.8864
Day 40				
GPT	28.2 ± 2.7[a]	23.4 ± 0.7[a]	15.3 ± 2.1[b]	0.0006
GOT	19.7 ± 3.5[a]	16.4 ± 3.9[a,b]	10.6 ± 2.9[b]	0.0444
BIL	0.7 ± 0.1[a]	0.6 ± 0.1[a]	0.7 ± 0.4[a]	0.8966
TG	85.9 ± 1.3[a]	72.9 ± 3.4[b]	71.9 ± 2.5[b]	0.0009
ALP	20 ± 1.0[a]	21 ± 1.0[a]	20 ± 1.0[a]	0.0000
HDLP	36.1 ± 2.9[b]	42.7 ± 1.9[a]	46.1 ± 2.6[a]	0.0078
CHOL	108.7 ± 1.2[a]	111.7 ± 1.5[a]	102.7 ± 1.5[b]	0.0007
Urea	35.6 ± 2.0[a]	34.4 ± 1.8[a]	35.9 ± 1.5[a]	0.5908

[1]Values are mean ± SD from three replicate groups of pigs ($n = 3$), where mean ± SD in each row with different superscripts (a,b) are statistically significant at the 5% level of significance ($P < 0.05$).
[2]GPT, glutamic pyruvic transaminase (mg/dL); [3]GOT, glutamic oxaloacetic transaminase (mg/dL); [4]BIL, total bilirubin (mg/dL); [5]TG, triglycerides (mg/dL); [6]ALP, alkaline phosphatase (mg/dL); [7]HDLP, high density lipoprotein (mg/dL); [8]CHOL, cholesterol (mg/dL).

TABLE 3 Dietary curcumin nanospheres (CN) on meat quality of neck muscle in finishing pigs for 40 days[1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Proximate composition				
Moisture (%)	53.2 ± 0.2[a]	51.2 ± 0.1[b]	52.3 ± 0.9[ab]	0.0161
Protein (%)	14.8 ± 2.1[a]	15.2 ± 4.5[a]	15.4 ± 1.5[a]	0.9718
Lipid (%)	4.8 ± 0.3[a]	5.1 ± 0.2[a]	5.0 ± 0.2[a]	0.3877
Ash (%)	0.7 ± 0.1[a]	0.7 ± 0.2[a]	0.7 ± 0.1[a]	0.7461
Meat color				
L∗ (lightness)	41.1 ± 2.5[a]	46.4 ± 3.3[a]	41.3 ± 2.3[a]	0.0899
a∗ (redness)	16.9 ± 2.3[a]	19.4 ± 2.6[a]	19.7 ± 1.1[a]	0.2906
b∗ (yellowness)	11.4 ± 1.2[a]	11.5 ± 1.4[a]	12.1 ± 1.7[a]	0.0861

[1]Values are mean ± SD from three replicate groups of pigs ($n = 3$), where mean ± SD in each row with different superscripts (a,b) are statistically significant at the 5% level of significance ($P < 0.05$).

Discussion

Phytogenic compounds as natural feed additives have been extensively used as growth promoter and immune booster in the feedstuffs of monogastric farm animals such as pigs, poultry and fish (4). Recently, researchers have emphasized the use of phytogenic compounds in the form of nanocapsules because most phytogenic materials are poorly bioavailable in biological systems. For this, incorporation of phytogenic compounds in nano encapsulation form with liposomal nano carrier showed most effective drug delivery systems in murine as a monogastric animal model for biomedical applications (22–25). Furthermore, it has been reported that liposomal nano carrier with its spherical form can easily entrap the lipophilic and hydrophobic curcumin,

TABLE 4 Dietary curcumin nanospheres (CN) on meat quality of longissimus dorsi muscle in finishing pigs for 40 days[1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Proximate composition				
Moisture (%)	50.9 ± 0.6[b]	53.4 ± 1.1[a]	52.5 ± 1.2[ab]	0.0523
Protein (%)	18.2 ± 2.9[a]	15.6 ± 3.4[a]	15.0 ± 1.4[a]	0.3764
Lipid (%)	5.3 ± 0.2[a]	4.9 ± 0.2[a]	5.1 ± 0.3[a]	0.1810
Ash (%)	0.8 ± 0.1[a]	0.7 ± 0.1[a]	0.6 ± 0.1[a]	0.1842
Meat color				
L∗ (lightness)	49.9 ± 4.8[a]	52.7 ± 4.4[a]	50.1 ± 4.3[a]	0.7223
a∗ (redness)	6.2 ± 1.3[b]	8.4 ± 0.8[a,b]	11.5 ± 3.2[a]	0.0550
b∗ (yellowness)	6.4 ± 0.7[a]	8.4 ± 0.8[a]	8.3 ± 4.1[a]	0.0752

[1]Values are mean ± SD from three replicate groups of pigs ($n = 3$), where mean ± SD in each row with different superscripts (a,b) are statistically significant at the 5% level of significance ($P < 0.05$).

TABLE 5 Dietary curcumin nanospheres (CN) on carcass quality and grading in finishing pigs for 40 days[1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Carcass weight (kg)	81.00 ± 1.25[b]	81.03 ± 1.63[b]	82.23 ± 1.40[a]	0.001
Backfat thickness (mm)	20.80 ± 1.14[b]	20.80 ± 0.35[b]	21.33 ± 0.85[a]	0.001
Grading				
1+ (%)	23.33	33.33	36.67	
1 (%)	36.67	40.00	43.33	
2 (%)	40.00	26.67	20.00	

[1]Values are mean ± SD from three replicate groups of pigs ($n = 3$), where mean ± SD in each row with different superscripts (a,b) are statistically significant at the 5% level of significance ($P < 0.05$).

TABLE 6 Dietary curcumin nanospheres (CN) on intestinal and fecal bacteria counts in finishing pigs for 40 days[1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Microbes in jejunum (CFU/g)				
Lactobacillus spp.	9.78 ± 0.51[a]	9.89 ± 0.70[a]	9.67±0.34[a]	0.880
Escherichia coli	3.67 ± 0.34[a]	3.89 ± 0.51[a]	3.89 ± 0.51[a]	0.801
Salmonella spp.	4.67± 0.58[a]	4.55± 0.69[a]	4.78± 0.19[a]	0.875
Microbes in feces (CFU/g)				
Lactobacillus spp.	6.89± 0.96[a]	6.11± 0.19[a]	6.33± 0.34[a]	0.332
Escherichia coli	7.45± 0.39[a]	5.55± 0.39[b]	4.44± 0.51[c]	0.001
Salmonella spp.	3.00± 0.33[a]	3.33± 0.34[a]	3.22± 0.19[a]	0.421

[1]Values are mean ± SD from three replicate groups of pigs ($n = 3$), where mean ± SD in each row with different superscripts (a,b,c) are statistically significant at the 5% level of significance ($P < 0.05$). CFU, colony forming unit.

and protect itself from external stress mediated by oxygen, pH and enzymatic degradation during digestion and absorption in gastrointestinal tract (26). Therefore, encapsulation of the phytogenic compounds in nano structure lead to the enahnced intraceullular uptake and effective delivery in the target organs through modifying the surface of nano carrier and increase the sensitivity of drug to the target areas (7). In line with previous studies on the utilization of nanotechnology in monogastric animals, the results of the current study showed the positive outcome of dietary curcumin nanospheres in terms of enhanced growth and feed utilization, improved serological indices, meat quality, and intestinal health status, as well as reduced pathogenic bacterial content and noxious gas in the feces of finishing pigs. Interestingly, the feeds administered with CN were well accepted by

TABLE 7 Dietary curcumin nanospheres (CN) on ammonia (NH_3) and hydrogen sulfide (H_2S) gas contents in the feces of finishing pigs for 40 days[1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Ammonia (ppm)	3.33 ± 1.04^{a}	1.17 ± 0.76^{b}	1.17 ± 0.29^{b}	0.0200
Hydrogen sulfide (ppm)	0.17 ± 0.06^{a}	0.13 ± 0.06^{a}	0.17 ± 0.06^{a}	0.7290

[1]Values are mean ± SD from three replicate groups of pigs (n=3), where mean ± SD in each row with different superscripts (a,b) are statistically significant at the 5% level of significance ($P < 0.05$).

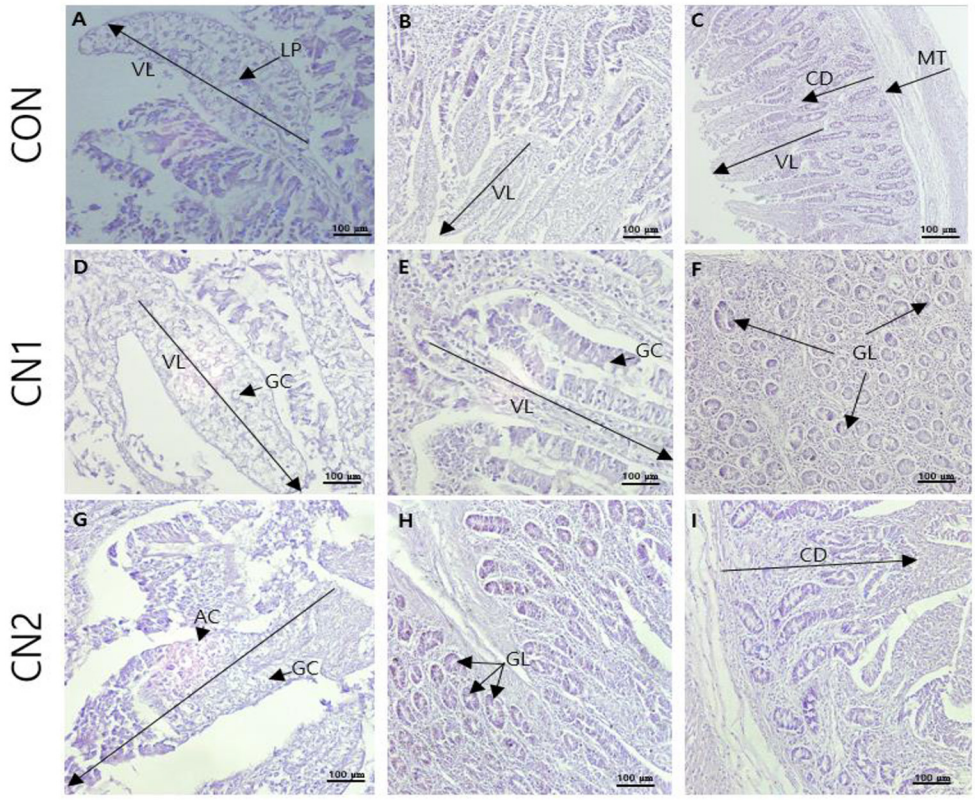

FIGURE 1
The representative photomicrographs from the jejunal sections of small intestine ($n = 6$) in finishing pigs fed **(A–C)** the control (CON) diet wihout curcumin nanospheres (CN); **(D–F)** the control diet supplemented with CN at 1.0 ml/kg diet (CN1); **(G–I)** the control diet supplemented with CN at 2.0 ml/kg diet (CN1); VL, villus length; LP, lamina propria; AC, absorptive cells; GC, goblet cells; CD, crypt depth; GL, mucosal glands; Hematoxylin and eosin **(H, E)** is used for tissue sections staining for viewing using bars 100 µm at different magnifications.

TABLE 8 Dietary curcumin nanospheres (CN) on jejunal histomorphology in finishing pigs for 40 days[1].

Items	Dietary treatments			*P*-value
	CON (no CN)	CN1 (1.0 ml/kg)	CN2 (2.0 ml/kg)	
Villus height, VH (µm)	686.22 ± 3.67^{b}	701.67 ± 2.65^{a}	707.89 ± 4.67^{a}	0.001
Crypt depth, CD (µm)	352.22 ± 3.56^{b}	361.44 ± 1.17^{a}	363.67 ± 2.91^{a}	0.005
VH/CD	1.95 ± 0.03^{a}	1.95 ± 0.03^{a}	1.95 ± 0.03^{a}	0.923
Muscular thickness (µm)	12.34 ± 1.15^{a}	14.00 ± 1.00^{a}	12.67 ± 2.34^{a}	0.456
Goblet cells per villus	413.55 ± 1.35^{c}	430.11 ± 1.65^{b}	434.22 ± 1.17^{a}	0.001

[1]Values are mean ± SD from three replicate groups of pigs ($n = 3$), where mean ± SD in each row with different superscripts (a,b,c) are statistically significant at the 5% level of significance ($P < 0.05$).

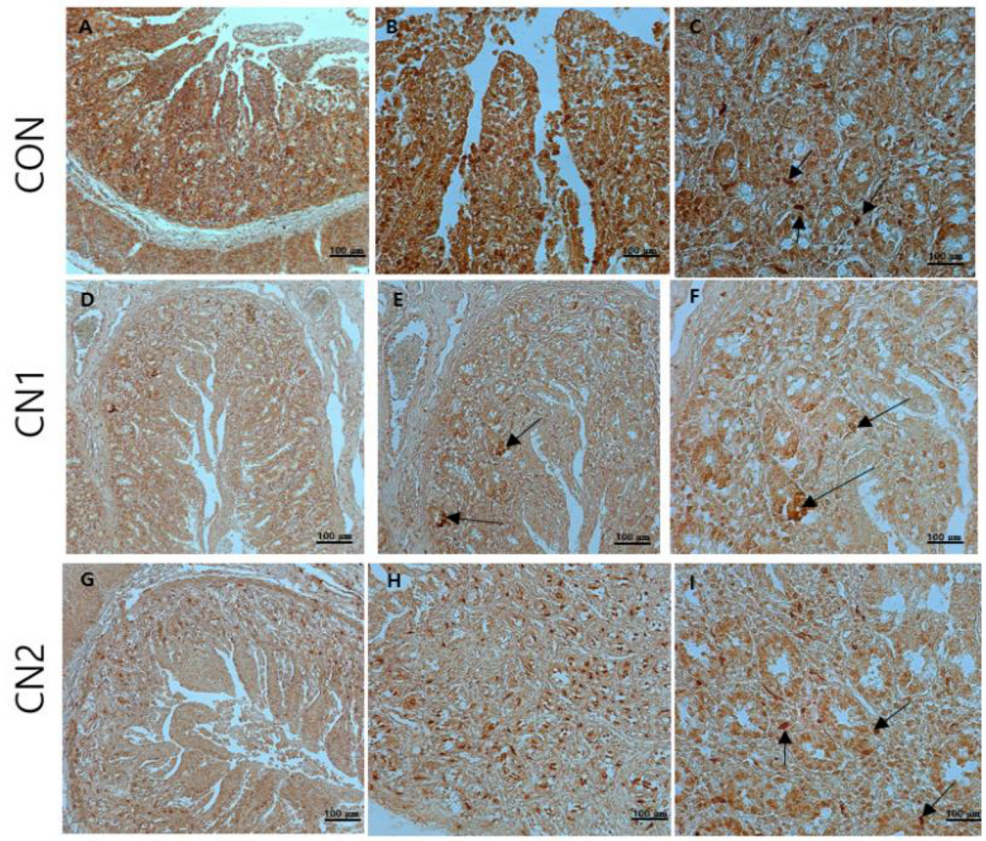

FIGURE 2
The immunohistochemical slides for the expressions of tumor necrosis factor-alpha (TNF-α) collected from the jejunal sections of small intestine (*n* = 6) in finishing pigs fed **(A–C)** the control (CON) diet wihout curcumin nanospheres (CN); **(D–F)** the control diet supplemented with CN at 1.0 ml/kg diet (CN1); **(G–I)** the control diet supplemented with CN at 2.0 ml/kg diet (CN1); The arrow heads represents the intensity of expressions of TNF-α cytokine. The 3,3′-diaminobenzidine (DAB) is used as the substrate for tissue sections staining for viewing using bars 100 μm at different magnifications.

the pigs, and no mortality or moribund pigs were observed during the experimental period.

In this study, pigs fed CN supplemented diets showed remarkably higher growth in terms of FW, WG, and ADG at the end of the 40 day long rearing period. Interestingly, after 28 days, we found significantly higher growth performance in pigs fed diets with higher CN content (CN2). However, from 29 to 40 days, no significant differences were found in the growth of pigs fed CN supplemented or control diets. The results of this study indicate that early stage pigs are more capable of utilizing CN for growth than late stage pigs. In our previous study, we demonstrated the potential of low doses of CN in a weaned piglet model (12). In agreement with the present study, Marcon et al. (10) reported that dietary ethyl polymethacrylate nanocapsules loaded with curcumin (N-CU) at 1.89 mg/kg diet could facilitate weight gain in Lacaune lambs. However, researchers also found that higher inclusion of nanoencapsulated curcumin (4 mg/kg diet) had no effect on growth and health improvement in lambs (10). In addition, Ashry et al. (27) observed that a 20–30 mg/kg diet of curcumin could improve the health of Gilthead seabream fish as a monogastric animal model, which indicates that curcumin administered at more than 10 times would be equally effective as dietary nanocurcumin in animal feeds. This is in agreement with Shaikh et al. (3) who compared the efficacy of native curcumin and nanocurcumin in an animal model. Furthermore, Marchiori et al. (9) reported that dietary nanocurcumin administered at a dosage three times lower than that of free curcumin enhanced the growth performance and egg quality in quails. However, Rahmani et al. (28) revealed that curcumin and nanocurcumin had equal effects at the same concentrations in terms of growth enhancement and heat resistance in broiler chickens. In addition, Bao et al. (29) did not find any significant effect on the dietary supplementation of nanocurcumin on overall performance and feed utilization in juvenile largemouth bass fish. In agreement with the present study, Taghavinia et al. (25) found nanoliposome-loaded phenolics from *Nasturtium officinale* improved the average daily weight gain in mice. In relation to the curcumin nanobiotechnology in the present study, other researchers have proposed that nanoformulation of bioactive compounds such as microencapsulated organic acids, zinc oxide nanoparticles and coated sodium butyrate can be beneficial for enhancing growth and immunity in pigs (30–32). Regarding feed utilization, the results of the present study showed no significant effects of dietary CN on ADFI and FCR in finishing pigs which is in agreement with Marcon et al. (10). In contrast,

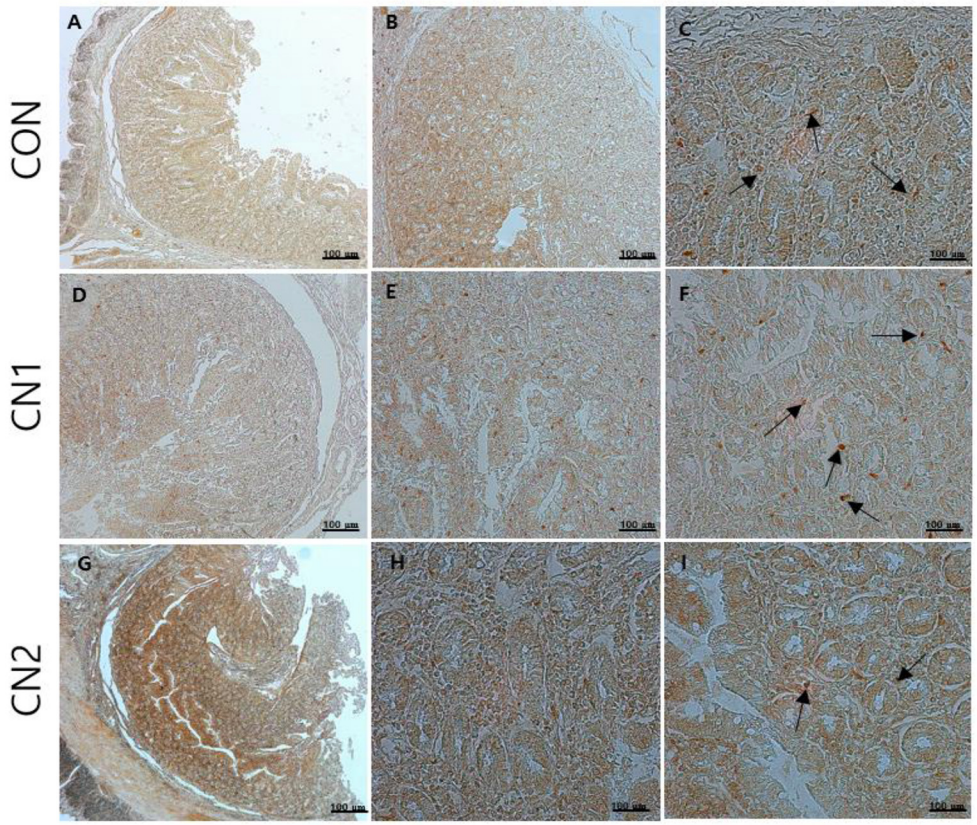

FIGURE 3
The immunohistochemical slides for the expressions of immunoglobulin A (IgA) collected from the jejunal sections of small intestine ($n = 6$) in finishing pigs fed **(A–C)** the control (CON) diet wihout curcumin nanospheres (CN); **(D–F)** the control diet supplemented with CN at 1.0 ml/kg diet (CN1); **(G–I)** the control diet supplemented with CN at 2.0 ml/kg diet (CN1); The arrow heads represents the intensity of expressions of IgA antibody. The 3,3′-diaminobenzidine (DAB) is used as the substrate for tissue sections staining for viewing using bars 100 μm at different magnifications.

in our previous study, we found positive effects of dietary CN on feed efficiency and FCR in weaned piglets, which was attributed to the fact that young pigs were more efficient in feed utilization than finishing pigs.

Serological information of blood is an important tool for ascertaining the health status of animals. In this study, at the end of 28 days, serological parameters, such as GPT, GOT, BIL, TG, ALP, CHOL, and urea were unaffected by dietary CN1 or CN2 supplementation in finishing pigs. However, GPT, GOT, TG and CHOL levels decreased significantly at the end of the finishing stage in pigs fed CN incorporated diets, which might be attributed to potential effects of dietary CN in terms of improving health condition of pigs. In accordance with the present study, Marcon et al. (10) corroborated that dietary curcumin nanocapsules can decrease the blood TG levels in lambs. Moreover, Reda et al. (15) postulated that dietary nanocurcumin can increase serum HDLP levels and decrease TG, CHOL, GPT (or ALT, alanine aminotransferase), and GOT (or AST, aspartate aminotransferase), and did not affect urea levels in Japanese quails which supports the data of the current study. Likewise, it is reported that phytogenic phenolic compounds loaded with nanoliposome can reduce blood ALT, AST and ALP levels on induced cadmium toxicity in mice (24).

In this study, we evaluated how dietary CN supplementation impact on the quality of meat in the neck and longissimus dorsi muscle of finishing pigs. The results showed that proximate compositions in terms of protein, lipid, and ash content of neck and longissimus dorsi muscles were not significantly affected by dietary CN. In addition, the meat color of the neck and longissimus dorsi muscles did not significantly change based on the lightness and yellowness of the meat samples. Interestingly, the redness of the longissimus dorsi muscle was highly increased upon increasing the dose of dietary CN in finishing pigs, which is in agreement with the data reported on the effects of dietary curcumin on the longissimus dorsi muscle of finishing pigs, as well as the breast muscles of broiler and duck meats (33–35). The carcass weight and backfat thickness of pigs fed the higher CN supplemented (2.0 ml/kg diet) diet were found to be higher than those of the control and low concentration CN groups, which endorsed the beneficial effects of CN supplementation on weight gain after slaughtering the pigs. Similarly, Reda et al. (15) observed an improvement in carcass traits upon supplementation with dietary curcumin nanoparticles in Japanese quails. In addition, in the present study, the grading percentage (1+) of pork meat also increased with dietary supplementation of CN, which was attributed to the upgradation of meat quality in pigs fed CN incorporated diets. The

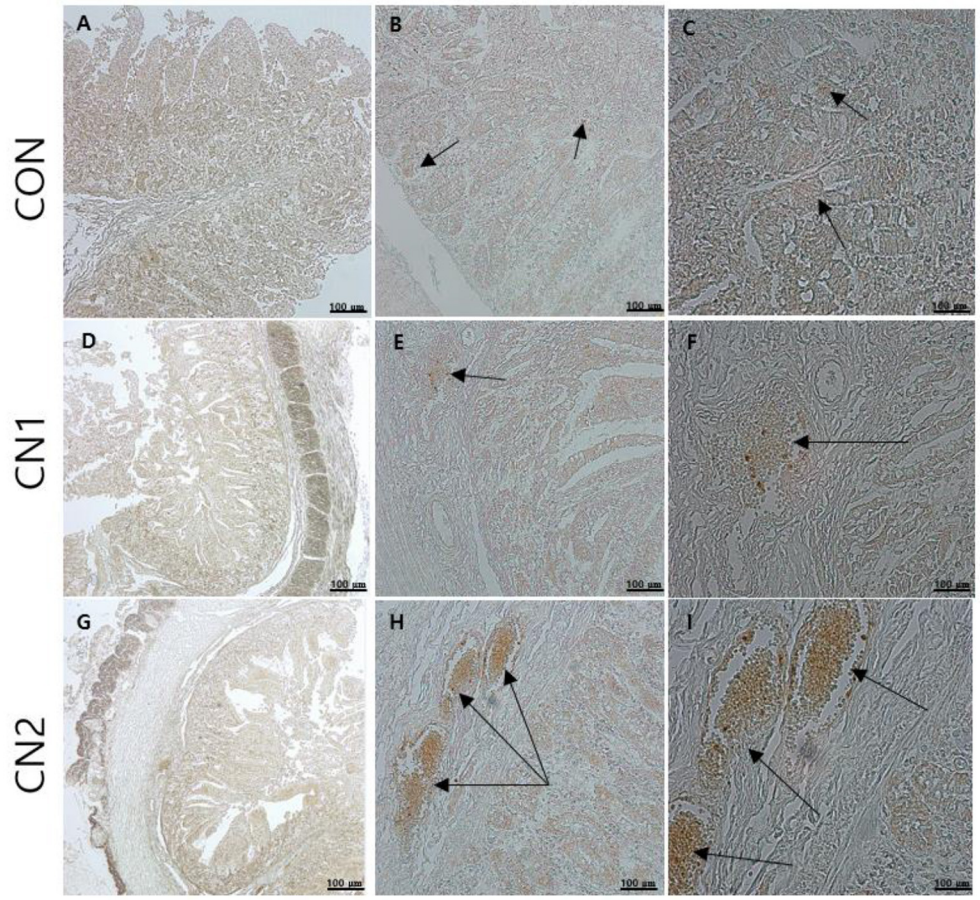

FIGURE 4
The immunohistochemical slides for the expressions of claudin 3 (CD3) collected from the jejunal sections of small intestine ($n = 6$) in growing to finishing pigs fed **(A–C)** the control (CON) diet wihout curcumin nanospheres (CN); **(D–F)** the control diet supplemented with CN at 1.0 ml/kg diet (CN1); **(G–I)** the control diet supplemented with CN at 2.0 ml/kg diet (CN1); The arrow heads represents the intensity of expressions of CD3 as the tight junction protein. Tissue sections were stained with 3,3′-diaminobenzidine (DAB) viewing using bars 100 µm at different magnifications.

findings of the current study regarding the meat quality of pork are consistent with those of Zhang et al. (33) and Jin et al. (35) for broiler and duck meat, respectively, which were supplemented with dietary curcumin.

In this study, we reported the effects of dietary supplementation with CN on the pathogenic and beneficial bacterial contents in fecal and intestinal samples collected from finishing pigs at the end of the experimental period. The results revealed that dietary CN had no effects on pathogenic bacteria, *Escherichia coli* and *Salmonella* spp., or the beneficial bacterium, *Lactobacillus* spp. in the jejunal samples after slaughtering the pigs. However, we observed that pathogenic *E. coli* bacterial content in feces was drastically reduced in pigs fed CN supplemented diets compared to those fed the control diet. These results are in accordance with those of Muniyappan et al. (31), who postulated that dietary microencapsulated organic acids can reduce the *E. coli* bacterial content in the feces of growing to finishing pigs. Furthermore, Reda et al. (15) confirmed that dietary nanocurcumin significantly depleted *E. coli* and *Salmonella* spp. and augmented lactic acid bacterial counts in the caeca of growing Japanese quails. In addition, Sampath et al. (36) reported that dietary black piper plant extract can linearly increase *Lactobacillus* and decrease *E. coli* bacterial counts in the fecal samples of finishing pigs. Lesschen et al. (37) reported that NH_3 and H_2S are important noxious air pollutants emitted from livestock farms. In the present study, we found that fecal gas content, such as ammonia, was significantly reduced in finishing pigs fed CN compared to the control diet. Likewise, Moniruzzaman et al. (12) found that dietary CN reduced fecal ammonia gas content in weaned piglets. In accordance with the present study, it has been reported that dietary plant extracts can reduce ammonia gas emissions from the feces of finishing pigs (38, 39). In contrast, Muniyappan et al. (31) observed an insignificant effect of microencapsulated organic acids on the ammonia gas content in the feces of growing to finishing pigs.

Gut morphology can serve as an important tool to evaluate the absorption and utilization of a feed additive in the intestine, which ultimately affect the growth and health status of animals. Abnormalities or changes in gastrointestinal tract (GIT) especially in small intestine as the major site for nutrient absorption may influence the overall growth of the animals. In the current study, pigs fed the CN supplemented diets showed remarkably enhanced villus length, crypt depth, and goblet cell number

in the jejunal part of intestine of finishing pigs, which was attributed to the higher surface area of the intestine for absorption of curcumin. These results are in accordance with those of Upadhaya et al. (32) and Lei and Kim (40), who reported that dietary coated sodium butyrate and coated zinc oxide could increase villus length and crypt depth in pigs, respectively. Likewise, Xun et al. (41) observed that dietary curcumin could enhance villus height, crypt depth, and goblet cell numbers in weaned piglets. In agreement of our study, Taghavinia et al. (21) and Beyrami et al. (24) found that plant derived phenolic compounds with liposomal nano carrier can improve the intestinal health on induced colorectal cancer or metal toxicity in murine model, respectively.

Immunohistochemistry (IHC) is the most frequently used method for immunostaining selective antigen proteins by binding with specific antibody proteins in animal tissues (42). Chromogenic IHC is a widely used method in which an antibody is conjugated with a peroxidase enzyme that executes a color producing reaction (42). The GIT of animals is composed of the outermost cellular barrier and the innermost immune functional barrier systems. For the intestinal epithelial cell barrier functions, tight junction proteins such as claudins, occludin and zona occludin-1 are key proteins to create a physiological and immunological barrier in the intestine. However, disruption or reduction of tight junction protein concentrations may cause a leaky gut, which may ultimately affect intestinal permeability in terms of digestion and absorption of nutrients (43). Gut associated lymphoid tissue (GALT) has the potential to mediate innate and adaptive mucosal immune responses. Tumor necrosis factor-α (TNFα) has an important intermediary role in GALT growth and antimicrobial defense mechanisms, and it functions as a pro-inflammatory regulator (44). Immunoglobulin A (IgA) is a major antibody class that plays a principal role in capturing pathogenic microorganisms and helps to maintain intestinal homeostasis as the primary defense system (41). In the present study, the chromogenic expression of TNFα was reduced in the jejunal intestine of pigs fed CN supplemented diets in relation to the control diet. On the other hand, the expression of IgA and CD3 proteins was increased in the jejunal intestine of pigs fed CN diets, which was attributed to the enhancement of gut immunity and intestinal permeability of curcumin in finishing pigs. In line with the present study, Xun et al. (41) found that dietary supplementation of curcumin at 300 mg/kg or 400 mg/kg diet could improve the intestinal barrier integrity and immune status in terms of increasing the expression of sIgA (secretory IgA) and interleukin 10 (IL-10) as well as decreasing the mRNA expression of TNFα and Toll-like receptor 4 (TLR4) in jejunal mucosa of weaned piglets. Likewise, it has been reported that quercetin at 25 mg/kg diet can improve intestinal oxidative status and inflammation by increasing villi height and enhancing the mRNA expression of occludin and zonula occudens-1 (ZO-1) as well as reducing the intestinal reactive oxygen species in the jejunum of finishing pigs on transport stress (45). Shi et al. (46) found that dietary single supplementation of curcumin at higher dose (200 mg/kg) or lower dose of curcumin (200 mg/kg) in combination with piperine (25 mg/kg) can improve the intestinal permeability in terms of increasing the mRNA expression of occludin, claudin-1 and zonula occludin-1 in the jejunal and ileal mucosa of weaned Wuzhishan piglets. In addition, Reda et al. (15) corroborated that dietary nanocurcumin can enhance immunoglobulin G (IgG) and immunoglobulin M (IgM) levels in growing Japanese quails, which supported the immunostaining features of IgA protein expression in the jejunum of finishing pigs fed CN supplemented diets in the present study.

Conclusion

In conclusion, the results of the present study showed that dietary supplementation with curcumin nanospheres can enhance the growth, serological indices, immunity, meat quality, gastrointestinal morphology, and reduce intestinal and fecal infectious bacterial colonies, as well as fecal noxious gas (ammonia) content in finishing pigs.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The study was approved by the Animal Care and Use Committee of Jeju National University, Jeju Island, Republic of Korea (approval no. 2021-0056).

Author contributions

MM has planned the experiment, determined the growth, somatic indices, hematology, meat quality, immunohistochemistry, microbiological analyzes, fecal gas analyzes, drafted the final article, and analyzed the statistical data and finalized the manuscript. DK did sample analyzes, immunohistochemistry, and drafting of the manuscript. HK, NK, SC, and AK growth, somatic indices, hematology, meat quality, and fecal gas analyses. KH and TM critically supervised and helped in experimental planning with the addition of manuscript drafting and finalizing. All authors read and approved the final manuscript.

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Conflict of interest

KH was employed by AT. Consulting.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effect of replacing whole wheat with broken rye as a sustainable grain in diets of fattening turkeys on growth performance, litter quality, and foot pad health

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Introduction: Rye is one of the most important cereal crops in Central Europe, thus attempts have been made to include it in the diets of birds to reduce production costs, since the cost of feed accounts for as much as 50 %—70 % thereof. Nevertheless, the use of rye has been limited to date, particularly in turkeys. This study aimed to test the effects of rye inclusion up to 10 % on growth, excreta, and/or litter dry matter, and foot pad health.

Methods: Four trials were performed with a total of 4,322, 4,307, 4,256, and 4,280 female turkeys (BUT BIG 6, Aviagen) for trials 1, 2, 3, and 4, respectively. All birds were fed commercial starter diets for the dietary phases 1 and 2 (up to d 35 of life). Thereafter, at the start of the study, the control group received commercial supplementary feed with 5 % or 10 % wheat until the end of the fattening period. The experimental group was offered supplementary feed to which instead of wheat increasing levels of rye were added stepwise from 5 % to 10 %.

Results: Using supplementary feed with rye showed no significant differences in the final body weight between the control and experimental groups (10.9 vs. 10.8 kg). The dry matter content of fresh excreta for turkeys during the experimental period did not show significant differences between both groups, except at weeks 10 and 14 of life. The feed type (either control diet or experimental diet) did not significantly affect litter dry matter content between the groups throughout the experimental period. No significant differences were noted in food pad dermatitis scoring between both groups throughout the experimental period, except at weeks 11 and 16 of life. Overall, this study showed that including proportions of rye up to 10% could replace conventional ingredients and may increase sustainability in poultry production regardless of the addition of supplementary feed.

KEYWORDS

turkey, rye, growth performance, litter quality, foot pad dermatitis

Introduction

The cost of feed accounts for as much as 50%−70 % of poultry production (1, 2). Due to the ongoing rise in the price of feed ingredients, producers are being forced to reconsider how best to allocate their resources for feeding efficiency (3, 4). Additionally, the International Feed Industry Federation (5) and the Food and Agriculture Organization of the United Nations (6) predict that by 2050 the production of livestock will have doubled. Therefore, it is crucial to find adequate substitutes for traditional feed sources in order to satisfy the nutritional needs of poultry. Efficient use of feedstuffs which have connections between poultry production, nutrition and a sustainable ecosystem is essential for production of sustainable human food (3, 7). One of the most relevant cereal crops in Central Europe is rye (*Secale cereale* L.). As it is affordable, simple to cultivate, and has lower soil and agro-technical needs than other cereals, rye is crucial to sustainable agriculture (7, 8). Rye has a high output yield, is resistant to fungi, tolerant to low temperatures, droughts, and unbalanced soil pH (2). In addition, rye has a small quantity of crude fiber and a lot of important proteins (9, 10). However, rye is not frequently included in poultry nutrition due to high quantities of anti-nutrients such as non-starch polysaccharides (NSP) primarily in the form of arabinoxylans, pentosans, and glucans (11). Due to the limited ability of poultry to digest these NSP, the digesta becomes more viscous, the nutrients are less digestible, and the digesta passing through the gastrointestinal tract tends to be slower (12–15). The high concentration of soluble carbohydrates in rye, which partly dissolve in the gastrointestinal tract, result in a thick, viscous solution in the digesta and cause extremely wet excreta (14, 16, 17). For environmental and economic sustainability, strategies to increase feed efficiency are especially crucial, and there are growing investments and efforts being undertaken to reduce anti-nutritional elements in wheat types (18). Due to the typically high levels of alkylresorcinols, which have been significantly reduced from over 1,000 mg kg^{-1} in the old varieties (19) to 815 mg kg^{-1} in the new ones (20), or even to 401 mg kg^{-1} in new types of hybrid rye, the amount of anti-nutritive compounds is limited and a reduction in feed palatability is no longer observed (20). This suggests that hybrid rye cultivars could be incorporated into the diet of broiler chickens, which primarily consists of cereal grains like wheat and corn (up to 65 %) in accordance with Alquaisi et al. (21). Other types of cereals, including traditional rye cultivars, are less common because of their high anti-nutrient content, which reduces nutrient conversion and digestion and, as a result, lowers performance (22). In diets supplied in mash form, there have been attempts to incorporate ground or whole rye grain in varying amounts. Recent research on the nutritional value of intact rye as a feed ingredient for broilers showed that using significant proportions of intact rye (gradual increase from 2 % at d 8 up to 20 % at d 29-d 33) could replace traditional ingredients in the chicken industry (23).

The prevalence and severity of foot pad dermatitis (FPD) are influenced by a wide range of circumstances which may affect litter quality and consequently the foot pad health. However, the most significant contributor to FPD is poor litter quality, namely moist litter (23–27). For the health and welfare of animals, it is therefore particularly important to achieve acceptable litter quality and a low prevalence of FPD. Therefore, the aim of this experiment was to determine whether rye could effectively substitute wheat in the diets of turkeys that were being raised for meat. The study also sought to determine the impact on the excreta and/or litter quality as well as foot pad health when gradually adding broken rye levels of up to 10 % to diets for growing turkeys.

Materials and methods

The fattening turkeys in this study were raised under standardized husbandry conditions and subjected to a standard fattening procedure on the Farm for Education and Research Ruthe, University of Veterinary Medicine Hannover, Foundation, Sarstedt, Germany. Animal experiments were carried out in accordance with German regulations. Since no relevant interventions according to the Animal Protection Act (§ 7, paragraph 2, sentence 3) had been carried out on live animals, the study was not an animal experiment, and thus did not require approval from the competent authority. This has been checked by the animal welfare officer of the University of Veterinary Medicine Hannover.

Experimental design and housing

Four trials were performed with a total of 4,322, 4,307, 4,256, and 4,280 female turkeys (B.U.T. Big 6, Aviagen Turkeys Ltd., Tattenhall, UK). These four trials (trial 1, 2, 3, and 4, respectively) together correspond to four repetitions. The birds were obtained commercially from the same hatchery in each trial (Moorgut Kartzfehn Turkey Breeder GmbH, Bösel, Germany). In all trials, the birds were divided into two groups fed either the SF with whole wheat as control diet or the SF with broken hybrid rye as experimental diet. Every group has considered as an experimental unit and the four trials were considered as the number of replications. Each group was allocated to a floor pen of 472 m^2 in all four trials. The location of groups was swapped between the four trials to avoid any effects regarding housing conditions. Each pen was littered with wood shavings to a depth of ∼4–6 cm above the concrete floor (7.60 kg/m^2) at the start of the trials. In all groups, fresh litter was added repeatedly after the 5th week of life in identical amounts for each group (without removing the old litter). Automatic chain feeding and watering systems *via* the troughs and drinkers were used (Big Dutchman International GmbH, Vechta, Germany). The temperature (mean = 15°C during experimental period), humidity (mean = 75% during experimental period), and light in the stables were controlled automatically (ViperTouch; Big Dutchman International GmbH). The stall was heated with a gas air-heating system and controlled by an automatic control assembly for temperature and humidity.

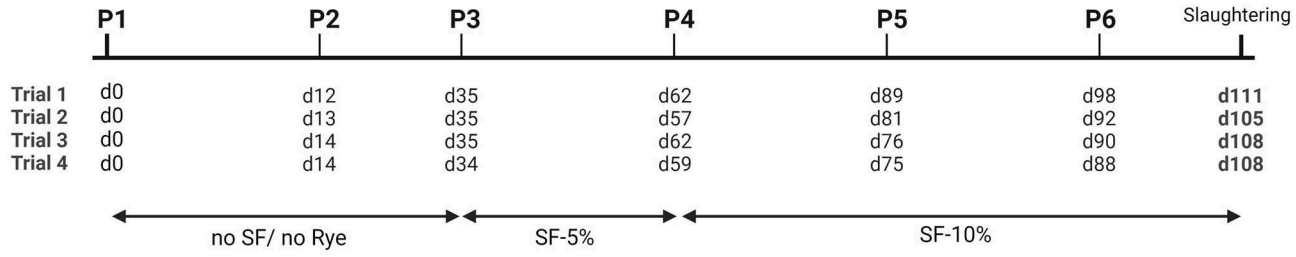

FIGURE 1
Overview of the timing of the change of feeding phases and feeding diets. P, Phase of feeding; d, day of life; SF, supplementary feed to whole wheat or broken rye; the figure was created with Biorender.com.

Diets

Feed and water were available *ad libitum* for all groups. All birds in the four trials were fed identical commercial complete diets for the first five weeks of life (P1 and P2 dietary phases). For the P3-P6 dietary phases the diets for control group and experimental group were based on the same supplementary feed (SF) for each phase. To the SF of each feeding phase whole wheat was added in the control diets and broken rye was added in the experimental diets in concentrations of 5 % (Phase 3) or 10 % (Phase 4–6) respectively (Figure 1). With the beginning of the sixth week of life (P3-P6 dietary phases), the experimental diets were fed to the experimental groups and control diets to control groups (Figure 1).

Four different types of SF were offered to the birds during the trials: SF-5 % was used from d 34 to d 58 (trial 4); d 35 to d 56 (trial 2)/d 61 (trials 1 & 3), while SF-10 % for P4 was used from d 57 (trial 2)/d 59 (trial 4)/d 62 (trials1& 3). The SF-10 % for P5 was used from d 75/ d 76/d 81/d 89 (for trials 1, 2, 3, and 4, respectively), while SF-10 % for P6 was offered from d 88/ d 90/ d 92/ d 98 (for trials 1, 2, 3, and 4, respectively) and onwards (Figure 1).

The commercial complete diets as well as pelleted SF were based on wheat grain, yellow corn, soybean meal, sunflower meal, and rapeseed meal (see Supplementary Tables 1–4 for details of feed ingredients) produced and delivered to the farm in a silo from BEST 3 Geflügelernährung GmbH, Twistringen, Germany. The broken rye (KWS Trebiano) grain was provided by KWS LOCHOW GmbH, Bergen, Germany in another silo. The SF-5 % and SF-10 % diets were formulated and 5 % or 10 % of the whole wheat (control diet) or broken rye (experimental diet) were added afterwards. For control diets the feed producer added whole wheat to the SF, while for experimental diets broken rye was mixed in the feeding lines just directly before filling the feeders. Moreover, it has to be mentioned that all diets in the four trials contained enzymes which were included in the SF (see Supplementary Tables 5–8 for details of feed analysis and composition).

Feed analysis

The Association of German Agricultural Analytic and Research Institutes (VDLUFA) methodologies were used to determine the chemical composition of the commercial diets as well as the SF (Table 1) for the four trials (36). Weighing the samples before and after they had been dried at 103°C allowed to calculate the dry matter (DM) content. Weighing the samples prior to and following the 600°C combustion allowed the researchers to determine the crude ash content using the muffle furnace. The crude fiber content was assessed by washing the samples in diluted acids and alkalis using Foss Fibertec 2010 Hot Extractor. The crude fat content was quantified using automatic hydrolysis and extraction with Hydrotherm and Soxtherm 416 (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Additionally, the total nitrogen concentration was determined using the rapid MAX N exceed nitrogen and protein analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany). The LuffSchoorl method was employed to test the sugar levels in the samples, and atomic absorption spectrometry was utilized to analyze the minerals (Zeenit 700P, Analytik Jena GmbH, Jena, Germany). The analysis of amino acid contents was performed using ion exchange chromatography (Biochrom 30+, Biochrom Ltd., Cambridge, United Kingdom). Finally, a polarimetrical approach was used to assess the starch content of the diets (Schmidt und Haensch GmbH & Co., Berlin, Germany). All experimental diets represented typical commercial formulations, since they were designed to be isocaloric and isonitrogenous, and the essential amino acids were calculated to be almost identical between all of the diets (see Supplementary Tables 1–4 for details).

Performance parameters

Farm data, including feed and water intake, were automatically recorded in all four experiments. Additionally, the farm measured body weight (BW) every day using hanging automatic scales (Swing 70, Big Dutchman International GmbH, Vechta, Germany) that were placed in the middle of the barn (near to the ground where birds could jump over them). Weekly BW measurements were taken on digital scales (BAT 1, VEIT Electronics, Moravany, the Czech Republic) after randomly selecting 50 birds from each group and catching them. Each group's animal losses were noted. The feed conversion ratio (FCR) was calculated by dividing the total flock feed intake by the total flock weight gain during fattening. The cumulative feed intake of the dead birds was calculated to estimate the corrected FCR.

TABLE 1 Analyzed chemical composition of control diet and experimental diets for dietary phase 3–6 (mean + SD first to fourth trial).

Item [g/kg DM]	P3		P4		P5		P6	
	Control (SF + 5% wheat)	Experimental (SF + 5% broken rye)	Control (SF + 10% wheat)	Experimental (SF + 10% broken rye)	Control (SF + 10% wheat)	Experimental (SF + 10% broken rye)	Control (SF + 10% wheat)	Experimental (SF + 10% broken rye)
Dry matter	885 ± 7.05	883 ± 8.46	878 ± 4.03	877 ± 3.95	878 ± 3.42	877 ± 1.71	880 ± 1.00	877 ± 1.50
Crude ash	64.1 ± 1.81	64.6 ± 1.09	54.2 ± 1.18	54.4 ± 0.70	49.5 ± 3.43	52.1 ± 3.84	46.4 ± 2.78	48.4 ± 1.88
Crude fat	49.7 ± 3.36	51.5 ± 3.12	53.4 ± 5.07	54.3 ± 4.35	67.8 ± 3.77	68.5 ± 2.21	87.8 ± 5.16	87.0 ± 5.64
Crude fiber	30.7 ± 1.73	29.9 ± 2.68	31.8 ± 1.93	32.4 ± 1.99	33.5 ± 2.96	33.3 ± 2.29	32.8 ± 1.25	34.9 ± 1.87
Crude protein	263 ± 6.45	262 ± 3.32	221 ± 10.4	218 ± 9.60	196 ± 4.19	193 ± 8.34	180 ± 5.89	174 ± 4.65
Starch	426 ± 9.18	420 ± 6.00	496 ± 10.1	486 ± 4.20	507 ± 1.91	506 ± 5.83	517 ± 8.14	510 ± 11.[a]
Sugar	52.0 ± 5.94	53.8 ± 5.25	43.4 ± 3.41	48.2 ± 6.61	39.3 ± 1.38	41.5 ± 2.22	37.4 ± 2.00	39.9 ± 1.03
Calcium	11.8 ± 0.41	11.6 ± 0.39	9.80 ± 0.86	9.75 ± 0.34	8.75 ± 0.82	8.88 ± 0.96	8.30 ± 0.87	8.17 ± 0.73
Magnesium	2.23 ± 0.15	2.24 ± 0.13	1.90 ± 0.11	1.90 ± 0.11	1.80 ± 0.05	2.05 ± 0.11	1.77 ± 0.11	2.05 ± 0.10
Phosphors	7.63 ± 0.63	7.47 ± 1.11	6.56 ± 0.18	6.60 ± 0.16	6.12 ± 0.22	6.07 ± 0.31	5.65 ± 0.17	5.61 ± 0.05
Sodium	1.62 ± 0.07	1.67 ± 0.04	1.54 ± 0.20	1.64 ± 0.08	1.78 ± 0.04	1.80 ± 0.09	1.85 ± 0.10	1.85 ± 0.10
Potassium	10.1 ± 0.46	10.1 ± 0.29	7.91 ± 0.32	8.03 ± 0.26	6.66 ± 0.44	6.73 ± 0.43	6.01 ± 0.61	6.06 ± 0.55
Copper [mg/kg DM]	24.3 ± 7.00	24.6 ± 7.46	22.1 ± 7.71	24.0 ± 9.19	23.2 ± 7.43	23.2 ± 6.96	22.6 ± 2.47	23.7 ± 3.85
Zinc [mg/kg DM]	131 ± 20.9	128 ± 21.2	133 ± 14.7	99.8 ± 55.5	117 ± 12.9	118 ± 8.46	121 ± 4.57	116 ± 8.66
Iron [mg/kg DM]	293 ± 58.7	290 ± 57.5	228 ± 13.7	226 ± 13.6	215 ± 27.9	235 ± 37.3	201 ± 23.5	229 ± 18.4
Manganese [mg/kg DM]	159 ± 21.0	142 ± 21.9	145 ± 21.5	137 ± 19.5	139 ± 18.3	135 ± 16.3	125 ± 17.5	130 ± 19.2
AME_N [MJ/kg]	13.6 ± 0.13	13.5 ± 0.17	14.1 ± 0.45	14.0 ± 0.26	14.3 ± 0.17	14.3 ± 0.14	14.9 ± 0.15	14.8 ± 0.13
Arginine	16.9 ± 0.37	17.0 ± 0.62	13.9 ± 1.18	13.7 ± 1.04	12.1 ± 1.39	11.6 ± 1.53	10.7 ± 1.16	10.2 ± 1.28
Cysteine	4.18 ± 0.66	4.22 ± 0.59	3.73 ± 0.32	3.68 ± 0.49	3.44 ± 0.45	3.48 ± 0.50	3.37 ± 0.49	3.17 ± 0.55
Isoleucine	10.8 ± 0.45	10.8 ± 0.38	8.76 ± 0.90	8.60 ± 0.75	7.32 ± 0.30	7.06 ± 0.58	6.66 ± 0.55	6.36 ± 0.68
Leucine	18.8 ± 0.65	18.9 ± 0.26	15.6 ± 1.21	15.3 ± 1.25	13.4 ± 0.50	12.8 ± 0.80	12.4 ± 0.83	11.9 ± 1.01
Lysine	16.3 ± 0.26	16.6 ± 0.56	14.3 ± 0.90	14.2 ± 0.72	13.2 ± 0.53	12.8 ± 0.60	11.9 ± 0.28	11.5 ± 0.33
Methionine	7.06 ± 0.94	6.89 ± 0.78	6.04 ± 0.66	6.11 ± 0.53	5.71 ± 0.87	5.84 ± 0.32	5.18 ± 0.81	5.02 ± 0.68
Phenylalanine	12.5 ± 0.37	12.5 ± 0.24	10.2 ± 0.67	9.99 ± 0.62	8.57 ± 0.18	8.23 ± 0.46	7.87 ± 0.33	7.42 ± 0.41
Threonine	10.5 ± 0.80	10.6 ± 0.61	9.48 ± 0.69	9.70 ± 1.20	8.31 ± 0.81	8.01 ± 0.47	6.77 ± 0.52	7.18 ± 0.99
Valine	12.3 ± 0.50	12.3 ± 0.24	10.4 ± 0.93	10.2 ± 0.80	9.09 ± 0.40	8.83 ± 0.75	8.30 ± 0.60	8.01 ± 0.79

SF, supplementary feed for dietary phase 3–6 to whole wheat inclusion (5% or 10% respectively) for control diets or broken rye inclusion (5% or 10% respectively) for experimental diets.
P3 (d 35-61); P4 (d 62-88); P5 (d 89-97); P6 (d 98-111).
[a] AME_N (MJ/kg) = 0.01551 × g/kg crude protein+0.03431 × g/kg crude fat+0.01669 × g/kg starch+0.01301 × g/kg sugar.

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Litter quality and foot pad dermatitis scoring

Weekly litter samples were taken from nine locations/spots in each stable for all trials to measure the DM content. Briefly, the stable was divided mathematically into nine squares then the central area of each square was chosen for collecting the samples. In order to assess the DM, nine fresh excreta samples from each group were also obtained (and pooled) on the same day and from the same areas as for litter samples. From the litter surface, only the fresh, pure excreta (free of litter debris) were collected. In accordance with Mayne et al. (24), the foot pads of the birds (50 randomly selected birds/group) were graded weekly on a scale from 0 to 7: Score 0 denoted healthy skin, whereas 7 denoted necrosis in more than 50 % of the foot pad region. For each bird, the average of the two legs' scores was calculated. In a prior study, the footpad score was demonstrated (28).

Statistical analyses

Statistical analysis was performed using the Statistical Analysis System for Windows, the SAS® Enterprise Guide® version 9.3 (SAS Institute Inc., Cary, NC, USA). For all parameters, mean values as well as the standard deviation of the mean were calculated. For the individual bird's BW, data and FPD Scores the individual data of 50 random sample birds out of each stable at each time point were used. For data of water intake, feed intake, water to feed ratio, corrected feed conversion ratio (cFCR), slaughterhouse BW and mortality rate the group data of the stables were basis of the calculation. For DM content of litter and excreta, nine samples in each stable were taken according to a standardized system for statistical calculation. For all data except the FPD scores, a Shapiro-Wilk test for normal distribution was performed and normally distributed data were checked for significant differences with the Ryan-Einot-Gabriel-Welsch Test (simple ANOVA). Not normally distributed data and FPD scores were checked for significant differences with a Kruskal-Wallis test followed by a Wilcoxon two-sample test. Differences with a significance level of $p \leq 0.05$ were considered significant.

TABLE 2 Water intake, feed intake, and water-to-feed ratio of turkeys for the four trials per bird.

Item	Feed		*P*-value
	Control	Experimental	
Water intake [g], P1-P6	50,458 ± 2,330	51,120 ± 1,163	0.6295
Water intake [g], P3-P6	44,066 ± 2,062	44,745 ± 1,349	0.6016
Feed intake [g], P1-P6	27,235 ± 2,086	27,192 ± 1,162	0.9721
Feed intake [g], P3-P6	25,469 ± 948	25,468 ± 624	0.9987
W:F, P1-P6	1.86 ± 0.13	1.88 ± 0.08	0.7862
W:F, P3-P6	1.73 ± 0.05	1.76 ± 0.04	0.3968

P1–P6 = dietary phases; W:F = water:feed intake ratio; n = 4.

TABLE 3 Average body weight [g] of turkeys for the four trials.

Week of life	Feed		*P*-value
	Control	Experimental	
5 (d35)	1,690 ± 192	1,718 ± 184	0.1336
6	2,497 ± 260	2,495 ± 241	0.9331
7	3,268 ± 288[b]	3,366 ± 304[a]	0.0010
8	4,321 ± 365	4,277 ± 348	0.2210
9	5,042 ± 497	5,057 ± 487	0.7560
10	6,243 ± 739	6,349 ± 777	0.1635
11	7,296 ± 769	7,290 ± 802	0.9359
12	8,350 ± 747	8,271 ± 732	0.2847
13	9,252 ± 753	9,289 ± 856	0.6425
14	10,176 ± 666	10,066 ± 747	0.1192
15	11,229 ± 725[a]	10,913 ± 804[b]	0.0004
16	11,692 ± 686	11,484 ± 823	0.1717

[a,b]Means in a row with different superscripts differ significantly ($p < 0.05$); n = 200 in week 5–14; n = 150 for week 15; n = 50 in week 16.

Results

The water intake did not differ significantly between the groups either during the entire fattening period (dietary phase: P1–P6) or during the experimental period (dietary phase: P3–P6) as presented in Table 2. No significant differences were noted in the feed intake between both groups in both periods (Table 2). Also, the water-to-feed intake ratio did not significantly differ between the groups either for the entire fattening period (dietary phase: P1–P6) or the experimental period (dietary phase: P3–P6).

Growth performance

Table 3 shows the average BW during the entire experimental period from the 6th week until the 17th week of life (dietary phase: P3–P6). No significant differences in BW during the experimental period were noted, except at weeks 7 and 15 of life. The average BW at the 16th week of life was 11,692 vs. 11,484 g for the control and experimental groups, respectively.

The performance parameters and mortalities recording by the farm as well as by the slaughterhouse for the four trials are presented in Table 4. The cFCR during the experimental period (P3–P6) did not significantly differ between the groups (2.82 vs. 2.93 for control and experimental groups, respectively). Furthermore, the final BW according to the slaughterhouse data did not differ significantly between the groups (control group=10.9 vs. 10.8 kg for the experimental group). Additionally, the mortality rate did not differ significantly between the groups either during the entire period (dietary phase: P1–P6) or during the experimental period (dietary phase: P3–P6).

TABLE 4 Corrected feed conversion ratio (cFCR), slaughterhouse BW as well as the mortality rate of turkeys for the four trials.

Item	Feed		*P*-value
	Control	Experimental	
cFCR, P1-P6	2.515 ± 0.217	2.564 ± 0.144	0.7141
cFCR, P3-P6	2.820 ± 0.143	2.925 ± 0.088	0.2584
BW at slaughterhouse [kg]	10.930 ± 0.382	10.753 ± 0.300	0.4930
Mortality rate [%], P1-P6	1.855 ± 0.193	1.960 ± 0.693	0.7803
Mortality rate [%], P3-P6	1.118 ± 0.263	1.475 ± 0.641	0.3420

cFCR, corrected feed conversion ratio; n = 4.

TABLE 5 Dry matter content [%] of excreta for the four trials during the experimental period (dietary phase: P3–P6).

Week of life	Feed		*P*-value
	Control	Experimental	
5 (d35)	21.3 ± 1.86	21.2 ± 1.86	0.7594
6	21.3 ± 1.42	21.1 ± 1.61	0.5630
7	21.1 ± 1.75	20.5 ± 1.32	0.0612
8	20.4 ± 3.26	20.6 ± 0.95	0.7921
9	21.1 ± 1.95	21.3 ± 1.30	0.5204
10	20.4 ± 1.02[b]	20.9 ± 1.06[a]	0.0318
11	21.1 ± 1.47	20.6 ± 1.52	0.2108
12	21.7 ± 1.21	21.3 ± 1.17	0.2709
13	22.3 ± 1.07	22.1 ± 1.41	0.5028
14	22.7 ± 0.94[a]	22.2 ± 1.03[b]	0.0165
15	21.9 ± 1.43	21.6 ± 1.40	0.4989
16	22.0 ± 1.21	21.7 ± 1.42	0.5628

[a,b]Means in a row with different superscripts differ significantly ($p < 0.05$); n = 36 in week 5–14; n = 27 for week 15; n = 9 in week 16.

Excreta dry matter

The DM content of fresh excreta for turkeys during the experimental period did not show significant differences between the two groups, except at weeks 10 and 14 of life (Table 5).

Litter quality

The feed type (either control diet or experimental diet) did not significantly affect the litter DM content between the groups throughout the experimental period from P3 until P6 (Table 6).

The final litter DM content or the amount of final litter in fresh/DM basis did not significantly differ between both groups (Table 7).

TABLE 6 Dry matter content [%] of litter for the four trials during the experimental period (dietary phase: P3–P6).

Week of life	Feed		*P*-value
	Control	Experimental	
5 (d35)	77.6 ± 5.55	76.7 ± 5.98	0.5261
6	72.4 ± 6.47	72.5 ± 5.33	0.9117
7	69.5 ± 7.29	67.7 ± 6.45	0.6114
8	61.4 ± 10.4	61.5 ± 8.49	0.9397
9	57.4 ± 10.3	56.3 ± 9.89	0.6784
10	57.5 ± 9.14	58.4 ± 10.64	0.7134
11	54.7 ± 8.27	52.9 ± 6.29	0.3076
12	56.2 ± 10.7	55.6 ± 9.94	0.7845
13	61.6 ± 8.20	58.8 ± 8.54	0.1558
14	61.3 ± 9.55	60.2 ± 8.08	0.6003
15	57.7 ± 6.42	59.5 ± 6.70	0.3177
16	52.4 ± 5.84	53.6 ± 3.39	0.6079

n = 36 in week 5–14; n = 27 for week 15; n = 9 in week 16.

TABLE 7 Parameters of final litter for the four trials.

Item	Feed		*P*-value
	Control	Experimental	Feed
Final litter DM [%]	58.1 ± 6.23	57.5 ± 3.50	0.8534
Amount of final litter in fresh basis [kg]	41,671 ± 2,486	43,468 ± 3,600	0.4428
Amount of final litter in DM basis [kg]	24,288 ± 3,542	24,957 ± 2,787	0.7764

The values in each item are the means of 4 analyzed samples.

Foot pad scoring

No significant differences were noted in the FPD scoring between both groups throughout the experimental period, except at weeks 11 and 16 of life (Table 8). At week 11, the broken rye group (experimental group) showed significantly higher FPD scores compared to the control group (4.97 vs. 4.70). However, at week 16 of life, the birds in the control group showed significantly higher FPD scores compared to the broken rye group (4.96 vs. 4.65).

Discussion

Maximizing the effectiveness of all feedstuffs that highlight the link between poultry production, nutrition and sustainable ecosystem services is crucial to ensure the continuity of good and sustainable contributions to a stable human food supply as well as to animal feed supply (3). Therefore, a detailed assessment of the nutritional effectiveness of new rye hybrids in poultry studies is required. Furthermore, this current study could be the first research study dealing with the addition of hybrid broken rye, particularly

TABLE 8 Foot pad dermatitis scoring of turkeys for the four trials.

Week	Feed		*P*-value
	Control	Experimental	Feed
5 (d35)	2.51 ± 0.93	2.38 ± 0.78	0.3989
6	3.21 ± 1.22	3.14 ± 1.10	0.3876
7	3.69 ± 1.11	3.51 ± 0.98	0.0673
8	3.74 ± 0.94	3.68 ± 0.80	0.3877
9	4.13 ± 0.84	4.25 ± 0.94	0.4230
10	4.22 ± 0.97	4.37 ± 1.12	0.2598
11	4.70 ± 0.97[b]	4.97 ± 1.25[a]	0.0254
12	5.19 ± 0.95	5.27 ± 1.01	0.3289
13	5.21 ± 0.94	5.17 ± 0.96	0.7173
14	5.36 ± 1.02	5.31 ± 0.88	0.6071
15	5.34 ± 0.93	5.49 ± 0.91	0.1170
16	4.96 ± 0.71[a]	4.65 ± 0.82[b]	0.0208

[a,b]Means in a row with different superscripts differ significantly ($p < 0.05$); n = 200 in week 5-14; n = 150 for week 15; n = 50 in week 16.

to the fattening turkey diet under practical field conditions (four consecutive trials).

At the 16th week of life, the average final BW (individual bird data) for the control and experimental groups was 11,692 g and 11,484 g, respectively, almost above the available B.U.T. 6 female performance objectives for turkeys of 11.29 kg (29). Moreover, in the current study, the calculated cFCR was 2.52 for the control group vs. 2.57 for the experimental group during the entire fattening period (dietary phase P1–P6). The evaluation of slaughterhouse data showed a mean daily BW gain for the rye group of 99.75 g and for the control group of 101.5 g. The monthly mean of the slaughterhouse data of comparison of all monthly slaughtered turkeys was 94 g. It seems that although the cFCR was slightly lower compared to the performance objectives for B.U.T. Big 6 female turkeys, the daily gain was higher compared to the monthly farm comparison of the slaughterhouse (29).

To the best of our knowledge, we did not find any literature regarding the effects of including broken rye in the diet of fattening turkeys on performance parameters. However, in broilers, Abd El-Wahab et al. (23) observed in two trials that the average BW (slaughterhouse data) was about 2,059 and 1,947 g for control and experimental groups, respectively at d 33 of life in the first trial, exceeding the available Ross 308 performance objectives of 1946 g (30). However, in the second trial in this previous study, this was not the case (1,883 and 1,747 g for control and experimental groups, respectively). Additionally, the corrected FCR in the first trial was identical for the two groups (1.50), whereas in the second trial, the calculated corrected FCR was 1.49 for the control group vs. 1.51 for the experimental group (23).

The results of additional studies on broilers concerning the effects of rye inclusion on broiler performance are consistent (2, 31). According to these findings, Teirlynck et al. (32) found no effect on BW of broiler diets containing 5 % rye from days 1 to 42. Similar to this, a study by Arczewska-Wlosek et al. (17) reported no detrimental effects of ground rye (20 %) on performance during the grower-finisher period in older broilers (d 22-d 42). However, according to Van Krimpen et al. (16), broiler diets containing 10 % rye from days 15 to 28 of life had a negative impact on performance when compared to diets containing 5 % rye (1,096 g for 10 % rye vs. 1,127 at day 21; 1,816 vs. 18,77 g at day 28 for 5 % and 10 %, respectively). According to Tellez et al. (332), the growth performance of broilers given a rye-based diet (58.3% rye) decreased by approximately 43 % as a result of increased intestinal viscosity. According to our results, rye should generally be introduced gradually to the diets of fattening turkeys, especially during their early fattening phase, and then gradually increased as they get older.

Feed composition as well as feed ingredients are considered to be important factors affecting excreta and litter conditions. Although the viscosity of excreta was not determined in the present study, it is well known that rye contains high levels of soluble carbohydrates, which can dissolve in the digesta, producing a thick viscous solution and consequently very wet excreta (23, 34). According to Silva and Smithard (35), birds fed rye produced a very wet excreta, this affecting the litter DM content. In the present study, the litter DM content was not affected by the inclusion of broken rye broken rye up to 10 % in the diets of fattening turkeys. Furthermore, it seems that using broken rye or its inclusion level had no impact on the excreta quality.

The mean results in terms of foot pad health (FPD scores) were better in the experimental group compared to those in the control group (4.65 vs. 4.96) at the end of the trials (16th week of life). Most of the previous studies investigated the effect of hybrid rye on foot pad health in broilers. Abd El-Wahab et al. (28) found that feeding broiler chickens either a wheat diet (control) or a SF with broken/squashed rye resulted in low FPD scores, which might be attributed to the good litter quality as well as to the comparable digesta viscosity in the wheat group.

Standing on wet litter brings the broilers' feet in constant contact with moisture and has been suggested to induce FPD (24, 27, 37). In a recent study by Abd El-Wahab et al. (23), it was observed that in the first trial, feeding SF with rye led to significantly higher FPD scores (3.80) compared to broilers fed the control diets (2.70).

However, these significant differences were not observed in the second trial, although both groups had high FPD scores (> 4). Feed technology, enzyme concepts on feed ingredients, and/or stable enrichment may not have been sufficiently taken into account here (25, 38). It is still to consider that including rye in higher amounts in the diets of poultry results in an increasing intestinal viscosity (33). Consequently, when, also in the interest of environmental sustainability, higher than 10 % of rye should be included in the diets of fattening poultry, more research is needed to better understand the opportunities of new feeding technology and of enzymes as well as the type of rye (intact or broken) on excreta viscosity, litter DM content and FPD scores.

Conclusion

The results of this study support the view that including a relative high proportion of broken rye (10 %) in diets of fattening

turkeys did not affect growth performance negatively, as no relevant differences could be observed between the rye and wheat diets during the trial in the case of sample point weighing. Moreover, it seems that excreta and litter dry matter contents were not influenced by adding broken rye to diets of fattening turkeys. Consequently, the foot pad health of fattening turkeys was not affected negatively by broken rye inclusion. Therefore, the result of this study is that broken rye can be included up to 10 % in diets for turkey hens without negative effects on performance parameters and food pad health.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Animal experiments were carried out in accordance with German regulations. Since no relevant interventions according to the Animal Protection Act (§ 7, paragraph 2, sentence 3) had been carried out on live animals, the study was not an animal experiment, and thus did not require approval from the competent authority. This has been checked by the animal welfare officer of the University of Veterinary Medicine Hannover, whose statement we sent to the editor.

Author contributions

Conceptualization: CV, VW, and AA. Data curation, formal analysis, validation, visualization, and writing—original draft preparation: JL. Funding acquisition: CV. Investigation: JL and AA. Methodology: JL, CS, and AA. Project administration and supervision: CV and VW. Resources: RG, AF, CV, and CS. Writing—review and editing: JL, CV, RG, AF, CS, VW, and AA. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

RG and AF are employed by the company KWS LOCHOW GmbH, Bergen, Germany. The company had no role in the collection, analyses, interpretation of the data, writing of the manuscript or decision to publish the results. KWS LOCHOW GmbH provided the rye for the diets.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1142500/full#supplementary-material

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Blood lipid profiles, fatty acid deposition and expression of hepatic lipid and lipoprotein metabolism genes in laying hens fed palm oils, palm kernel oil, and soybean oil

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The palm oil, palm kernel oil and soybean oil have unique and distinctive fatty acid chain length and saturation profiles, and how they affect lipid peroxidation, fatty acid intake and metabolism is worth exploring in poultry. This study elucidated the influence the dietary oils on lipid peroxidation, blood lipid profiles, fatty acid deposition of liver, serum and yolk and the expression of liver genes related to lipid and lipoprotein metabolism in laying hens. About 150 Hisex brown laying hens were fed diets containing crude palm oil (CPO), red palm oil (RPO), refined palm oil (RBD), palm kernel oil (PKO) or soybean oil (SBO) for 16 weeks. Serum, liver and yolk lipid peroxidation were not different between dietary oils. The PKO increased liver, serum and yolk medium-chain fatty acids (MCFA). There was no difference in liver saturated fatty acids (SFA). The CPO and RPO reduced serum SFA, but the PKO increased yolk SFA. The SBO increased polyunsaturated fatty acids (PUFA) in liver serum and yolk. No difference in liver elaidic acid (C18:1-trans), but SBO lowered elaidic acid (C18:1-trans) in serum. Higher very-low density lipoprotein (VLDL) in CPO than RPO and SBO and greater serum lipase in CPO, RBD and PKO than SBO. There was no difference in sterol regulatory element-binding protein 2 (SREBP-II) between oils. Apolipoprotein VLDL-II (APOVLDL2) was upregulated in palm oils and apolipoprotein B-100 (APOB) in RBD. Downregulation in peroxisome proliferator-activated receptor-alpha (PPAR-α), peroxisome proliferator-activated receptor gamma (PPAR-γ) and low-density lipoprotein receptor (LDLR) was observed in palm oils and PKO. In conclusion, different dietary oils greatly influence several aspects of fatty acid metabolism, deposition and lipoprotein profiles but have no influence on reducing lipid peroxidation.

KEYWORDS

crude palm oil, red palm oil, refined palm oil, palm kernel oil, soybean oil, medium-chain fatty acids, poultry, chicken

1. Introduction

Oils or fats are included in the diet of commercial laying hens at 2–3% to achieve 5% crude fat, as recommended by most commercial breeds. High oil inclusion contributed to excessive fat deposition, which reduced reproductive performance in laying hens (1, 2). Although oil is added at a small percentage in poultry feed, it significantly impacts poultry performance as it supplies a better composition of fatty acids in the diet (3). In addition, it improves the physical quality of feed by reducing dustiness, enhancing feed palatability, and supplying essential fat-soluble vitamins (4–6). Lipids can be sourced either from plant or animal based. Plant-based oil has advantages over animal-based oil due to the lower price with higher polyunsaturated fatty acids (PUFA), particularly omega-3 fatty acid and contains naturally occurring phytonutrients such as polyphenols, squalene, carotenoids and vitamins (7, 8).

The inclusion of oils supplies metabolically essential fatty acids such as omega-3 and omega-6 fatty acids to the poultry. The fatty acid profiles of the feed contributed by the oils are the critical factor in influencing the profile of fatty acid in the blood and fatty acid deposition in the body tissues and poultry products such as meat and eggs (3, 4, 9–14). Eggs enriched with omega-3 fatty acids through hens' diets produce value-added eggs for human consumption that deliver anti-inflammatory properties to reduce health risks (15, 16). The composition of fatty acids characterized the oil, and fatty acid composition is the primary predictor of oxidative stability (17). The higher the amount of unsaturated fatty acids (USFA) fraction in oils, the higher and faster the oxidation process occurs compared to saturated fatty acids (SFA) (18). Lower USFA in oil is expected to reduce lipid peroxidation. Since different properties of oil sources have other effects on lipid metabolism, deposition and oxidation, selecting a source of oil to be added to the poultry feed is important.

Palm oils such as crude palm oil (CPO), red palm oil (RPO) and refined palm oil (RBD) are produced from the mesocarp of the palm fruit. It contains palmitic (C16:0) and oleic (C18:1)-rich fatty acids and is balanced in the SFA to USFA fraction. It also contains high levels of antioxidants contributed by vitamin E in RBD and both vitamin E and carotenoids in CPO and RPO. On the other hand, palm kernel oil (PKO) is extracted from the kernel of the palm fruit. PKO is rich in medium-chain fatty acids (MCFA), mainly lauric acid (C12:0) and myristic acid (C14:0) and contains up to 80% SFA. Soybean oil (SBO) is a polyunsaturated fatty acid (PUFA)-rich oil extracted from soybeans and undergoes refining, bleaching and deodorization to produce refined SBO. The summary of the source of oils and the fatty acid characteristics are displayed in Figure 1, which is adapted from (19–21).

Recently, the effects of dietary CPO, RPO, RBD, and PKO on production performance, egg quality, serum biochemicals and profiles of beta-carotene, retinol and tocopherols in laying hens were reported (22). Oils produced from oil palms, such as CPO, RPO, RBD, and PKO, were not previously studied with respect to fatty acid metabolism and serum lipid and lipoprotein profiles in laying hens. The unique properties of the fatty acid composition, saturation profiles, and naturally occurring antioxidant compounds such as vitamin E and carotenoids are worth exploring. Shorter and saturated fatty acids had higher antioxidant activity and reduced the degree of oxidation. Sengupta et al. (23) reported rice bran oil containing caprylic (C8:0), caproic (C10:0) or lauric (C12:0) increased antioxidant activity in all antioxidant assays. However, they had a lower thiobarbituric acid reactive substance (TBARS) value and conjugated diene as compared with the control rice bran oil, which contained a higher fraction of a longer chain of SFA mainly palmitic acid (C16:0) and USFA mainly oleic (C18:1) and linoleic (C18:2) acids. Hence, this study focused on comparing the dietary palmitic-rich CPO, RPO and RBD, MCFA-rich PKO and PUFA-rich SBO on the serum lipid profiles, the fatty acid composition of feed and its deposition in the serum, liver and yolks, and the liver lipid metabolism gene expression. In addition, the SBO was included as a reference oil with high PUFA as a comparison. It is hypothesized that the fatty acid profiles of oil will influence the blood lipid profiles, lipid peroxidation and deposition of fatty acid profiles in feeds, serum, liver and yolk in laying hens.

2. Materials and methods

2.1. Ethic approval, dietary treatments, and hens' management

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (AUP No: UPM/IACUC/AUP-R013/2020). The experimental location was at the Poultry Unit, Department of Animal Science, Universiti Putra Malaysia. A total of 150 Hisex Brown laying hens were randomized into five treatment groups (30 hens per group), which contained six biological replicates per treatment and five hens per biological replicate. The dietary treatments contained either 3% of CPO, RPO, RBD, PKO, or SBO. The diets were formulated to be isocaloric and isonitrogenous (Table 1) and to achieve the nutrient requirements of Hisex Brown laying hens. The feeds were prepared monthly. Each hen received 120 g feed daily in mash form, as recommended by the Hisex Brown nutritional guide. The water was given *ad libitum* through a nipple drinker. The feeding trial was from week 22 to week 37 (16 weeks). The housing system was an open-sided house, and temperature and humidity were 24 to 32°C and 80 ± 5%, respectively. Hens were placed in two-tier A-type battery cages (30 cm width, 50 cm depth and 40 cm height) individually and received a total of 16 h (±12 h of natural light and 4 h of LED fluorescent light) and 8 h of darkness (Figure 2).

2.2. Sample collection and analysis

Feed samples were collected and kept in a −80°C freezer until analysis. Two eggs from each biological replicate were collected randomly at the end of the experimental period for egg yolk collection. Two yolks were homogenized, combined, freeze-dried at −84°C in a freeze dryer (Labconco, Kansas City, MO, USA), and kept at −80°C until analysis. At the end of the experimental period, six birds per treatment were randomly selected for sacrifice through Halal slaughter and sample collection. At the bleeding point, about 8 ml of blood was collected into a 10 ml BD Vacutainer® Serum Tubes tube (BD, Franklin Lakes, NJ, USA) and allowed to clot on ice. The serum was separated from the blood through centrifugation (3,000 RCF for 20 min at 4°C) and stored at −80°C. A portion of lower right lobe of the liver sample were collected at evisceration into a cryotube and snap-frozen before being kept at −80°C (Figure 3).

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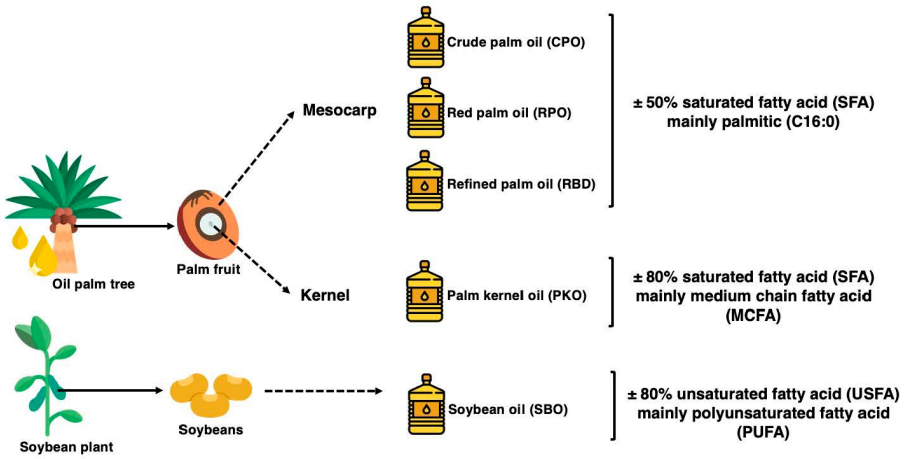

FIGURE 1
The summary of the source of the palm oils, palm kernel oil, and soybean oil and its saturation profiles adapted from (19–21).

2.3. Serum lipid and lipoprotein profiles

The serum biochemistry was analyzed at the Veterinary Hematology and Clinical Biochemistry Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Serum samples were analyzed for cholesterol (TC), triacylglycerol (TAG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and lipase using the Roche/Hitachi 902 clinical chemistry automatic analyzer (Roche, Basel, Switzerland) using appropriate kits. The very-low-density lipoprotein (VLDL) in serum was determined using a Chicken VLDL ELISA kit (FineTest, Wuhan, Hubei, China) according to the protocol provided by the manufacturer. The ELISA kit was based on a double antibody to capture and detect the target protein. The absorbance was determined at 450 nm using an ELx800™ microplate reader (BioTek™, Winooski, Vermont, USA) equipped with Gen5 Microplate Reader and Imager Software (BioTek™, Winooski, VT, USA). The concentration of VLDL was interpolated based on the constructed standard curve of different concentrations of VLDL (μg/ml) against absorbance at 450 nm.

2.4. Lipid peroxidation

Lipid peroxidation was determined using a thiobarbituric acid reactive substance (TBARS) assay by measuring malondialdehyde (MDA) as the product of lipid peroxidation in serum, liver and yolk samples. One gram of sample or 1 ml of MDA standard was combined with 4 ml of 1.15% (w/v) potassium chloride and mixed by the vortex. The mixture was added to 2.5 ml TBARS solution comprising 2 ml of 0.8% (w/v) thiobarbituric acid in 20% (v/v) acetic acid at pH 3.5, 300 μl of deionized water, 165 μl of 8.1% (w/v) SDS and 35 μl 7.0 ethanolic butylated hydroxytoluene. The mixture was mixed by vortex, incubated in a water bath at 95°C for 60 min, and cooled down to room temperature. The cooled mixture was added to 3 ml of n-butanol, mixed by vortex and centrifuged at 5000 rpm for 10 min at 24°C. The supernatant was collected into a cuvette to read absorbance at 532 nm using a SPECORD® 250 PLUS UV/Vis spectrophotometer (Analytic Jena, Jena, Germany). The lipid peroxidation was calculated from the linear regression derived from a standard curve of MDA and expressed as the concentration of MDA per gram sample.

2.5. Fatty acid profile of oil, feed, liver, serum, and egg yolk

Fifty milligrams of the oil sample were combined with 950 μl hexane and 50 μl of 1 N methanolic sodium methoxide and mixed by a vortex. The mixture was allowed to stand for 5 min and centrifuged at 3000 × *g* for 5 min for complete separation. The upper layer was collected into a 2.0 ml glass vial with a PTFE screw cap for fatty acid methyl ester (FAME) separation. For feed, liver, egg yolk (1 g) and serum (1 ml), the sample was mixed with 10 ml of 2:1 (v/v) chloroform: methanol containing butylated hydroxytoluene in a glass tube with a PTFE-lined screw cap, mixed by inversion and stood for 12 h in the dark at 4°C. Five milliliters of 0.9% (w/v) sodium chloride were added, mixed by vortex and centrifuged at 3000 × *g* for 5 min at 24°C. The lower phase was collected into a fresh glass tube with a PTFE-lined screw cap containing 100 μl of 12 mM methanolic heneicosanoic acid (C21:0) (Sigma-Aldrich, St. Louis, MI, USA) before incubating in a 70°C water bath under a constant and mild flow of nitrogen gas to evaporate chloroform. The tubes were cooled to room temperature, and 2 ml of 0.66 N methanolic potassium hydroxide was added into the tubes, mixed by vortex, screwed tightly and incubated in 90°C water for 10 min. The tubes were cooled to room temperature, and 2 ml of 20% methanolic boron trifluoride was added into the tubes, mixed by vortex, screwed tightly and incubated in a 90°C water bath for 20 min. The tubes were cooled to room temperature, and 4 ml of deionized water and 4 ml of petroleum ether were added. The tubes were mixed by vortex and centrifuged at 3000 × *g* for 5 min at 24°C. About 1 ml of upper phase (petroleum ether) was aliquoted into a 2.0 ml glass vial with a PTFE-lined screw cap to be injected into gas chromatography.

Fatty acids were determined using an Agilent 6,890 N Network Gas Chromatograph (Agilent, Santa Clara, CA, USA) equipped with an autosampler and injector. About 1 μl of the sample was injected into the inlet set at 250°C with a split mode of 30:1. The flame ionization detector was set at 250°C with a hydrogen gas flow of 40 ml/min, air

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TABLE 1 Ingredients and nutrient profiles of feeds containing different oils.

	CPO	RPO	RBD	PKO	SBO
Ingredients (%)					
Corn	48.90	48.90	48.90	48.90	48.90
Soybean meal	28.00	28.00	28.00	28.00	28.00
Wheat pollard	8.000	8.000	8.000	8.000	8.000
CPO	3.000	-	-	-	-
RPO	-	3.000	-	-	-
RBD	-	-	3.000	-	-
PKO	-	-	-	3.000	-
SBO	-	-	-	-	3.000
DL-Methionine	0.300	0.300	0.300	0.300	0.300
MDCP	2.300	2.300	2.300	2.300	2.300
Calcium carbonate	8.350	8.350	8.350	8.350	8.350
Choline chloride	0.200	0.200	0.200	0.200	0.200
Salt	0.350	0.350	0.350	0.350	0.350
Mineral mix	0.200	0.200	0.200	0.200	0.200
Vitamin mix	0.200	0.200	0.200	0.200	0.200
Antioxidants	0.100	0.100	0.100	0.100	0.100
Toxin binder	0.100	0.100	0.100	0.100	0.100
TOTAL	100.0	100.0	100.0	100.0	100.0
Calculated nutrient (in % unless stated)					
ME (kcal/kg)	2,790	2,790	2,790	2,790	2,790
CP	17.17	17.17	17.17	17.17	17.17
EE	4.980	4.98	4.98	4.98	4.98
CF	3.800	3.80	3.80	3.80	3.80
Ca	4.000	4.00	4.00	4.00	4.00
Total phosphorus	0.840	0.84	0.84	0.84	0.84
Avail. phosphorus	0.460	0.46	0.46	0.46	0.46
Methionine	0.581	0.581	0.581	0.581	0.581
Lysine	0.933	0.933	0.933	0.933	0.933

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; MDCP, mono dicalcium phosphate; ME, metabolizable energy; CP, crude protein; EE, ether extract; CF, crude fiber; Ca, calcium.

flow of 450 ml/min and make up the flow of 45 ml/min. The nitrogen gas was used as a carrier gas with a constant flow rate of 1 ml/min. The oven temperature gradient was set at 80°C and held for 2 min, increased at 5°C/min to 150°C and held for 10 min, increased at 4°C/min to 220°C and held for 10 min at 220°C with a total runtime of 53 min. The fused-silica capillary column used was a J&W HP-88 GC Column (112-8867), 60 m, 0.25 mm, and 0.20 µm in film thickness (Agilent, CA, USA). The heneicosanoic acid (C21:0; Sigma-Aldrich, St. Louis, MI, USA) was used as an internal standard, and the 37 Component FAME Mix (Merck, Darmstadt, Germany) was used as an external standard to identify the target fatty acids. The SFA is the sum of caprylic (C8:0), caproic (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids. The USFA is the sum of palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids. The MUFA is the sum of palmitoleic (C16:1) and oleic (C18:1) acids. The PUFA is the sum of linoleic (C18:2) and linolenic (C18:3) acids.

2.6. Liver's lipid metabolism genes

Total RNA was extracted using a NucleoSpin® RNA plus kit (Machery Nagel, Dueren, Germany). The quantity and quality of extracted RNA were confirmed using the spectrophotometry method on a Multiskan™ Go spectrophotometer (Thermo Scientific, Waltham, MA, USA). The reverse transcription (1 µg RNA) was conducted using the cDNA Synthesis Kit (Biotechrabbit, Berlin, Germany). The qPCR reaction comprised 1 µl cDNA, 1 µl each for forward and reversed primers, 5 µl 4× CAPITAL™ qPCR Green Master Mix (Biotechrabbit, Berlin, Germany) and 12 µl nuclease-free water. The reaction was conducted using the LightCycler® 480 Instrument (Roche, Basel, Switzerland). The cycling program setting was initially activated at 95°C for 2 min and 30 s, followed by 45 cycles of quantification steps containing a denaturation step at 95°C for 15 s. Then, it was followed by combined annealing and extension for 30 s at a temperature specific to the primer. The specificity of the amplification was confirmed using a melt curve. The information for housekeeping and target genes is available in Table 2. The expression of the target gene was calculated using Livak's $2^{-\Delta\Delta Ct}$ method (24).

2.7. Experimental design and statistical analysis

The feeding trial was subjected to a completely randomized design (CRD). The data analysis was performed on the SAS software package, version 9.4 (SAS Inst. Inc., Cary, NC, USA). The data normality was determined using PROC UNIVARIATE and determined based on Shapiro–wilk. All data obtained were normally distributed and analyzed using one-way analysis of variance (ANOVA) using the General Linear Model (GLM) procedure and paired with Duncan's multiple-range test for comparing the treatment means. The difference was considered significant at $p < 0.05$.

3. Results

3.1. Fatty acid profiles of oils

Different types of oils showed significant differences ($p < 0.05$) in their fatty acid profiles (Table 3). The PKO contained the significantly highest ($p < 0.05$) fraction of medium-chain fatty acids (MCFA), which include caprylic (C8:0), caproic (C10:0), lauric (C12:0) and myristic (C14:0) acids compared to other treatments. The significantly highest ($p < 0.05$) SFA was present in PKO (81.86%), followed by CPO (60.87%), RPO (57.04%), RBD (53.58%) and SBO (28.54%). However, USFA and PUFA fractions were contrary to the SFA, in which SBO had the significantly highest ($p < 0.05$) and PKO the lowest ($p < 0.05$). MUFA had the significantly highest ($p < 0.05$) percentage in RBD (40.79%), followed by RPO (37.73%), CPO (34.39%), SBO (29.88%)

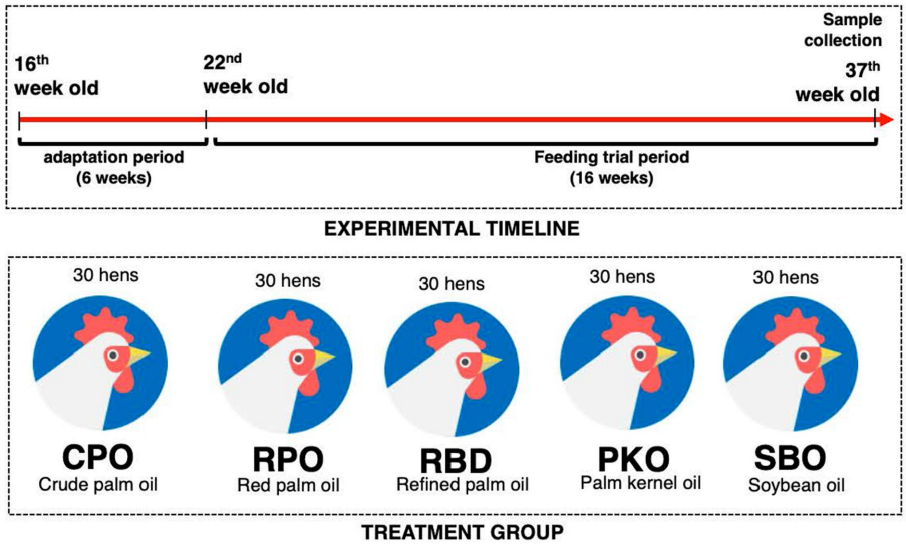

FIGURE 2
The summary of treatment groups and experimental timeline.

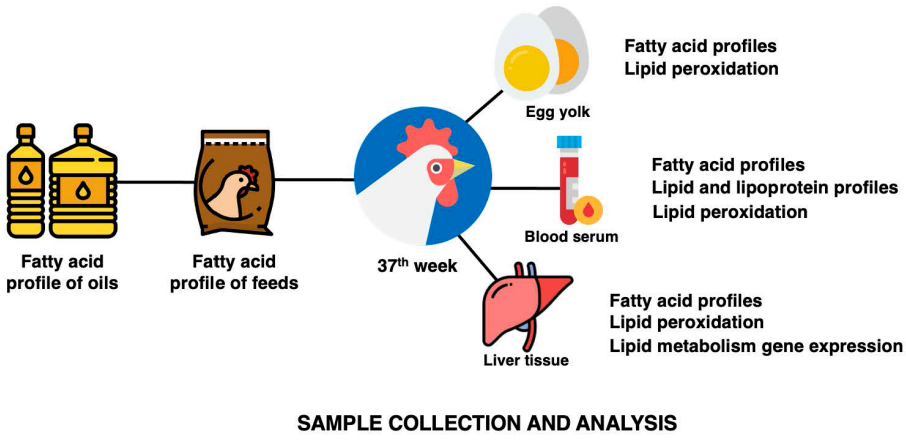

FIGURE 3
The summary of sample collection from oils, feeds, and hens and its analysis.

and the lowest ($p < 0.05$) in PKO (16.72%). The ratio of USFA to SFA, MUFA to SFA and PUFA to SFA was significantly highest ($p < 0.05$) in SBO, followed by RBD, RPO, CPO and PKO.

3.2. Fatty acid profiles of feeds

There were significant differences ($p < 0.05$) in the fatty acid profiles of the feed containing different oils (Table 4). The SFA of feed was significantly highest ($p < 0.05$) in PKO, followed by CPO, RPO and RBD, and the lowest ($p < 0.05$) fraction was in SBO. The USFA had an inverse trend to the SFA in which SBO had the significantly highest ($p < 0.05$) fraction, followed by RBD and RPO, CPO and the lowest ($p < 0.05$) in PKO. The significantly highest ($p < 0.05$) fraction of MUFA was found in RPO and RBD, with no difference ($p > 0.05$) between each other, followed by CPO, SBO and PKO. Total PUFA and linoleic acid (C18:2n-6) concentrations were significantly higher (P0.05) in SBO and lower (P0.05) in PKO. In palm oils (CPO, RPO and RBD), there is a significantly higher ($p < 0.05$) total PUFA and linoleic acid (C18:2n-6) in RPO compared to CPO, with no difference ($p > 0.05$) between RPO and RBD or between CPO and RBD. The linolenic acid (C18:3n-3) had the significantly highest ($p < 0.05$) concentration in SBO compared to other oils. The ratio of USFA to SFA was significantly highest ($p < 0.05$) in SBO, followed by RBD and RPO, CPO and the lowest ($p < 0.05$) ratio in PKO.

3.3. Lipid profiles and lipase enzyme activity of serum

There were no significant differences ($p > 0.05$) in the serum cholesterol (TC), triacylglycerol (TGL), low-density lipoprotein (LDL)

TABLE 2 The forward and reverse of primer sequence, product size, accession number of target genes.

Target gene	Primer sequence	Product size (bp)	Accession number
GAPDH F	CTGGCAAAGTCCAAGTGGTG	275	NM_204305.1
GAPDH R	AGCACCACCCTTCAGATGAG		
APOB F	AGGTGGTGGTGAAGAGGTGGAGAG	97	NM_001044633.1
APOB R	GAGCAGCAAGAGCCGCACAG		
PPAR-α F	TGCTGTGGAGATCGTCCTGGTC	166	NM_001001464.1
PPAR-α R	CTGTGACAAGTTGCCGGAGGTC		
PPAR-γ F	TACATAAAGTCCTTCCCTCTGACC	470	NM_001001460.1
PPAR-γ R	TCCAGTGCATTGAACTTCACAGC		
SREBP-2 F	CCCAGAACAGCAAGCAAGG	108	XM_040660556.1
SREBP-2 R	GCGAGGACAGGAAAGAGAGTG		
apoVLDL2 F	ATGGTGCAATACAGGGCATT	196	NM_205483.2
apoVLDL2 R	GGGAAACATCCAGCAAGAAC		
LDLR F	CGCGTCCGGCTCCATATC	457	NM_204452.1
LDLR R	CTCGCAGCCCCACTCATCC		

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; APOB, apolipoprotein B-100; PPAR-α, peroxisome proliferator-activated receptor-alpha; PPAR-γ, peroxisome proliferator-activated receptor gamma; SREBP2, sterol regulatory element-binding protein 2; APOVLDL2, apolipoprotein VLDL-II; LDLR, low-density lipoprotein receptor; F, forward; R, reverse.

TABLE 3 The fatty acid profiles of oils.

	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
Caprylic acid (C8:0)	0.019^{b}	0.014^{b}	0.010^{b}	2.680^{a}	0.009^{b}	0.285	<0.001
Caproic acid (C10:0)	0.021^{b}	0.019^{b}	0.015^{b}	2.776^{a}	0.006^{b}	0.296	<0.001
Lauric acid (C12:0)	0.521^{b}	0.452^{b}	0.392^{b}	44.61^{a}	0.422^{b}	4.722	<0.001
Myristic acid (C14:0)	0.900^{b}	0.856^{b}	0.843^{b}	16.20^{a}	0.152^{c}	1.660	<0.001
Palmitic acid (C16:0)	55.54^{a}	51.85^{b}	49.80^{c}	13.44^{e}	22.37^{d}	4.606	<0.001
Stearic acid (C18:0)	3.833^{b}	3.812^{b}	3.547^{c}	2.373^{d}	5.529^{a}	0.270	<0.001
Oleic acid (C18:1-cis)	34.37^{c}	37.71^{b}	40.76^{a}	16.71^{e}	29.84^{d}	2.249	<0.001
Linoleic acid (C18:2n-6)	4.606^{d}	5.087^{c}	5.564^{b}	1.420^{e}	37.22^{a}	3.554	<0.001
Linolenic acid (C18:3n-3)	0.126^{c}	0.139^{b}	0.069^{d}	0.003^{e}	4.302^{a}	0.451	<0.001
SFA	60.87^{b}	57.04^{c}	53.58^{d}	81.86^{a}	28.54^{e}	4.557	<0.001
USFA	39.13^{d}	42.96^{c}	46.42^{b}	18.14^{e}	71.46^{a}	4.557	<0.001
MUFA	34.39^{c}	37.73^{b}	40.79^{a}	16.72^{e}	29.88^{d}	2.251	<0.001
PUFA	4.735^{d}	5.230^{c}	5.637^{b}	1.424^{e}	41.58^{a}	4.010	<0.001
USFA:SFA	0.643^{d}	0.753^{c}	0.867^{b}	0.222^{e}	2.504^{a}	0.210	<0.001
MUFA:SFA	0.565^{d}	0.662^{c}	0.762^{b}	0.204^{e}	1.047^{a}	0.073	<0.001
PUFA:SFA	0.078^{d}	0.092^{c}	0.105^{b}	0.017^{e}	1.457^{a}	0.148	<0.001

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; SFA, saturated fatty acid; USFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. ^{a,b,c,d,e}Means with different superscripts in the same row depict significant differences ($p < 0.05$). Experimental unit, $n = 3$.

and high-density lipoprotein (HDL; Table 5). There were significant differences ($p < 0.05$) in the concentration of very low-density lipoprotein (VLDL) and lipase enzyme activity. Significantly higher ($p < 0.05$) lipase enzyme activity was observed in the CPO, RBD, and PKO compared to the SBO. There was a significant difference ($p < 0.05$) in lipase enzyme activity between RPO and SBO. The serum VLDL was significantly affected ($p < 0.05$) by different dietary supplementations of oils. Significantly higher ($p < 0.05$) serum VLDL concentrations were observed in CPO as compared to RPO and SBO but not different ($p > 0.05$) to RBD and PKO. Serum VLDL did not significantly differ ($p > 0.05$) between RPO, RBD, PKO and SBO.

3.4. Lipid peroxidation in serum, yolk, and liver

The TBARS value of serum, yolk and liver did not differ between different oils (Table 6).

TABLE 4 Fatty acid profiles of feeds containing different oils.

	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
Caprylic acid (C8:0)	0.012^{b}	0.000^{b}	0.000^{b}	2.245^{a}	0.036^{b}	0.240	<0.001
Caproic acid (C10:0)	0.010^{b}	0.000^{b}	0.000^{b}	1.976^{a}	0.035^{b}	0.210	<0.001
Lauric acid (C12:0)	0.264^{b}	0.206^{b}	0.171^{b}	27.95^{a}	0.476^{b}	2.962	<0.001
Myristic acid (C14:0)	0.637^{c}	0.600^{c}	0.578^{c}	9.527^{a}	5.654^{b}	0.973	<0.001
Palmitic acid (C16:0)	45.57^{a}	41.80^{b}	41.82^{b}	18.26^{d}	23.62^{c}	2.962	<0.001
Stearic acid (C18:0)	3.948^{b}	3.852^{b}	3.851^{b}	3.226^{b}	4.875^{a}	0.167	0.0086
Oleic acid (C18:1)	33.85^{b}	35.85^{a}	36.46^{a}	21.85^{d}	28.89^{c}	1.467	<0.001
Linoleic acid (C18:2n-6)	14.91^{c}	17.80^{b}	16.27^{bc}	14.18^{c}	34.95^{a}	2.090	<0.001
Linolenic acid (C18:3n-3)	0.798^{b}	0.894^{b}	0.850^{b}	0.780^{b}	3.234^{a}	0.260	<0.001
SFA	50.44^{b}	46.46^{c}	46.42^{c}	63.19^{a}	34.10^{d}	2.507	<0.001
USFA	49.56^{c}	53.54^{b}	53.58^{b}	36.81^{d}	65.90^{a}	2.507	<0.001
MUFA	33.85^{b}	35.85^{a}	36.46^{a}	21.85^{d}	28.40^{c}	1.480	<0.001
PUFA	15.71^{cd}	18.20^{b}	17.12^{bc}	14.96^{d}	37.50^{a}	2.278	<0.001
USFA:SFA	0.983^{c}	1.155^{b}	1.154^{b}	0.583^{d}	1.934^{a}	0.118	<0.001

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; SEM, standard error of means; SFA, saturated fatty acid; USFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; PUFA3, omega-3 fatty acids; PUFA6, omega-6 fatty acids. ^{a,b,c,d}Means with different superscripts in the same rows depict significant differences ($p < 0.05$). Experimental unit, $n = 3$.

TABLE 5 Serum lipid profiles and lipase in laying hens fed different oils.

Treatment	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
TC (mmol/L)	3.463	3.079	2.521	3.184	2.883	0.158	0.4610
TAG (mmol/L)	8.741	8.714	8.622	8.389	8.731	0.076	0.6243
LDL (mmol/L)	0.974	0.915	0.803	0.977	0.871	0.049	0.8365
HDL (mmol/L)	0.999	0.859	0.846	1.093	0.925	0.055	0.6692
VLDL (μg/ml)	0.803^{a}	0.579^{b}	0.611^{ab}	0.659^{ab}	0.539^{b}	0.033	0.0490
Lipase (U/L)	41.33^{a}	38.66^{ab}	41.92^{a}	41.95^{a}	34.68^{b}	0.901	0.0131

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil. SEM, standard error of means; TC, cholesterol; TAG, triacylglycerol; LDL, low-density lipoprotein; HDL, high-density lipoprotein. ^{a,b}Means with different superscripts in the same rows depict significant differences ($p < 0.05$). Experimental unit, $n = 6$.

TABLE 6 Thiobarbituric acid reactive substance (TBARS) of serum, yolk, and liver in laying hens fed different oils.

Treatment	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
Serum (μg/ml MDA)	3.118	4.929	5.169	2.802	4.935	0.411	0.185
Yolk (μg/g MDA)	53.36	53.36	43.41	43.74	54.02	1.835	0.099
Liver (μg/g MDA)	42.03	40.85	32.14	45.95	36.28	1.768	0.089

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; SEM, standard error of means. Experimental unit, $n = 6$.

3.5. Fatty acid profiles of liver

There were significant differences ($p < 0.05$) in most of the profiles except for palmitic (C16:0) and elaidic (C18:1-trans) acids, total SFA, USFA, and the ratio of USFA to SFA (Table 7). Dietary supplementations of PKO significantly increased ($p < 0.05$) lauric (C12:0) and myristic (C14:0) acids. There was no significant difference ($p > 0.05$) in lauric acid (C12:0) between CPO, RPO and RBD or between SBO and RPO. There was no significant difference ($p > 0.05$) in myristic acid (C14:0) between CPO, RPO, RBD and SBO. Significantly higher ($p < 0.05$) stearic (C18:0), linoleic (C18:2n-6) and linolenic (C18:3n-3) acids were found in SBO than other oils. Significantly higher ($p < 0.05$) MUFA fraction in RPO with no difference ($p > 0.05$) in RBD and lowest ($p < 0.05$) in PKO and SBO. The SBO showed higher total PUFA, linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) than other dietary supplementations of oils.

3.6. Fatty acid profiles of serum

All fatty acid profiles showed a significant difference ($p < 0.05$) between treatment groups (Table 8). In MCFA, lauric acid (C12:0) was

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TABLE 7 Fatty acid profiles of the liver in laying hens fed different oils.

	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
Lauric acid (C12:0)	0.138^{c}	0.191^{bc}	0.118^{c}	0.691^{a}	0.333^{b}	0.045	<0.0001
Myristic acid (C14:0)	0.544^{b}	0.558^{b}	0.492^{b}	2.215^{a}	0.503^{b}	0.124	<0.0001
Palmitic acid (C16:0)	41.15	38.83	41.13	40.76	38.31	0.414	0.0569
Palmitoleic acid (C16:1)	1.206^{abc}	1.000^{bc}	1.239^{ab}	1.582^{a}	0.753^{c}	0.081	0.0101
Stearic acid (C18:0)	13.60^{b}	12.39^{b}	13.29^{b}	13.61^{b}	16.35^{a}	0.381	0.0138
Elaidic acid (C18:1-trans)	0.613	0.476	0.765	0.669	0.779	0.070	0.6754
Oleic acid (C18:1)	33.37^{bc}	37.61^{a}	34.91^{ab}	31.48^{cd}	29.46^{d}	0.708	0.0003
Linoleic acid (C18:2n-6)	5.872^{b}	7.388^{b}	6.311^{b}	6.539^{b}	11.814^{a}	0.464	<0.0001
Linolenic acid (C18:3n-3)	0.129^{b}	0.188^{b}	0.142^{b}	0.132^{b}	0.484^{a}	0.031	<0.0001
SFA	55.43	51.96	55.02	57.58	54.67	0.661	0.1054
USFA	44.57	48.04	44.98	42.88	45.33	0.661	0.1853
MUFA	35.35^{b}	39.09^{a}	36.91^{ab}	33.73b^{c}	30.99^{c}	0.710	0.0005
PUFA	7.656^{b}	8.948^{b}	8.069^{b}	8.685^{b}	14.34^{a}	0.511	<0.0001
USFA:SFA	0.812	0.925	0.821	0.737	0.846	0.022	0.1116

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; SEM, standard error of means; SFA, saturated fatty acid; USFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. PUFA3, omega-3 fatty acids; PUFA6, omega-6 fatty acids; TRANS, trans fatty acids. ^{a,b,c,d,e}Means with different superscripts in the same rows depict significant differences ($p < 0.05$). Experimental unit, $n = 6$.

TABLE 8 Fatty acid profiles of serum in laying hens fed different oils.

	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
Lauric acid (C12:0)	0.094^{c}	0.582^{b}	0.133^{bc}	1.862^{a}	0.355^{bc}	0.137	<0.0001
Myristic acid (C14:0)	0.481^{c}	0.692^{bc}	0.429^{c}	2.035^{a}	0.911^{b}	0.123	<0.0001
Palmitic acid (C16:0)	40.78^{bc}	39.75^{c}	42.18^{ab}	43.28^{a}	41.57^{abc}	0.361	0.0145
Palmitoleic acid (C16:1)	1.456^{ab}	1.276^{bc}	1.156^{bc}	1.788^{a}	0.869^{c}	0.087	0.0061
Stearic acid (C18:0)	11.16^{b}	11.15^{b}	13.66^{a}	13.30^{a}	13.94^{a}	0.365	0.0117
Elaidic acid (C18:1-trans)	1.050^{a}	0.981^{a}	0.913^{a}	1.026^{a}	0.740^{b}	0.029	0.0008
Oleic acid (C18:1)	39.12^{a}	39.27^{a}	35.68^{ab}	33.01^{bc}	30.28^{c}	0.813	<0.0001
Linoleic acid (C18:2n-6)	5.710^{b}	6.146^{b}	5.678^{b}	6.017^{b}	9.420^{a}	0.278	<0.0001
Linolenic acid (C18:3n-3)	0.146^{b}	0.154^{b}	0.172^{b}	0.156^{b}	0.410^{a}	0.020	<0.0001
SFA	52.52^{b}	52.18^{b}	56.40^{a}	58.45^{a}	58.28^{a}	0.717	0.0013
USFA	47.48^{a}	47.82^{a}	43.60^{b}	43.27^{b}	41.72^{b}	0.619	0.0004
MUFA	41.62^{a}	41.52^{a}	37.75^{b}	35.44^{b}	31.89^{c}	0.849	<0.0001
PUFA	5.856^{b}	6.300^{b}	5.850^{b}	6.117^{b}	9.830^{a}	0.297	<0.0001
USFA:SFA	0.904^{a}	0.918^{a}	0.775^{b}	0.719^{b}	0.723^{b}	0.022	0.0004

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; SEM, standard error of means; SFA, saturated fatty acid; USFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; PUFA3, omega-3 fatty acids; PUFA6, omega-6 fatty acids. ^{a,b,c}Means with different superscripts in the same rows depict significant differences ($p < 0.05$). Experimental unit, $n = 6$.

significantly higher ($p < 0.05$) in SBO, and myristic acid (C14:0) was the highest ($p < 0.05$) in PKO. The stearic acid (C18:0) was significantly higher ($p < 0.05$) in RBD, PKO and SBO than in CPO and RPO. The SFA was significantly higher ($p < 0.05$) in RBD, PKO and SBO than in CPO and RPO. The USFA was significantly higher ($p < 0.05$) in CPO and RPO and lower ($p < 0.05$) in RBD, PKO and SBO. The MUFA was

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TABLE 9 Fatty acid profiles of egg yolk in laying hens fed different oils.

	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
Lauric acid (C12:0)	0.521^{b}	0.562^{b}	0.490^{b}	0.833^{a}	0.434^{b}	0.047	0.0348
Myristic acid (C14:0)	0.493^{b}	0.445^{b}	0.415^{b}	2.949^{a}	0.399^{b}	0.269	<0.001
Palmitic acid (C16:0)	40.62^{a}	39.87^{ab}	38.84^{c}	40.16^{a}	39.18^{ac}	0.196	0.0029
Palmitoleic acid (C16:1)	2.085^{b}	1.926^{b}	1.862^{b}	2.511^{a}	1.546^{c}	0.088	<0.001
Stearic acid (C18:0)	7.366^{b}	7.182^{b}	7.492^{b}	7.591^{b}	8.601^{a}	0.150	0.0024
Oleic acid (C18:1)	42.41^{b}	43.03^{b}	44.09^{a}	39.07^{c}	36.54^{d}	0.757	<0.001
Linoleic acid (C18:2n-6)	6.346^{b}	7.102^{b}	6.637^{b}	6.679^{b}	12.62^{a}	0.644	<0.001
Linolenic acid (C18:3n-3)	0.158^{c}	0.188^{bc}	0.174^{bc}	0.207^{b}	0.678^{a}	0.053	<0.001
SFA	49.00^{b}	48.06^{c}	47.24^{d}	51.53^{a}	48.61^{bc}	0.397	<0.001
USFA	51.00^{c}	51.94^{b}	52.76^{a}	48.47^{d}	51.39^{bc}	0.397	<0.001
MUFA	44.50^{b}	44.96^{b}	45.95^{a}	41.58^{c}	38.09^{d}	0.771	<0.001
PUFA	6.504^{b}	6.985^{b}	6.811^{b}	6.886^{b}	13.30^{a}	0.701	<0.001
USFA:SFA	1.041^{c}	1.081^{b}	1.117^{a}	0.940^{d}	1.057^{bc}	0.016	<0.001

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; SEM, standard error of means; SFA, saturated fatty acid; USFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; PUFA3, omega-3 fatty acids; PUFA6, omega-6 fatty acids. ^{a,b,c,d}Means with different superscripts in the same rows depict significant differences ($p < 0.05$). Experimental unit, $n = 6$.

TABLE 10 Liver lipid metabolism gene expression in laying hens fed different oils in relative to SBO.

Treatment	CPO	RPO	RBD	PKO	SBO	SEM	*p*-value
APOB	1.228^{bc}	1.386^{b}	1.973^{a}	0.266^{c}	1.000^{bc}	0.225	0.005
PPAR-α	0.257^{b}	0.273^{b}	0.395^{b}	0.479^{b}	1.000^{a}	0.079	0.001
PPAR-γ	0.022^{c}	0.120^{c}	0.129^{c}	0.311^{b}	1.000^{a}	0.096	<0.001
SREBP-II	1.143	1.199	1.304	0.819	1.000	0.121	0.805
apoVLDL2	7.780^{a}	5.088^{a}	6.325^{a}	2.029^{b}	1.000^{b}	0.894	0.001
LDLR	0.052^{c}	0.130^{c}	0.222^{bc}	0.380^{b}	1.000^{a}	0.095	<0.001

CPO, crude palm oil; RPO, red palm oil; RBD, refined palm oil; PKO, palm kernel oil; SBO, soybean oil; SEM, standard error of means; APOB, apolipoprotein B-100; PPAR-α, peroxisome proliferator-activated receptor-alpha; PPAR-γ, peroxisome proliferator-activated receptor gamma; SREBP2, sterol regulatory element-binding protein 2; APOVLDL2, apolipoprotein VLDL-II; LDLR, low-density lipoprotein receptor. ^{a,b,c}Means with different superscripts in the same rows depict significant differences ($p < 0.05$). Experimental unit, $n = 6$.

significantly higher ($p < 0.05$) in CPO and RPO, followed by RBD and PKO and lowest ($p < 0.05$) in SBO. The total PUFA, linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) were significantly higher ($p < 0.05$) in SBO compared to other oils. The trans fatty acid (elaidic acid; C18:1-trans) was significantly lower ($p < 0.05$) in SBO. Serum USFA:SFA was significantly higher ($p < 0.05$) in CPO and RPO than in RBD, PKO and SBO.

3.7. Fatty acid profiles of egg yolk

There were significant differences ($p < 0.05$) in the yolk fatty acid profiles between the treatments (Table 9). The lauric (C12:0) and myristic (C14:0) acids were significantly higher ($p < 0.05$) in PKO. The SFA was significantly higher ($p < 0.05$) in PKO, followed by CPO, SBO, RPO, and the lowest ($p < 0.05$) in RBD, with no difference ($p > 0.05$) between SBO with CPO and RPO. The USFA was significantly highest ($p < 0.05$) in RBD, followed by CPO, RPO and lowest ($p < 0.05$) PKO. The SBO had no difference ($p > 0.05$) from the CPO and RPO in the USFA. The MUFA was significantly highest ($p < 0.05$) in RBD, followed by CPO and RPO, PKO, and the lowest ($p < 0.05$) in SBO. The total PUFA, linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) were significantly higher ($p < 0.05$) in SBO than in other oils. The USFA:SFA was significantly higher ($p < 0.05$) in RBD and lowest ($p < 0.05$) in PKO. There was no significant difference ($p > 0.05$) in SBO compared to CPO and RPO. No trans fatty acids (elaidic acid; C18:1-trans) were detected in the egg yolk.

3.8. Liver lipid metabolism genes

The significant difference ($p < 0.05$) in the regulation of gene expression between oils was observed in apolipoprotein B-100 (APOB), peroxisome proliferator-activated receptor-alpha (PPAR-α), peroxisome proliferator-activated receptor gamma (PPAR-γ), apolipoprotein VLDL-II (apoVLDL2) and low-density lipoprotein receptor (LDLR) genes (Table 10). However, the sterol regulatory element-binding protein 2 (SREBP-II) gene did not significantly differ

($p > 0.05$) in expression. The APOB was significantly upregulated ($p < 0.05$) in RBD and had no difference ($p > 0.05$) in regulation in other types of oils. Significantly lower ($p < 0.05$) expression of PPAR-α, PPAR-γ and LDLR was observed in the palm oil groups (CPO, RPO and RBD) relative to SBO. The apoVLDL2 was significantly upregulated ($p < 0.05$) in CPO, RPO and RBD compared to PKO and SBO. There was no significant difference ($p > 0.05$) in apoVLDL2 expression between PKO and SBO.

4. Discussion

4.1. Fatty acid profiles of oils and feeds

There was a reduction in the percentage of palmitic (C16:0), stearic (C18:0), linolenic (C18:3) and SFA and an increase in oleic (C18:1-cis), linoleic (C18:2), USFA, MUFA and PUFA across the CPO, RPO and RBD. Removal of the solid fraction (palm stearin) from CPO to obtain the liquid fraction (palm olein) to produce RPO and RBD would be the primary cause of the fatty acid composition shift. Palm stearin fraction had a higher SFA and lower USFA fraction than palm olein (25). The further fractionation processes of CPO to RPO through molecular distillation and CPO to RBD through refining, bleaching and deodorization also impacted the fatty acid profiles of the oils. It is worth noting that PKO had the highest SFA, mainly in the form of MCFA, compared to palm oils (CPO, RPO and RBD). The PKO originated from the kernel and has a different fatty acid profile composition than oil extracted from the mesocarp of the palm fruit such as CPO, RPO and RBD. The composition of omega-3 (linolenic acid; C18:3n-3) and omega-6 (linoleic acid; C18:2n-6) fatty acids in the oils and their inclusion in the diet of poultry to promote performance, health, and healthier products are receiving great attention. The SBO had superior characteristics in terms of n-3 and n-6 fatty acid content compared to palm oils (CPO, RPO and RBD) and PKO. Therefore, the presence of higher omega-3 and omega-6 fatty acids in oils and the poultry diet is beneficial to the birds and may yield poultry products such as meat and eggs enriched with n-3 and n-6 fatty acids and concurrently deliver the benefits to consumers (26).

The fatty acid profile of feeds was greatly dependent on the oil source. Khatun et al. (9) reported that adding palm oil and SBO to the diet of poultry influences the fatty acid composition of the feeds. Feed containing palm oils (CPO, RPO and RBD) had higher SFA contributed by higher palmitic (C16:0) and stearic (C18:0) acids. The increment was contributed by the higher percentage of palmitic acid (C16:0) in about 50–55% of palm oil (19, 27). Khatun et al. (9) also reported an increase in SFA and palmitic acid (C16:0) in the feed containing palm oil compared to SBO. Feed with PKO inclusion had a higher 63% SFA contributed by palmitic acid (C16:0) and MCFA such as lauric acid (C12:0) and myristic acid (C14:0). It was contributed by PKO, which contains higher MCFA, mainly lauric acid (C12:0) and myristic acid (C14:0) (27). The addition of SBO in the feed enhanced the USFA and PUFA fractions up to 66 and 38%, respectively, mainly due to oleic acid (C18:1), linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3). The increment was contributed by higher USFA, PUFA, oleic acid (C18:1) and linoleic acid (C18:2n-6) in the SBO (19). Khatun et al. (9) also reported an increase in USFA, PUFA, oleic acid (C18:1) and linoleic acid (C18:2n-6) in the feed containing SBO as compared to palm oils.

4.2. Lipid profile and lipase enzyme activity of serum

The dietary supplementation of different oils did not influence the lipid biomarkers such as TC, TAG, LDL and HDL but affected VLDL. Our results corroborated Agboola et al. (28), who reported no difference in serum TAG, HDL, LDL and TC between laying hens fed 1.5% palm oil and 1.5% SBO. Furthermore, Agboola et al. (29) reported no difference in egg yolk TC, TAG, LDL, HDL, and VLDL between laying hens fed 1.5% palm oil and 1.5% SBO. However, Yifei et al. (30) reported no difference in serum cholesterol between 3% RPO and 3% SBO but lower serum TAG in 3% RPO compared to 3% SBO in laying ducks. A previous study showed that the increment in cholesterol increased as the level of oil increased. Kolani et al. (31) offered RBD at different levels from 0, 1, 2, to 3% in laying hens and found no effects on serum TAG, but TC was higher at 3% compared to 0 and 1% inclusion levels. Thus, the lack of difference in serum CHOL concentration in the current study could be linked to the similar inclusion of oil in the diet. Dietary fatty acids contribute to cholesterol synthesis, and an increase in dietary fatty acids contributes to higher cholesterol synthesis (31).

The liver synthesizes the VLDL and carries triacylglycerols and cholesterol in the blood for supply to the body's tissues. The serum VLDL concentrations in the current study were higher in CPO than in RPO and SBO. The reasons for the higher serum VLDL between CPO and SBO could be related to the higher SFA contributed by long-chain fatty acids such as palmitic (C16:0) and stearic (C18:0) in CPO compared to the lower SFA in SBO. Similarly, Khatun et al. (4) found higher serum VLDL concentrations in broiler chickens fed 6% palm oil compared to 6% SBO. Crespo and Esteve-Garcia (32) also found higher serum VLDL concentrations in broiler chickens supplemented with tallow rich in SFA (50%), mainly palmitic (C16:0) and stearic (C18:0) acids compared to high USFA and PUFA-rich oils such as olive, sunflower and linseed oils. Despite higher SFA in the diet of PKO, there was no difference in serum VLDL concentration compared to SBO. The SFA in PKO was comprised of MCFA such as caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0) which may contribute to the lack of difference in serum VLDL in the SBO. Khatun et al. (4) suggested dietary PUFA reduced chylomicron secretion in intestinal cells and reduced the synthesis of fatty acids and triacylglycerols in the liver. Thus, an increase in the fatty acid saturation in the diet of chickens contributed to the increase in fatty acid synthesis and fat deposition (9).

In addition, the serum lipase was higher in palm oils (CPO and RBD) and PKO than in SBO. The contribution of the increment in serum lipase enzyme activity may be correlated to the SFA in palm oils and PKO. The SFA in palm oil is rich in C16:0 (palmitic acid) and the SFA in PKO is rich in MCFA in the form of lauric acid (C12:0) and myristic acid (C14:0). Lipase is an enzyme in the blood plasma that hydrolyses triacylglycerol into free fatty acids and glycerol. Dietary SFA increased the serum VLDL that carries triacylglycerols in the

blood (9, 32) and increased serum lipase enzyme activity for the deposition of triacylglycerols in tissues.

4.3. Lipid peroxidation in serum, yolk, and liver

The secondary lipid peroxidation products, such as MDA, indicate lipid oxidation and oxidative stress (33). A higher concentration of lipid oxidation products indicates higher oxidation, leading to an increase in inflammation and oxidative stress (34). Naturally occurring antioxidant compounds, such as vitamins, react with oxidants to prevent further oxidation and reduce oxidative stress. Tavárez et al. (35) The addition of in-feed antioxidants prevented further oxidizing of lipids, improved broiler performance and enhanced the meat's shelf life even with dietary oxidized oil. Abdulla et al. (36) reported a higher concentration of MDA in the breast meat of broiler chickens fed high USFA oils such as SBO and linseed oil than palm oil.

However, the current study revealed that different oils did not affect the lipid peroxidation in serum, yolk and liver despite the differences in fatty acid composition and the presence of antioxidants such as vitamin E and carotenoids in the feed. It could be linked to the similar concentration of antioxidants such as retinol and tocopherol in the serum, liver and yolk of laying hens fed CPO, RPO, RBD, PKO and SBO (22). The lack of effects may also be attributed to a similar inclusion level of oil and the sufficient protection capacity provided by the antioxidants and vitamins in the feed. However, the increase in the lipid content in the diet increased the lipid oxidation of the yolk, as Yeasmin et al. (37) reported that the increase in dietary CPO from 1.5, 3 to 5% markedly increased the lipid peroxidation of the egg yolk.

4.4. Fatty acid profiles of liver

Higher SFA mainly from palmitic acid (C16:0) in palm oil and higher MCFA from lauric acid (C12:0) and myristic acid (C14:0) in PKO did not contribute to the difference in liver SFA and palmitic acid (C16:0). Conversely, higher USFA in the diet of SBO did not contribute to higher liver USFA. The lack of difference in the palmitic acid (C16:0) in the liver was similar to that in the serum. However, Khatun et al. (38) found higher liver SFA and palmitic acid (C16:0) in broiler chickens fed palm oil compared to SBO. The fatty acids supplied by dietary oil influence the fatty acid deposition in the liver tissue of broiler chickens (9, 38). Our finding on the lack of effects despite the difference in fatty acid saturation of different oils was attributed to *de novo* fatty acid production in the liver, which determines the regulation of fatty acid production. The predominant fatty acids in the liver are palmitic (C16:0) and stearic (C18:0) acids for SFA, oleic (C18:1) and palmitoleic (C16:1) acids for MUFA and linoleic (C18:2n-6) and linolenic (C18:3n-3) acids for PUFA (39).

Dietary supplementation of PKO increased the MCFA in the liver, such as lauric acid (C12:0) and myristic acid (C14:0). The increment of MCFA in the liver was similar to the higher MCFA in the serum. No previous study reported the effects of dietary PKO on liver fatty acid profiles in laying hens. The increase could be related to higher lauric acid (C12:0) and myristic acid (C14:0) in PKO (27), which contributed to the higher deposition of such fatty acids in the liver.

There was no difference in USFA in the liver between different oils, but PUFA, linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3) were higher in SBO. The increment of liver PUFA, linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3) in the liver was similar to that in the serum. Similarly, Khatun et al. (38) found higher liver PUFA, linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3) in broiler chickens fed SBO compared to palm oil. Despite SBO contributing high USFA to the diet, the lack of difference in liver USFA was contributed by the higher values of liver palmitoleic acid (C16:1) and oleic acid (C18:1) in palm oils and PKO and the higher values of liver stearic acid (C18:0) in SBO that balanced the USFA. The increase in liver PUFA, linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3) in the SBO group was contributed by the higher percentages of linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3) in SBO (9).

4.5. Fatty acid profiles of serum

Fatty acids present in the serum are composed of digested and absorbed fatty acids from the intestinal tract and *de novo* synthesis of fatty acids by the liver. Dietary supplementation of palm oils (CPO, RPO and RBD) did not affect serum palmitic acid (C16:0) compared to SBO. However, serum SFA and stearic acid (C18:0) were higher in PKO and SBO than in palm oil (CPO and RPO). Higher feed SFA and stearic acid (C18:0) in PKO contributed to the increase in serum SFA and stearic acid (C18:0). However, no difference in serum palmitic acid (C16:0) between oils and higher serum stearic acid in SBO was contributed by *de novo* synthesis of fatty acids in the liver. The liver synthesizes palmitic (C16:0) and stearic (C18:0) acids for SFA, oleic (C18:1) and palmitoleic (C16:1) acids for MUFA, and linoleic (C18:2n-6) and linolenic (C18:3n-3) acids for PUFA as the primary fatty acids for the body (39).

The PKO contributed to an increment in serum lauric (C12:0) and myristic (C14:0) acids. The increment of MCFA in the serum was similar to the higher MCFA in the liver. No previous study reported the effects of dietary PKO on serum fatty acid profiles in laying hens. The increase could be related to higher levels of lauric acid (C12:0) and myristic acid (C14:0) in PKO (27) that were absorbed into the blood and contributed to the higher levels of these fatty acids in the serum. Dietary supplementation of SBO contributed to lower elaidic acid (C18:1-trans) and higher PUFA, linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) than palm oils (CPO, RPO and RBD) and PKO. The increase in fatty acids in the serum was consistent with the higher levels of fatty acids in the diet and in the liver. The SBO contained higher USFA, PUFA, oleic acid (C18:1) and linoleic acid (C18:2n-6) (19) and contributed them to the diet. Khatun et al. (9) also reported an increase in USFA, PUFA, oleic acid (C18:1) and linoleic acid (C18:2n-6) in the feed containing SBO as compared to palm oils.

4.6. Fatty acid profiles of yolk

There were no differences in yolk palmitic acid (C16:0) between palm oil (CPO, RPO and RBD) and PKO to SBO despite the higher palmitic acid (C16:0) in the feed containing palm oils. Khatun et al. (9) reported higher levels of palmitic acid (C16:0) in feed containing palm oil than SBO. The lack of difference in yolk palmitic acid (C16:0)

was consistent with the lack of difference in serum and liver palmitic acid (C16:0). Similar to serum and liver; there was no difference in yolk palmitic acid (C16:0) between oils and the higher yolk palmitic acid in SBO was contributed by the *de novo* synthesis of fatty acids in the liver that regulates the production of each fatty acid. The liver is synthesizing palmitic (C16:0) and stearic (C18:0) acids for SFA, oleic (C18:1) and palmitoleic (C16:1) acids for MUFA and linoleic (C18:2n-6) and linolenic (C18:3n-3) acids for PUFA as the main fatty acids for the body (39).

Dietary-specific fatty acids and *de novo* regulation of fatty acid production and packaging of lipoprotein in the liver determined the composition of fatty acid profiles in the egg yolk. Dietary supplementation with PKO increased the deposition of the lauric (C12:0) and myristic (C14:0) acids in egg yolk. The increment in the deposition of lauric (C12:0) and myristic (C14:0) acids in the yolk was consistent with the high lauric (C12:0) and myristic (C14:0) acids in the feed, liver and serum of PKO. The PKO is known to contain high levels of lauric acid (C12:0) and myristic acid (C14:0) (27).

Similarly, dietary supplementation of SBO contributed to the increment of egg yolk stearic acid (C18:0), PUFA, linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6). The increase was consistent with the higher PUFA, PUFA, linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) in the feed, serum and liver containing SBO. The SBO contained higher levels of stearic acid (C18:0), PUFA, oleic acid (C18:1) and linoleic acid (C18:2n-6) (19), and the inclusion of SBO increased USFA, PUFA, oleic acid (C18:1) and linoleic acid (C18:2n-6) in the feed (9). However, Agboola et al. (29) reported no difference in yolk palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2n-6) and linolenic (C18:3n-3) acids, total SFA, MUFA and PUFA in laying hens fed 1.5% palm oil (RBD) and 1.5% SBO. The lower inclusion level of RBD and SBO might have less effect on the yolk fatty acid profiles due to the liver's *de novo* regulation of fatty acid production (39).

4.7. Liver lipid metabolism genes

The PPAR-α regulates lipid metabolism in the liver, including fatty acid oxidation to produce energy. The presence of fatty acids activates the PPAR-α, which triggers β-oxidation for ketogenesis enhancement and adenosine triphosphate production (40). The PPAR-γ is highly expressed in adipocytes and lower in the liver and muscle. High expression of PPAR-γ in the liver may induce the emergence of lipid droplets due to the regulation of several proteins related to the uptake and storage of triacylglycerols (40, 41). In this study, liver PPAR-α and PPAR-γ showed a similar trend of downregulation in palm oils and PKO relative to the SBO. Higher regulation of PPAR-α and PPAR-γ in SBO might be attributed to the higher PUFA fraction in the diet contributed by SBO. Ramiah et al. (42) observed upregulation of liver PPAR-α, PPAR-γ, and liver fatty acid-binding protein (L-FABP) genes in broiler chickens supplemented with conjugated linoleic acids. The results suggested the prominent role of PPARs as a vital regulator in the chicken's liver lipid metabolism.

Apolipoprotein B-100 is a major lipoprotein in chicken VLDL that permits the attachment of VLDL, intermediate lipoprotein (IDL) and LDL in the bloodstream to specific receptors on the cell surface (43, 44). The attachment allows the lipoprotein content to be endocytosed into the cell. The current study found that the liver APOB gene was highly regulated in RBD and lowest regulated in PKO, suggesting the contribution of long-chain SFA in inducing higher surface APOB protein production for VLDL, IDL and LDL. This finding was concurrent with higher expression of apoVLDL-2 in palm oils, which co-exists with APOB on VLDL but at a higher number. ApoVLDL-2 is the major apoprotein in VLDL. In laying hens, VLDL is primarily transported from the liver to supply triacylglycerols and cholesterol to the oocytes for subsequent use in the development of the embryo, and apoVLDL2 protein is present in a larger amount than APOB on the VLDL surface (45). Our results revealed that palm oils contributed to higher expression of the liver apoVLDL2 gene and similar gene regulation between PKO and SBO. Therefore, palmitic acid-rich diets of CPO, RPO and RBD contributed to the higher production of apoVLDL2 mRNA and would be linked to the higher production of VLDL. Previous studies showed that broiler chicken fed dietary palm oil (4) and tallow (32) increased serum VLDL compared to a diet with high PUFA.

The SREBP-II regulates the synthesis and cellular uptake of fatty acids and cholesterol. The SREBPB activates the LDLR for cholesterol uptake, and SREBP activates the acetyl-CoA carboxylase and fatty acid synthase for fatty acid synthesis (46). We found similar regulation in the SREBP-II gene between different dietary oils, indicating no effect of fatty acid composition on the SREBP-II gene regulation. However, the current study showed a difference in LDLR expression between different dietary oils. LDLR presents on the cell surface and recognizes ApoB 100 and Apo E and is an essential mediator of the cell to endocytose LDL, chylomicron remnants and IDL that determine the blood plasma concentration of LDL (47). Despite similar regulation of the SREBP-II gene, the LDLR gene was downregulated in palm oils and PKO, with the lowest regulation in CPO and RPO. Therefore, higher regulation of the LDLR gene in SBO might be associated with greater uptake of LDL by the liver to regulate the cholesterol concentration in blood plasma and cholesterol metabolism (48).

5. Conclusion

This study contributed to the knowledge of the influence of feeding palm oil, palm kernel oil and soybean oil with different fatty acid compositions on fatty acid metabolism, and blood lipid profiles in laying hens. It can be concluded that the inclusion of oil greatly influenced the fatty acid composition of the feed, and dietary supplementation with different oils had similar trends in determining fatty acid profiles in the serum, liver and egg yolk. All sources of oils are suitable to be used in laying hens' diet, as no negative effects were observed in the fatty acid profiles, blood lipid profile and lipid peroxidation. The SBO has the advantage of increasing omega-3 and omega-6 fatty acids in the body's tissues. Palm oils did not affect the SFA profiles, but PKO increased the SFA profiles contributed by MCFA. The selection of oils in the diet should be influenced by the target of the producer, such as increasing specific fatty acids in eggs and meats or reducing the cost of feed by choosing cheaper oils such as CPO.

Data availability statement

The original contributions presented in the study are included in the article/supplementary files. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Universiti Putra Malaysia (AUP No: UPM/IACUC/AUP-R013/2020).

Author contributions

WI, TL, HA, NN, AN, and HF designed the experiment. WI carried out the experiment, laboratory work, data analysis and manuscript writing. TL, HA, NN, AN, and HF were involved with manuscript revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of crude protein and non-essential amino acids on growth performance, blood profile, and intestinal health of weaned piglets

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This study investigated the effect of crude protein (CP) and non-essential amino acid (NEAA) supplementation on the growth performance, blood profile, intestinal morphology, mRNA relative abundance of inflammatory and antioxidant markers, and tight junction proteins in piglets over the first 2 weeks after weaning. Ninety 21-day-old piglets (7.55 ± 0.72 kg) were assigned in a randomized block design to one of three dietary treatments: (1) high CP, a diet with 24% CP; (2) low CP, a diet with 18% CP; and (3) low CP + NEAA, a diet with 18% CP supplemented with 5 g/kg Arg (L-arginine; purity >99%) and 10 g/kg Glu + Gln (minimum 10% L-glutamine and minimum 10% L-glutamate). Piglets were fed with corn-soybean meal basal diets in a 14-day trial. There was an improvement ($p < 0.05$) in the feed conversion ratio of piglets fed the high-CP diet compared to treatments with low CP or low CP + NEAA. Serum urea nitrogen was higher ($p < 0.05$) in piglets fed high CP compared to other dietary treatments. In the duodenum, the villus height of animals fed the low-CP + NEAA diets was greater ($p < 0.05$) than those fed with the high- and low-CP diets. The goblet cell proportion of piglets fed low CP + NEAA or high CP was higher ($p < 0.05$) compared to low CP. In the jejunum, the crypt depth of the piglets with the high-CP dietary treatment was greater ($p < 0.05$) in comparison with low CP + NEAA. In the jejunum, IFN-γ mRNA expression was higher ($p < 0.05$) in animals fed the high-CP diets compared to other dietary treatments. However, superoxide dismutase and occludin mRNA expression were higher ($p < 0.05$) in animals fed low CP + NEAA than in piglets on the high-CP diets. In the ileum, the number of Peyer's patches in piglets fed high CP was higher ($p < 0.05$) compared to other dietary treatments. In conclusion, the high-CP diet (24% CP) improves the feed conversion of piglets in the first 2 weeks after weaning compared to the low-CP diet (18% CP) supplemented or not with NEAA. However, the low-CP diet supplemented with NEAA (Arg, Gln, and Glu) improves intestinal health in piglets by promoting greater villus height and proportion of goblet cells in the duodenum, reducing jejunal crypt depth, and reducing Peyer's number patches in the ileum. In addition, piglets that received the low-CP + NEAA diet showed an increase in superoxide dismutase and occludin and a lower expression of IFN-γ mRNA.

KEYWORDS

amino acids, crude protein, intestinal health, non-essential amino acids, weaned piglets

1 Introduction

The gastrointestinal tract of the piglet undergoes several changes during the post-weaning period until it is able to digest plant-based feed ingredients. Therefore, it is crucial for the gastrointestinal tract to regulate the changes caused by the introduction of a solid diet, such as gastric-intestinal pH regulation, enzymatic secretion, and intestinal motility, with the aim of improving digestion and nutrient absorption processes.

Soybean meal (SBM) is the most widely used plant protein source for weaned piglets' diets. The amino acid (AA) profile, balance, and digestibility of SBM are better than any other plant protein source used in swine diets. However, growth performance, intestinal morphology, and immunological status of weaned piglets may be negatively affected due to the presence of antinutritional compounds in this ingredient (1, 2). The reduction of dietary crude protein (CP) coupled with supplementation of industrial AA classified as nutritionally essential (EAA) and non-essential (NEAA) are alternatives to reduce the impacts reported in the post-weaning phase (3–5).

EAA cannot be synthesized by pigs from materials ordinarily available in cells at a rate matching the demands for maintenance, growth, development, and health, which must be provided in the diet to meet the requirements (6). In contrast, NEAAs are AAs that can be synthesized in adequate amounts by the animal organism to meet the requirements for maintenance, growth, development, and health and, therefore, do not need to be provided in the diet (7). During stress, such as health challenges, the synthesis of adequate amounts of NEAAs can be limited by the availability of appropriate amounts of metabolic nitrogen (N) (8). However, NEAAs have a physiological function, and thus the animal may have, in some specific conditions, dietary requirements for NEAAs to support their maximal growth and health (2). Because of an incomplete understanding of AA biochemistry, nutrition, and physiology, the concept of "nutritional non-essentiality" has led to a disregard for the importance of NEAAs in the practice of nutrition (9), resulting in reduced growth performance (5).

Among the NEAAs, arginine (Arg) (10), glutamine (Gln) (11), and glutamate (Glu) (12) can improve the intestinal health of weaned piglets by reducing inflammation and improving the integrity of the intestinal epithelial mucosa. Wu et al. (13) suggested that the effects of Arg are mediated by nitric oxide production and regulation of gene expression related to cell proliferation and differentiation in the intestinal mucosa. Glutamine is the main source of energy for enterocytes, and it is important to maintain the structural and functional integrity of the intestinal mucosa (14). Similarly, Glu is related to increasing the rate of cell proliferation and differentiation and reducing the oxidative stress of intestinal cells by increasing glutathione synthesis (12).

On the other hand, studies have also shown that higher levels of CP in diets for piglets can be beneficial due to the greater contribution of NEAA, peptides, and total N (15–17). According to these authors, CP levels as high as 24% would not compromise piglets' growth performance, although they could reduce gut health. Moreover, according to Rocha et al. (2), there is a minimum CP level after which the growth performance of pigs can be compromised. For weaned piglets, the proposed minimum CP level was 18.4%. Apparently, below this minimum level, other nutrients such as NEAAs, bioactive compounds, and others become limiting for maximal growth performance.

Based on this knowledge, the hypothesis of this study is that supplementation with NEAAs in low-CP diets can improve the performance, intestinal health, and immune response of weaned piglets. Thus, the study investigated the effect of CP and NEAA supplementation on the growth performance, blood profile, intestinal morphology, mRNA relative abundance of inflammatory and antioxidant markers, and tight junction proteins in piglets over the first 2 weeks after weaning.

2 Materials and methods

2.1 Animals and housing

Ninety piglets [PIC 337 (Large White × Landrace × Duroc × Pietrain) × Camborough (Large White × Landrace)], castrated male and female, weaned at 21 days old and with 7.55 ± 0.72 kg body weight (BW), were used over the first 2 weeks after weaning. Piglets were housed in suspended pens (0.54 m^2/piglet) at an experimental facility at the Universidade Federal de Viçosa, MG, Brazil. Each pen houses three piglets with free access to feed and water. For increased microbial pressure, piglets were raised in rooms that were not disinfected or cleaned after the previous occupation by piglets from the same herd (18, 19). This procedure was adopted to simulate the commercial condition of a production unit. The minimum and maximum temperatures inside the nursery room were 27.4 ± 0.7°C and 30.9 ± 0.8°C, respectively.

2.2 Diets and experimental design

Diets were formulated according to the nutritional recommendations of the Brazilian Tables for Poultry and Swine (20) (Table 1) and provided in mash form. At 21 days, piglets were assigned in a randomized block design based on BW to one of three dietary treatments: (1) high CP, a diet with 24% CP; (2) low CP, a diet with 18% CP; and (3) low CP + NEAA, a diet with 18% CP supplemented with 5 g/kg Arg (L-arginine; purity >99%) and 10 g/kg Glu + Gln (minimum 10% L-glutamine and minimum 10% L-glutamate). There were 10 pen replicates for each of the three dietary treatments.

2.3 Performance and diarrhea incidence

Throughout the trial, feed was weighed before feeding, and feed wastage was collected and weighed daily to determine the average daily feed intake (ADFI). At 21 and 35 days, piglets were individually weighed to estimate BW, average daily weight gain (ADG), and feed conversion ratio (FC). In addition, diarrhea incidence was visually assessed by the same technician at 7:00 h when piglets were 25, 27, 29, 31, and 33 days of age and were classified as 0 = absence or 1 = presence for each pen (5).

2.4 Sample collection

At 35 days of age, blood was collected from one piglet whose BW was closest to the average weight of the piglets within its respective

TABLE 1 Ingredients and calculated nutritional composition of diets fed to weaned piglets (g/kg, as-fed basis).[a]

Ingredients, g/kg	High CP	Low CP	Low CP + NEAA
Corn, 7.8% CP	318.5	495.5	495.4
Soybean meal, 46.0% CP	261.5	73.5	73.5
Dried whey, 12.5% CP	150.0	150.0	150.0
Soybean micronized, 36.0% CP	100.0	100.0	100.0
Extrude corn, 7.6% CP	55.0	55.0	55.0
Plasma protein, 78.0% CP	40.0	40.0	40.0
Sugar	30.0	30.0	30.0
Dicalcium phosphate	11.7	13.3	13.3
Limestone	8.5	9.1	9.1
Soybean oil	11.0	3.0	3.0
Anti-caking[b]	3.0	3.0	3.0
Zinc oxide	2.5	2.5	2.5
Choline chloride	2.0	2.0	2.0
L-lys, 78.0%	1.3	7.0	7.0
DL-met, 99.0%	1.4	3.1	3.1
L-thr, 98.5%	1.1	3.8	3.8
L-trp, 99.0%	–	1.0	1.0
L-val, 96.5%	–	2.6	2.6
L-ile, 98.0%	–	1.8	1.8
L-leu, 99.5%	–	0.6	0.6
L-his, 98.0%	–	0.7	0.7
L-arg, 98.0%	–	–	5.0
Gln + Glu, 98.0%	–	–	10.0
Salt	0.4	0.4	0.4
Copper sulfate	0.6	0.6	0.6
Vitamin–mineral premix	1.4	1.4	1.4
Calculated and analyzed[c] composition			
Metabolizable energy, kcal/kg	3,400	3,400	3,453
Crude protein, %	24.0 (23.4)	18.0	19.6 (19.5)
SID[d] lys, %	1.45 (1.52)	1.45	1.45 (1.51)
SID met, %	0.43 (0.48)	0.52	0.52 (0.52)
SID met + cys, %	0.81 (0.84)	0.81	0.81 (0.83)
SID thr, %	0.97 (1.11)	0.97	0.97 (1.05)
SID trp, %	0.27 (0.29)	0.27	0.27 (0.28)
SID val, %	1.06 (1.29)	1.00	1.00 (1.19)
SID ile, %	0.94 (0.96)	0.79	0.79 (0.83)
SID leu, %	1.84 (1.93)	1.45	1.45 (1.55)
SID his, %	0.59 (0.57)	0.47	0.47 (0.50)
SID arg, %	1.44 (1.30)	0.90	1.37 (1.22)
Total calcium, %	0.85	0.85	0.85
Available P, %	0.50	0.50	0.50
Sodium, %	0.28	0.28	0.28
Lactose, %	11.2	11.2	11.2

[a]Dietary treatment: high CP, diet with 24% CP; low CP, diet with 18% CP; low CP + NEAA, diet with 18% CP supplemented with 5 g/kg Arg (L-arginine, purity > 99%) and 10 g/kg Glu + Gln (minimum 10% L-glutamine with minimum 10% L-glutamate).
[b]Tixosil® (Solvay, Brazil) prevents the formation of lumps (caking).
[c]Total amino acids analyzed are included in the parenthesis.
[d]Standardized ileal is digestible.

pen. Blood was collected by orbital sinus puncture with a hypodermic needle (40 × 1.6 mm) into 10 mL tubes without anticoagulants for the determination of serum urea N (SUN; Ureal Cobas C311, Linklab, software PNCQ) and immunoglobulin G concentrations (IgG Atellica CH IgG_2, CH Analyzer, Siemens Healthineers). In addition, blood samples were collected in 10 mL tubes containing sodium heparin Now the whole sentence is: In addition, blood samples were collected in 10 mL tubes containing sodium heparin and sent to commercial laboratory (Viçosa Lab, Viçosa-MG, Brazil) to assess the plasma amino acid profile using liquid chromatography–tandem mass spectrometry. to assess the plasma amino acid profile using liquid chromatography–tandem mass spectrometry.

The same blood donor piglet was electrically stunned, followed by exsanguination to collect samples. Fragments measuring 2 cm were sampled from the duodenum (10 cm from the pylorus), jejunum (mid-section), and ileum (5 cm to the ileocecal junction) for histological evaluation (21). The histological sections were then washed in a physiological solution and fixed in 4.0% paraformaldehyde solution for 24 h at room temperature. Another 2 cm of jejunum was collected, immediately frozen in liquid nitrogen, and stored at −80°C for RNA extraction and gene expression analysis.

2.5 Intestinal morphology, Peyer's patches, and goblet cells

After 24 h of fixation, the tissues of the duodenum, jejunum, and ileum were transferred to a 70% (v/v) ethanol solution. Next, they were cross-sectionally cut and dried in ethyl crescent gradients, diaphanized in HistoChoice®, and embedded in liquid Paraplast® at 65°C. Five transverse cuts with 5 µm thickness each were placed per slide and stained with hematoxylin and eosin. The cuts were semi-serial, using 1 in 10 cuts. For morphological readings of villus height and crypt depth in the duodenum, jejunum, and ileum, an EVOS M5000 Imaging System (Invitrogen, Thermo Fisher Scientific) optical microscope with a 10-objective lens was used. Afterward, the images were analyzed using the image analyzer ImageJ 1.50i; java1.6.0_20 (National Institutes of Health). The heights of 20 villi and their 20 crypts were selected and measured. Villus:crypt ratios using the length data were then calculated. All measurements were made by a single individual. In the ileum segment, the total count of Peyer's patches was performed with a magnification of 4×.

For evaluation of goblet cells in the duodenum, jejunum, and ileum, 10 fields per slide were photographed at a magnification of 20×. Subsequently, the Image J program was used, and perpendicular lines were inserted with markings in uniformly sized quadrants under each image. Then, the total count of intersections in the image and of the cells that touched the intersections was performed. The calculation followed the methodology proposed by Mandarim-de-Lacerda (22):

$$\text{Goblet cells}\,(\%) = \frac{\text{total number of goblet cells} \times 100}{\text{total number of intersections.}}$$

2.6 Relative mRNA abundance

Total RNA extraction was performed using a commercial kit (SV Total RNA isolation kit—Promega, Z3100) following the

manufacturer's instructions. The RNA concentration was estimated using NanoDrop™ Lite (Thermo Fisher Scientific), and RNA integrity was evaluated through 1% agarose gel electrophoresis. Complementary DNA synthesis was performed according to the GoScript™ Reverse Transcription System protocol (Promega Corporation). GenBank numbers to access the primers for the genes are shown in Table 2. Primers were used for reverse transcription quantitative PCR with GoTaq® qPCR Master Mix (Promega) in QuantStudio® 3 (Applied Biosystems, Thermo Fisher Scientific). Geometric mean of the Ct value of β-actin was used to normalize target gene expression in the jejunum samples. Gene of interest relative expression was calculated by $2^{-\Delta\Delta Ct}$ (23) for glutathione peroxidase (*GPX*), superoxide dismutase (*SOD*), catalase (*CAT*), occludin (*OCL*), zonula occludens-1 (*ZO-1*), interferon gamma (*IFN-γ*), tumor necrosis factor-alpha (*TNF-α*), interleukin 1 beta (*IL1-β*), and interleukin 10 (*IL-10*).

TABLE 2 List of primers used in reverse transcription quantitative-PCR gene expression analysis in weaned piglets.

Genes[a]	GenBank number	Sequence[b]
GPX	NM_214201.1	F: 5′-GCCCAACTTCATGCTCTTC-3′
		R: 5′-CAGGATCTCCCCATTCTTGGC-3′
SOD	NM_001190422.1	F: 5′-ATCAAGAGAGGCACGTTGGA-3′
		R: 5′-TCTGCCCAAGTCATCTGGTT-3′
CAT	NM_214301.2	F: 5′-GCTTTAGTGCTCCCGAACAG-3′
		R: 5′-AGATGACCCGCAATGTTCTC-3′
OCL	NM_001163647.1	F: 5′-TCCTGGGTGTGATGGTGTTC-3′
		R: 5′-CGTAGAGTCCAGTCACCGCA-3′
ZO-1	XM_003353439.2	F: 5′-AAGCCCTAAGTTCAATCACAATCT-3′
		R: 5′-ATCAAACTCAGGAGGCGGC-3′
IFN-γ	NM_213948	F: 5′-TGGTAGCTCTGGGAAACTGAATG-3′
		R: 5′-GGCTTTGCGCTGGATCTG-3′
TNF-α	NM_214022.1	F: 5′-CATCGCCGTCTCCTACCA-3′
		R: 5′-CCCAGATTCAGCAAAGTCCA-3′
IL1-β	NM_214055.1	F: 5′-TCTGCCCTGTACCCCAACTG-3′
		R: 5′-CCCAGGAAGACGGGCTTT-3′
IL-10	NM_214041.1	F: 5′-GAAGGACCAGATGGGCGACTT-3′
		R: 5′-CACCTCCTCCACGGCCCTTG-3′
β-actin	U07786.1	F: 5′-CTCTTCCATCGTGTCCTTCTAC-3′
		R: 5′-CCTCAGACTTGTCGATCTTCTG-3′

[a]*GPX*, glutathione peroxidase; *SOD*, superoxide dismutase; *CAT*, catalase; *OCL*, occludin; *ZO-1*, zonula occludens-1; *IFN-γ*, interferon gamma; *TNF-α*, tumor necrosis factor alpha; *IL1-β*, interleukin 1 beta; *IL-10*, interleukin 10.
[b]F and R indicate forward and reverse primers, respectively.

2.7 Statistical analysis

The pen was considered the experimental unit for growth performance and diarrhea incidence analysis. One piglet from each pen was considered the experimental unit for intestinal morphology, gene expression, and serum results. The statistical model included the fixed effect of treatment, and block and residual errors as random factors. The normality of experimental errors was evaluated using the Shapiro–Wilk test. The data were analyzed using the GLMMMIX procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC, United States) via one-way analysis of variance (ANOVA). When an effect was detected in the ANOVA ($p < 0.05$), means were compared using Tukey's *post-hoc* test. Data on diarrhea were analyzed using the FREQ procedure of SAS, and the effects were determined using the chi-squared test at $p < 0.05$.

3 Results

3.1 Growth performance and fecal consistency score

There was no effect ($p > 0.05$) of dietary treatments on ADFI, ADG, and final BW (Table 3). However, there was an improvement ($p < 0.05$) in the FC of piglets fed the high-CP diet compared to treatments with low CP or low CP + NEAA. Treatments did not alter ($p > 0.05$) the diarrhea incidence (Table 4).

TABLE 3 Effects of crude protein and non-essential amino acids on growth performance of piglets (at 35 days old).[1]

Item[2]	Dietary treatment[3]			SEM[4]	*p*-value
	High CP	Low CP	Low CP + NEAA		
Initial BW, kg	7.56	7.56	7.55	–	–
ADFI, g/day	399	395	424	22.28	0.78
ADG, g/day	345	298	333	19.32	0.31
FC, g:g	1.16[b]	1.33[a]	1.27[a]	0.02	<0.01
Final BW, kg	12.37	11.75	12.22	0.31	0.47

[a,b]Means with different superscript letters are different by Tukey's *post-hoc* test at 5% probability.[1]Data are means of 10 pens replicated per dietary treatment and 3 piglets per pen as an experimental unit.
[2]Average daily feed intake (ADFI, g/day), average daily weight gain (ADG, g/day), feed conversion ratio (FC).
[3]Dietary treatment: high CP, diet with 24% CP; low CP, diet with 18% CP; low CP + NEAA, diet with 18% CP supplemented with 5 g/kg Arg (L-arginine, purity > 99%), and 10 g/kg Glu + Gln (minimum 10% L-glutamine with minimum 10% L-glutamate).
[4]Pooled standard error of the mean.

TABLE 4 Effects of crude protein and non-essential amino acids on diarrhea incidence of piglets.[a]

Days of age	Dietary treatment[b]			*p*-value
	High CP	Low CP	Low CP + NEAA	
25	0	0	1	0.37
27	2	1	2	0.85
29	1	0	1	0.78
31	2	0	0	0.26
33	2	0	0	0.09

[a]Data are means of 10 pen replicates per dietary treatment and 3 piglets per pen as an experimental unit.
[b]Dietary treatment: high CP, diet with 24% CP; low CP, diet with 18% CP; low CP + NEAA, diet with 18% CP supplemented with 5 g/kg Arg (L-arginine, purity > 99%), and 10 g/kg Glu + Gln (minimum 10% L-glutamine with minimum 10% L-glutamate).

TABLE 5 Effects of crude protein and non-essential amino acids on the blood profile of piglets (at 35 days old).[1]

Item[2]	Dietary treatment[3]			SEM[4]	*p*-value
	High CP	Low CP	Low CP + NEAA		
SUN, mg/dL	21.0[a]	5.7[b]	7.0[b]	0.97	<0.01
IgG, mg/dL	203.3	161.6	170.0	23.09	0.37
Amino acids, μmol/L					
Glutamine + lysine	51.2[a]	37.1[b]	44.9[ab]	4.17	0.03
Methionine	39.6[b]	87.4[a]	61.0[ab]	11.61	0.01
Arginine	93.0[a]	40.0[b]	81.1[a]	3.53	<0.01
Threonine	57.2	88.3	79.8	14.24	0.28
Tryptophan	25.2	21.7	24.1	1.74	0.34
Valine	129.3[b]	144.1[ab]	181.6[a]	12.20	0.01
Leucine + isoleucine	146.1[a]	92.2[b]	97.5[b]	5.81	<0.01
Glycine	448.2	410.5	405.3	34.66	0.63
Tyrosine	74.4[a]	30.6[b]	25.2[b]	3.63	<0.01
Ornithine	72.2[a]	39.3[b]	58.1[a]	5.13	<0.01
Phenylalanine	40.0[a]	23.9[b]	16.5[b]	2.58	<0.01
Citrulline	46.4	34.2	36.9	4.32	0.12
Glutamate	146.1[a]	84.5[b]	129.8[a]	8.50	<0.01
Alanine	199.3[b]	173.7[b]	254.5[a]	11.65	<0.01

[a,b]Means with different superscript letters are different by Tukey's *post-hoc* test at 5% probability.[1]Data are means of 10 piglets per dietary treatment.
[2]SUN, serum urea nitrogen; IgG, immunoglobulin G.
[3]Dietary treatment: high CP, diet with 24% CP; low CP, diet with 18% CP; low CP + NEAA, diet with 18% CP supplemented with 5 g/kg Arg (L-arginine, purity > 99%) and 10 g/kg Glu + Gln (minimum 10% L-glutamine with minimum 10% L-glutamate).
[4]Pooled standard error of the mean.

3.2 Blood profile

The SUN was higher ($p < 0.05$) in piglets fed the high-CP treatment than those with the low-CP and low-CP + NEAA diets (Table 5). There was no effect ($p > 0.05$) of treatments on IgG concentrations. Plasma Gln + Lys concentration was higher ($p < 0.05$) in piglets on high-CP treatment than in low CP, while low CP + NEAA had intermediate results. Plasma Met concentration was higher ($p < 0.05$) in piglets fed low CP than those piglets that received high CP, while low CP + NEAA had intermediate results. Plasma Arg, Orn, and Glu concentrations were higher ($p < 0.05$) in piglets fed high-CP and low-CP + NEAA dietary treatment compared to low CP. Plasma Val concentration was higher in piglets receiving low-CP + NEAA dietary treatment than those with high CP, while low CP had intermediate results. Plasm Leu + Ile, Tyr, and Phe concentrations were higher ($p < 0.05$) in piglets on high-CP dietary treatment compared to others. Plasma Ala concentration was higher ($p < 0.05$) in piglets from low-CP + NEAA treatment compared to others. Plasma Thr, Try, Gly, and Cit concentrations were not influenced ($p > 0.05$) by dietary treatments.

3.3 Intestinal morphology, Peyer's patches, and goblet cells

In the duodenum, the villus height of animals fed the low-CP + NEAA diets was greater ($p < 0.05$) than those fed with the high- and low-CP diets (Table 6). Moreover, the goblet cell proportion of piglets fed high CP or low CP + NEAA was higher ($p < 0.05$) compared to low CP. However, there were no effects ($p > 0.05$) of treatments on the crypt depth or villus:crypt ratio. In the jejunum, the crypt depth of the piglets with the high-CP dietary treatment was greater ($p < 0.05$) in comparison with low CP + NEAA, while low CP had intermediate results. However, dietary treatments had no effect ($p > 0.05$) on villus height, villus:crypt ratio, or proportion of goblet cells. In the ileum, dietary treatments had no effects ($p > 0.05$) on villus height, crypt depth, villus:crypt ratio, or proportion of goblet cells. However, the number of Peyer's patches in piglets fed high CP was higher ($p < 0.05$) compared to other dietary treatments.

3.4 Relative mRNA abundance

In the jejunum, *IFN-γ* mRNA expression was higher ($p < 0.05$) in animals fed the high-CP diets compared to other dietary treatments (Figure 1). However, *SOD* and *OCL* mRNA expression were higher ($p < 0.05$) in animals fed low CP + NEAA than in piglets on high-CP diets. There was no effect ($p > 0.05$) of dietary treatments on mRNA expression of *GPX*, *CAT*, *TNF-α*, *ZO-1*, *IL1-β*, and *IL-10*.

4 Discussion

The reduction of dietary CP balanced with EAA has been used as part of a strategy to improve intestinal health in pigs and, consequently, improve growth performance (4). However, under stress such as the post-weaning period, there is a greater demand for NEAAs because tissue production does not meet the systemic needs (9). Thus, it has been suggested that the generation of NEAAs from EAAs may become a limiting factor for the normal growth performance of weaned pigs (2, 7, 24). In this way, studies have shown that high-CP levels or

TABLE 6 Effects of crude protein and non-essential amino acids on the intestinal morphology of piglets (at 35 days old).[1]

Item	Dietary treatment[2]			SEM[3]	*P*-value
	High CP	Low CP	Low CP + NEAA		
Duodenum					
Villus height, μm	381[b]	383[b]	427[a]	11.49	0.01
Crypt depth, μm	208	213	229	8.83	0.40
Villus:crypt ratio	1.8	1.8	1.9	0.07	0.58
Goblet cells, %	53.2[a]	45.3[b]	53.4[a]	2.24	0.01
Jejunum					
Villus height, μm	365	324	299	22.00	0.16
Crypt depth, μm	161[a]	143[ab]	141[b]	5.05	0.01
Villus:crypt ratio	2.3	2.2	2.1	0.12	0.67
Goblet cells, %	45.3	44.8	43.6	2.88	0.45
Ileum					
Villus height, μm	249	239	244	15.97	0.86
Crypt depth, μm	135	131	132	6.07	0.95
Villus:crypt ratio	1.9	1.9	1.9	0.07	0.90
Goblet cells, %	40.9	44.7	43.0	1.99	0.57
Peyer's patches, *n*	48[a]	38[b]	41[b]	2.20	0.02

[a,b]Means with different superscript letters are different by Tukey's *post-hoc* test at 5% probability.[1]Data are means of 10 piglets per dietary treatment.
[2]Dietary treatment: high CP, diet with 24% CP; low CP, diet with 18% CP; low CP + NEAA, diet with 18% CP supplemented with 5 g/kg Arg (L-arginine, purity > 99%) and 10 g/kg Glu + Gln (minimum 10% L-glutamine with minimum 10% L-glutamate).
[3]Pooled standard error of the mean.

supplementation of NEAAs in diets for newly weaned piglets may be beneficial due to the higher intake of NEAAs (15–17).

In the present study, three experimental diets were fed to piglets in the first 2 weeks after weaning. The first diet contained 24% CP, supplemented with Lys, Met, and Thr. The second diet contained 18% CP, supplemented with Lys, Met, Thr, Trp, Val, Ile, Leu, and His. The third diet was similar to the second and supplemented with NEAA Arg, Gln, and Glu. All diets were formulated with the EAA at or above the recommended ratio to Lys (20). The hypothesis of the study was that low-CP diets supplemented with NEAAs would improve the growth performance, gut health, and immune response of weaned piglets.

High-CP levels may be associated with a higher incidence of diarrhea (25) and worse growth performance in weaned piglets (26). However, in the present study, the high-CP diets had no negative effects on the incidence of diarrhea, ADG, ADFI, and BW at 35 days of age. In addition, high-CP diets improved the FC of piglets. Others also demonstrated improved growth performance associated with higher levels of dietary CP (27–29). According to Silva et al. (30), reducing dietary CP levels decreases the supply of dietary N and NEAAs, as well as the expression of digestive enzyme genes for carbohydrates and proteases in pigs (31). Therefore, it is assumed that in the present study, inadequate endogenous NEAA synthesis limited the growth of piglets fed the low-CP diets. Moreover, the supplementation of NEAAs in the low-CP + NEAA treatment may not have been sufficient to recover growth performance, probably because the animals required a higher level of NEAAs or other non-supplemented NEAAs. According to Gloaguen et al. (32), the rate of NEAA synthesis can be limited by the availability of dietary or metabolic N, originating from the deamination of EAA, which will further limit the growth performance of animals.

The SUN is indicative of the efficiency of N utilization by the animals. In the present study, piglets fed the low-CP and low-CP + NEAA diets had lower SUN concentrations compared to the high-CP diets. According to Heo et al. (25), AA absorbed beyond what is necessary for biosynthesis cannot be stored and undergoes catabolism, which has urea as its final product. The present result indicated that there was an excess of AA in the high-CP diet. Thus, animals fed the low-CP diets may have been more efficient in N utilization, corroborating the results reported by other authors (5, 16, 17).

Plasma concentrations of AA can be influenced either by the uptake of AA from the diet or by the tissue absorption of circulating AA (33). The lower plasma concentrations of Arg and Glu in piglets fed the low-CP diet may be related to the lower level in the diet and lower availability of N for the synthesis of these AAs as compared to low CP + NEAA and high CP. Piglets fed the high-CP diet had lower concentrations of Met and Val in the plasma, which can be explained by the lower dietary supplementation of these AAs in industrial form. Supplemented industrial AAs are readily available for absorption and are promptly absorbed in the proximal small intestine, while CP-bound AAs need to be broken down by luminal and brush border enzymes before absorption (34, 35). Plasma concentrations of Leu + Ile, Phe, and Tyr were higher in piglets fed the high-CP diet, which is explained by the fact that this diet contained a higher concentration of those AAs as a result of the higher CP content. Moreover, low-CP + NEAA treatment increased plasma concentrations of Orn and Ala compared to low CP, showing that

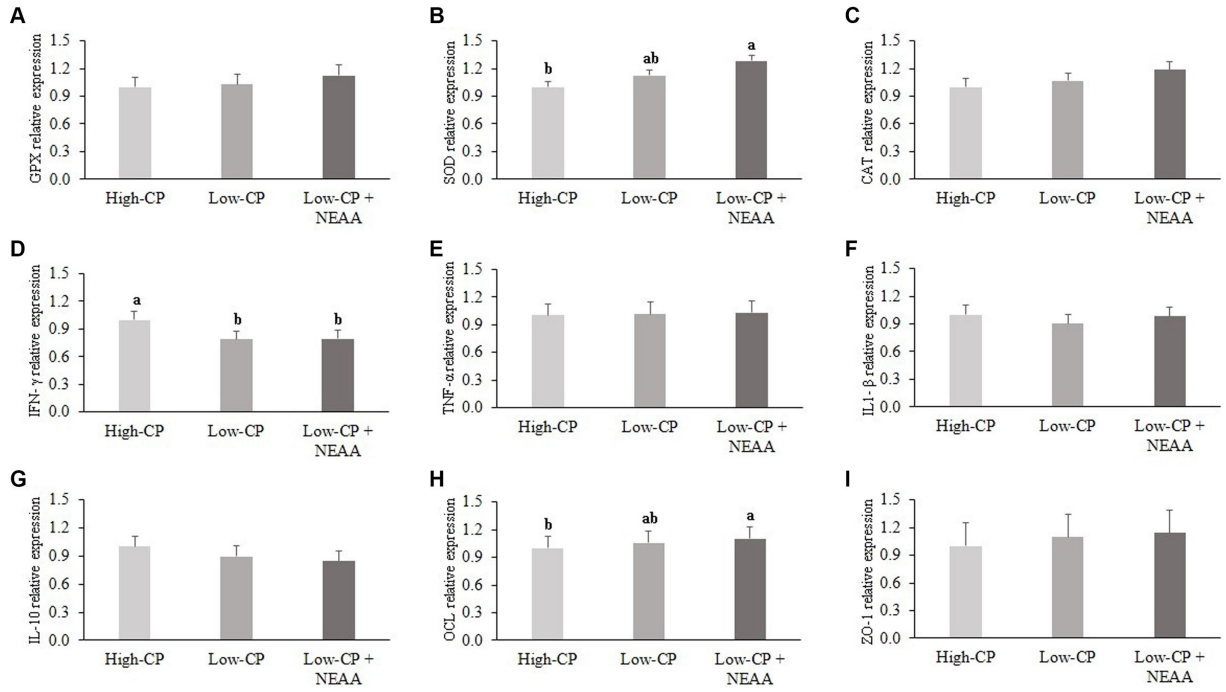

FIGURE 1
Effects of crude protein and non-essential amino acids on the mRNA relative abundance of inflammatory and antioxidant markers and tight junction proteins of the jejunum of piglets (at 35 days old). Dietary treatments: high CP, diet with 24% CP; low CP, diet with 18% CP; low CP + NEAA, diet supplemented with 5 g/kg Arg (L-arginine; purity >99%), and 10 g/kg Glu + Gln (minimum 10% L-glutamine with minimum 10% L-glutamate). Results are relative to the high-CP treatment. Data are means of 10 piglets per dietary treatment. [a,b]Means with different superscript letters are different by Tukey's *post-hoc* test at 5% probability. GPX, glutathione peroxidase **(A)**; SOD, superoxide dismutase **(B)**; CAT, catalase **(C)**; IFN-γ, interferon gamma **(D)**; TNF-α, tumor necrosis factor **(E)**; IL1-β, interleukin 1 beta **(F)**; IL-10, interleukin 10 **(G)**; OCL, occludin **(H)**; ZO-1, zonula occludens-1 **(I)**.

dietary NEAA supplementation may reduce the intestinal catabolism of other AA and elevate their entry into the portal vein, as reported by Yi et al. (3).

Gut health has significant implications for swine health status and nutrient utilization due to its various functions, including digestion and absorption of nutrients, secretion of mucins and immunoglobulins, and selective barrier protection against harmful antigens and pathogens (35). Thus, the evaluation of intestinal morphometry, goblet cells, and Peyer's patches associated with gene expression of tight junction proteins, pro- and anti-inflammatory cytokines, and antioxidant enzymes can be used as tools for assessing intestinal health (19).

In the present study, animals fed the low-CP + NEAA diet had higher villus height in the duodenum, indicating greater absorptive capacity for the available nutrients (36). This result may be related to the supplementation of Gln and Glu. Glutamine is a major metabolic fuel for rapidly dividing cells, such as enterocytes, and together with Glu, it is related to increasing the rate of cell proliferation and differentiation (12, 14). Piglets fed low-CP + NEAA also had shorter crypt depth in the jejunum, indicating decreased metabolic cost of epithelium turnover associated with inflammation response (35). In addition to shorter crypt depth, those piglets had reduced Peyer's patches in the ileum, suggesting less intestinal challenge compared to the high-CP diet. Peyer's patches are aggregated lymphoid follicles, with a protective function against pathogens (37). The high-CP content, as a result of the high SBM level, may have increased the proliferation of pathogenic bacteria in the ileum (although not evaluated in the present study), stimulating the immune system and increasing the number of Peyer's patches. These results are supported by a study conducted by Deng et al. (1), who reported that the higher the SBM content in the diet, the higher the content of indigestible carbohydrates (stachyose and raffinose) and antigenic proteins (glycinin and β-conglycinin) considered antinutritional factors. In addition, the high-CP content can increase the proliferation of pathogenic bacteria and their potential toxins for the gastrointestinal tract, such as ammonia and polyamines (38).

Goblet cells are responsible for the production of mucus that acts as a physical barrier against the invasion of pathogens, while tight junction proteins form a selective physical barrier to prevent endotoxin absorption (39). In the present study, it was demonstrated higher proportion of goblet cells and higher expression of OCLN in animals fed low-CP + NEAA diets, thus indicating improved intestinal integrity. Additionally, the expression of antioxidant enzymes and cytokines in the jejunum of weaned piglets was evaluated because the antioxidant capacity and the immune response are fundamental for the promotion of intestinal health. According to Yin et al. (40), weaning causes an increase in reactive oxygen species that can cause oxidative stress at the intestinal level and in other tissues. In this way, it has been shown that supplementation of Arg, Gln, and Glu in diets

can improve the intestinal antioxidant response in pigs (3, 41). Corroborating this report, animals fed a low-CP + NEAA diet showed increased SOD expression in the jejunum, which suggested greater antioxidant capacity associated with NEAA supplementation.

IFN-γ is a pro-inflammatory cytokine considered an immunological marker produced in response to inflammation (42). Animals fed the high-CP diet had higher expression of IFN-γ, which may be related to higher SBM levels compared to the low-CP treatments (261 × 73 g/kg). High levels of indigestible proteins in the diet might result in inflammatory response, especially by increasing pro-inflammatory cytokine levels, which might decrease gut integrity (43). Actually, in the present study, the higher IFN-γ in pigs fed high-CP diets was associated with reduced OCL expression.

Altogether, the results indicated that the low-CP + NEAA diet improves N utilization efficiency and intestinal architecture and modulates the response expression of genes related to the immune system and antioxidant capacity in piglets in the first 2 weeks after weaning. Therefore, supplementation with Arg, Gln, and Glu in diets for weaned piglets is a promising nutritional approach to support a formulation with low dietary CP levels.

5 Conclusion

The high-CP diet (24% CP) improves the feed conversion of piglets in the first 2 weeks after weaning compared to the low-CP diet (18% CP) supplemented or not with NEAAs. However, the low-CP diet supplemented with NEAA (5 g/kg of Arg and 10 g/kg of Gln + Glu) improves intestinal health in piglets by promoting greater villus height and proportion of goblet cells in the duodenum, reducing jejunal crypt depth, and reducing Peyer's number patches in the ileum. In addition, piglets that received the low-CP + NEAA diet showed an increase in *SOD* and *OCL* mRNA expression and a lower expression of *IFN-γ* mRNA.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Ethical Committee on Animal Use of Universidade Federal de Viçosa (UFV) protocol n° 066/2021. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AC, AS, and GR: conceptualization, design of the study, and writing—original draft preparation. AC: carrying out the project. AC, JG, and GR: methodology, statistical analysis, formal analysis, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Feeding sunflower meal with pullets and laying hens even at a 30% inclusion rate does not impair the ileal digestibility of most amino acids

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The use of locally available protein sources in poultry nutrition is challenging for feed manufacturers and farmers. Sunflower meal (SFM) is available in high quantities in several European countries and could be used as a poultry feedstuff at higher inclusion rates. However, its maximum inclusion rate in the diets of different poultry species and age categories is unknown. Pullets and laying hens can probably tolerate higher amounts of SFM, but only limited information is available on these poultry groups. Therefore, a digestibility trial was carried out with 8-week-old layer type pullets and 50-week-old laying hens. Beside a basal diet, SFM was fed at 10, 20 and 30% inclusion rates. Feeding SFM significantly improved the digestibility of essential amino acids (AA) of threonine, valine, lysine, tyrosine, glycine, aspartic acid, and arginine in the pullet diets. No such improvement was found in laying hens. Only the absorption of the two branch-chain AAs, leucin (pullets) and isoleucine (hens), declined due to SFM. The AA digestibility of the SFM itself was also calculated by linear regression. The coefficients were, in all cases, higher in hens than in pullets. Comparing the measured digestibility coefficients of SFM with table values, it can be concluded that high variance exists because of the differences in the methodology and the test animals in the digestibility trials. From the present trial, it can be concluded that SFM can entirely replace extracted soybean meal in pullet and layer diets, without negative effects on the protein digestion of birds.

KEYWORDS

sunflower meal, laying hens, pullet, amino acid, digestibility

1 Introduction

Protein is one of the most expensive components of animal diets and its amount is increasingly limited around the world (1). Soybean meal is the dominating protein source for farm animals in Europe. Because the cultivation of soybean is focused mostly in America, its transportation around the world has a high environmental impact (2–5). Therefore, the

importance of locally available protein sources, legume seeds, and industrial by-products will increase in the future (6).

Sunflower is a widely cultivated crop, the third biggest in global oil seed production (7, 8). Across the EU in 2021, the harvested production of sunflower seed was 10.4 million tons. Sunflower meal (SFM) is a byproduct of the oil industry and it can be used as an alternative protein source in farm animal nutrition (2, 3, 5, 9). The crude protein content of SFM shows high variance (23–44%), depending mainly on the quality of the dehulling procedure. The use of SFM in poultry diets is limited due to its high fiber and low energy content, its low concentration of lysine (LYS) and threonine (THR), and the presence of different polyphenolic compounds (10, 11).

Sunflower contains a very diverse fiber composition, including both structural and water-soluble fractions. Its structural, insoluble fiber, which can be found mainly in the hulls, can stimulate gizzard development and by this process, may increase the retention time of the digesta in the upper part of the GIT. Proper gizzard function also stimulates pancreatic enzyme secretion, improving the digestibility of starch, lipids, and other dietary components on the GIT (12). In the water-soluble fraction, β-glucans dominate. SFM's β-glucan, like the β-glucans found in cereals, can increase the viscosity of the gut content, which is associated with reduced nutrient absorption and imbalance of the microbiota in the small intestine (13, 14). For this reason, NSP-degrading enzymes are used also if SFM-containing diets are provided (15). The positive effect of this addition on nutrient utilization and production traits has already been demonstrated by numerous studies (12). Our knowledge of the specific effects of SFM's fiber on the digestion and gut health of birds is incomplete, and we do not know its maximal inclusion rates for the different poultry species and age categories (16). Using the last generation exogenous enzymes, we can also modify the negative effects of the different fiber fractions. Its considerably high fiber limits its use in broilers (8, 12, 17–19). However, according to several studies, SFM can be utilized in the diets of laying hens with no negative impact on egg quality parameters (9, 20, 21). This can be explained by the fact that layers have a more developed digestive system in terms of gut capacity compared to broilers. Laying hens have a lower protein requirement than broiler chickens, which makes it possible to replace soybean meal completely with SFM (22). In the case of pullets, the use of insoluble fiber has been shown to be beneficial for the development of the gastrointestinal tract (GIT) (23, 24). In the study of Abdallah and Beshara (23), supplementing the pullets' diet with 7 and 14% sunflower meal from 11 to 19 weeks resulted in significantly improved live weight and FCR compared to the SFM-free control diet. The protein evaluation of poultry feedstuffs is based on the so-called standardized ileal amino acid digestibility (SID). The determination of SID is based on the evaluation of the AA content of the whole or terminal ileum content, assuming that the amino acids of this gut segment are not digestible (25). This term is used to express the amino acid content of the feeds and the requirements of the birds. Rodehutscord et al. (26) developed a linear regression method as a tool to study the AA digestibility of raw materials in chickens. In this case, the test feedstuff is incorporated into the test diets at the expense of starch at graded levels. The increased protein content of the diets and the AA intake of animals is related only to the test feedstuff. Therefore, the slope of the linear regression between the AA intake and pre-cecally absorbed AA content means the digestibility of the AAs. A further advantage of this method is that it can also give information on the maximal inclusion rate of the feedstuffs without impairing digestion. In the present work, this method was used for AA digestibility determination.

Most of the animal experiments on SID measurements have been carried out in broiler chickens and limited research data are available regarding pullets and laying hens (25, 27). Pullets are reared with a restricted feeding and light program to achieve the optimal live weight at the start of the laying period. The low amount of daily feed intake is an important difference between broiler chickens and pullets, which could affect protein digestibility. In the case of laying hens, the protein, energy, and calcium requirements change during the day due to the synthesis of egg components, which modify the feeding habits of hens. The longer dark period and the restricted feeding means also difference from broiler chickens (28, 29). According to the current research intended to assess the effect of dietary inclusion of SFM as a complementary protein resource at 10, 20, and 30% on the ileal amino acid digestion of pullets and laying hens. According to the knowledge of the authors, no ileal amino acid digestibility result of SFM is available for pullets and layers. The measured values have been compared with table values.

TABLE 1 Nutrient content of the sunflower meal (g/kg).

Nutrient content of SFM	
Dry matter	920.8
Crude protein	349.5
Crude fat	8.0
Crude fiber	184.8
Ash	71.3
Predicted AMEn (MJ/kg)*	6.61
Amino acid content of SFM	
Cystine	6.0
Aspartic acid	33.8
Methionine	8.5
Threonine	13.7
Serine	15.7
Glutamic acid	72.9
Proline	15.0
Glycine	21.0
Alanine	15.9
Valine	18.2
Isoleucine	14.6
Leucine	21.9
Tyrosine	8.3
Phenylalanine	16.9
Histidine	9.3
Lysine	12.7
Arginine	31.8

*The predicted AMEn content of sunflower meal was calculated with the equation of the European Table of Energy Values for Poultry Feedstuffs (30).

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TABLE 2 Composition and measured nutrient contents of pullet diets (g/kg).

	C	SFM10	SFM20	SFM30
Composition of experimental diets				
Maize	415	415	415	415
Starch	300	200	100	0
Wheat	200	200	200	200
Ext. sunflower meal	0	100	200	300
Sunflower oil	50	50	50	50
Limestone	14	14	14	14
MCP[1]	7	7	7	7
Premix[2]	5	5	5	5
NaCl	3	3	3	3
$NaHCO_3$	1	1	1	1
TiO_2	5	5	5	5
	1,000	1,000	1,000	1,000
Measured nutrient contents				
Dry matter	891.7	891.7	891.9	892.4
Crude protein	53.0	84.5	118.2	146.6
Crude fat	67.3	68.2	73.8	72.9
Crude fiber	17.8	33.7	48.4	64.6
Ash	39.7	45.2	52.4	56.5
Ca	8.3	8.9	8.8	8.9
Predicted AMEn (MJ/kg)*	13.96	13.57	13.05	12.28
Cystine	1.2	1.7	2.3	2.8
Aspartic acid	3.2	6.2	9.4	12.2
Methionine	1.0	1.7	2.5	3.2
Threonine	1.8	3.0	4.3	5.4
Serine	2.5	3.9	5.4	6.7
Glutamic acid	12.4	18.8	25.8	31.6
Proline	5.0	6.4	8.0	9.2
Glycine	2.1	4.1	6.2	7.9
Alanine	3.0	4.4	6.0	7.2
Valine	2.4	4.0	5.8	7.3
Isoleucine	1.8	3.2	4.6	5.8
Leucine	5.1	7.2	9.5	11.3
Tyrosine	1.4	2.3	3.1	3.9
Phenylalanine	2.5	4.1	5.7	7.1
Histidine	1.4	2.2	3.1	3.9
Lysine	1.6	2.7	4.0	5.1
Arginine	2.6	5.3	8.2	10.7

C, control; SFM10, control diet supplemented with 10% extracted sunflower meal; SFM20, control diet supplemented with 20% extracted sunflower meal; SFM30, control diet supplemented with 30% extracted sunflower meal. [1]MCP, monocalcium phosphate. [2]Pullet premix was supplied by Agrofeed Ltd. (Győr, Hungary). The active ingredients contained in the premix were as follows (per kg of diet): vitamin A—2,000,000 NE, vitamin D3—600,000 NE, vitamin E—5,000 mg, menadione—450 mg, thiamine—450 mg, riboflavin—1,320 mg, pyridoxin HCl—720 mg, cyanocobalamin—4 mg, niacin—6,000 mg, pantothenic acid—1,680 mg, folic acid—216 mg, biotin—20 mg, betaine—14,060 mg, BHT—75 mg, BHA—75 mg, citric acid—67.5 mg, Zn (as ZnO)—14,000 mg, Cu (as $CuSO_45H_2O$)—1,600 mg, Fe (as $FeSO_4H2O$)—6,000 mg, Mn (as MnO)—20.000 mg, I [as $Ca(IO_3)_2$]—200 mg, Se (as Na_2SeO_3)—60 mg, endo-1.4-beta-xylanase—244,000 U, Endo-1.3(4)-beta-glucanase—30,400 U, 6-phytase—100,000 FTU. *The AMEn content of diets was calculated with the equation of McNab and Fisher (31).

TABLE 3 Composition and measured nutrient contents of layer diets (g/kg).

	C	SFM10	SFM20	SFM30
Composition of experimental diets				
Maize	331	331	331	331
Starch	300	200	100	0
Wheat	200	200	200	200
Extr. sunflower meal	0	100	200	300
Sunflower oil	50	50	50	50
Limestone	98	98	98	98
MCP	7	7	7	7
Premix[2]	5	5	5	5
NaCl	3	3	3	3
$NaHCO_3$	1	1	1	1
TiO_2	5	5	5	5
	1,000	1,000	1,000	1,000
Measured nutrient contents				
Dry matter	890.9	896.9	897.9	900.2
Crude protein	42.8	75.6	112.4	146.2
Crude fat	63.6	63.7	65.2	64.6
Crude fiber	16.2	35.7	59.9	76.3
Ash	121.2	123.1	127.1	133.2
Ca	51.6	48.8	48.6	4.92
Predicted AMEn (MJ/kg)*	13.10	12.31	11.51	10.73
Cystine	1.0	1.6	2.1	2.9
Aspartic acid	2.8	5.4	8.5	12.2
Methionine	0.9	1.5	2.5	3.0
Threonine	1.6	2.6	4.2	5.5
Serine	2.2	3.6	5.2	6.6
Glutamic acid	9.5	16.9	25.0	32.0
Proline	3.9	5.7	7.8	9.5
Glycine	1.8	3.5	6.0	8.0
Alanine	2.3	4.1	5.7	7.0
Valine	1.8	3.7	5.6	7.4
Isoleucine	1.6	2.9	4.3	5.9
Leucine	4.1	6.4	8.9	11.3
Tyrosine	1.3	2.1	2.9	3.8
Phenylalanine	2.1	3.5	5.3	7.2
Histidine	1.2	2.2	2.8	3.6
Lysine	1.5	2.4	3.9	5.2
Arginine	2.2	4.8	7.9	10.4

C, control; SFM10, control diet supplemented with 10% extracted sunflower meal; SFM20, control diet supplemented with 20% extracted sunflower meal; SFM30, control diet supplemented with 30% extracted sunflower meal; MCP—monocalcium phosphate; 1 Premix was supplied by Agrofeed Ltd. (Győr, Hungary). The active ingredients contained in the premix were as follows (NE per kg of diet): vitamin A—2,000,000 NE, vitamin D3—600,000 NE, vitamin E—6,000 mg, menadione—400 mg, thiamine—436 mg, riboflavin—1,200 mg, pyridoxin HCl—600 mg, cyanocobalamin—4 mg, niacin—6,254 mg, pantothenic acid—1825 mg, folic acid—300 mg, biotin—30 mg, betaine—30,000 mg, BHT—79.5 mg, BHA—79.5 mg, citric acid—71.5 mg, Zn (as ZnO)—8,000 mg, Zn (as 3b607)—8,000 mg, Cu (as 3b413)—2,000 mg, Fe (as $FeSO_4H2O$)—10,000 mg, Mn (as MnO)—10,000 mg, Mn (as 3b506)—10,000 mg, I [as $Ca(IO_3)_2$]—300 mg, Se (as $C_2H_{11}NO_2Se$)—40 mg, endo-1.4-beta-xylanase—244,000 U, Endo-1.3(4)-beta-glucanase—30,400 U, 6-phytase—100,000 FTU. *The AMEn content of diets was calculated with the equation of McNab and Fisher (31).

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TABLE 4 The ileal amino acid digestibility of pullet diets (%).

	Essential amino acids										Non-essential amino acids					
%	MET*	THR	CYS	VAL	ILE	LEU	PHE	HIS	LYS	ARG	TYR	GLY	ASP	SER	GLU	PRO	ALA
C	84.34	58.60^{b}	73.44	74.86^{b}	79.34	85.00^{a}	82.50	77.05	69.85^{b}	80.09^{b}	70.24^{b}	70.23^{b}	72.23^{b}	72.51	88.88	81.08	78.66
SFM10	84.80	64.40^{a}	73.88	76.56^{ab}	79.99	82.73^{ab}	82.94	77.58	73.04^{ab}	82.58^{ab}	76.33^{a}	73.11^{ab}	74.60^{ab}	73.12	87.55	81.67	77.74
SFM20	86.31	66.90^{a}	74.10	79.20^{a}	80.44	82.48^{ab}	82.96	77.52	73.20^{ab}	84.49^{a}	78.46^{a}	74.40^{a}	77.08^{a}	74.88	88.02	82.37	77.99
SFM30	84.38	66.75^{a}	73.91	78.31^{ab}	80.33	79.67^{b}	82.33	76.67	74.04^{a}	85.17^{a}	79.07^{a}	73.32^{ab}	75.18^{ab}	73.26	86.39	81.24	77.19
Pooled SEM	0.004	0.007	0.004	0.005	0.005	0.005	0.004	0.005	0.005	0.050	0.007	0.005	0.006	0.005	0.004	0.004	0.004
p-value	0.384	**0.000**	0.972	**0.030**	0.881	**0.001**	0.957	0.929	**0.027**	**0.001**	**0.000**	**0.031**	**0.007**	0.503	0.277	0.763	0.768

[a,b]Means with different superscripts of the same column are significantly different ($p < 0.05$).

*Methionine, threonine, cystine, valine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine, tyrosine, glycine, aspartic acid, serine, glutamine, proline, alanine. $n = 8$ for each diet.

The bold *p*-values show the significantly differences ($p<0.05$).

2 Materials and methods

The trials were carried out at the experimental farm of the Institute of Physiology and Nutrition, Hungarian University of Agriculture and Life Sciences (Georgikon Campus, Keszthely, Hungary). The animal experiments were approved by the Institutional Ethics Committee (Animal Welfare Committee, Georgikon Campus, Hungarian University of Agriculture and Life Sciences) with the number MÁB-11/2019.

2.1 Experiment 1

In the first experiment, a total of 32 Tetra SL pullets were individually housed in metabolic cages. The special feeders made possible the exact measurement of daily feed intakes. The water was available *ad libitum* through nipple drinkers. In the beginning, the pullets were 10 weeks old with an average body weight of 638 g. Alongside a corn, wheat, and cornstarch-based control diet (C), three diets containing graded levels of SFM were used. The proportions of SFM were 10, 20, and 30% (SFM10, SFM20, SFM30). All diets were fed in 8 replicate pullets. Sunflower meal was fed at the expense of wheat starch, and consequently, the increase in the AA concentrations of the experimental diets originated from SFM only. Titanium dioxide (TiO_2) was used as an indigestible marker at 0.5%. The nutrient content of SFM can be found in Table 1, while the composition and nutrient content of the experimental diets are shown in Table 2. The AMEn content of SFM and the diets were calculated with the equation of McNab and Fisher (31). As can be observed, the increased SFM incorporation increased both the crude protein and crude fiber contents of the diets. All diets were fed in mash form and the daily feed intake was adjusted to the breeder's nutritional guide (32). The length of the light and dark periods was 10 and 14 h, respectively. Computer-controlled climatic conditions were maintained during the trial according to the breeder's recommendations (33).

2.2 Experiment 2

In the second trial, a total of 32 Teta SL laying hens were used and housed in the same metabolic cages as described in the first experiment. At the beginning of the experiment, the hens were 50 weeks old, with an average body weight of 1,941 g. The composition and nutrient content of the hen diets are shown in Table 3. The lengths of the light and dark periods were in this case 16 and 8 h, respectively. All the housing and experimental conditions were the same as described in the first experiment.

2.3 Sample collection

During a 5-day adaptation period, the pullets were accommodated in metabolic cages and consumed their daily rations entirely. On the 6th and 7th days, the daily feed intake of the animals was measured. On the 7th day, the birds were slaughtered by asphyxiation with carbon dioxide, and the ileal contents were collected immediately. The samples were collected from the Meckel's diverticulum up to 1 cm before the ileocecal junction. The ileum was cut into short pieces, then

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TABLE 5 The ileal amino acid digestibility of layer diets (%).

Essential amino acids												Non-essential amino acids					
%	MET*	THR	CYS	VAL	ILE	LEU	PHE	HIS	LYS	ARG	TYR	GLY	ASP	SER	GLU	PRO	ALA
C	90.31	73.55	81.65	84.94	88.35^{ab}	88.10	88.77	87.38	81.49	88.45	80.83	80.16	83.00	82.35	93.58	88.23	86.78
SFM10	90.09	78.57	85.82	87.02	89.25^{a}	89.43	90.13	86.71	84.79	91.41	86.85	82.73	85.81	84.16	92.56	89.20	87.67
SFM20	90.07	73.69	81.97	83.73	82.70^{b}	84.77	85.84	82.25	79.00	86.74	81.59	79.46	80.22	76.89	90.19	85.34	82.71
SFM30	87.77	74.53	83.04	83.13	84.68^{ab}	86.32	87.75	82.95	80.29	89.64	86.01	79.55	82.42	81.32	90.44	85.30	82.88
Pooled SEM	0.006	0.017	0.005	0.011	0.009	0.009	0.007	0.009	0.014	0.008	0.013	0.013	0.004	0.012	0.005	0.007	0.009
p-value	0.539	0.737	0.581	0.693	**0.025**	0.363	0.250	0.094	0.450	0.298	0.296	0.875	0.519	0.214	0.087	0.154	0.098

[a,b]Means with different superscripts of the same column are significantly different ($p < 0.05$).
*Methionine, threonine, cystine, valine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine, tyrosine, glycine, aspartic acid, serine, glutamine, proline, alanine. $n = 8$ for each diet.
The bold *p*-values show the significantly differences ($p<0.05$).

TABLE 6 Linear regression equation parameters and their SE of estimates, describing the response of daily digested amino acids up to the terminal ileum (y) depending on the respective daily amino acid intake (x).

	Pullets			Laying hens		
	Slope	Constant	r^2	Slope	Constant	r^2
Cystine	0.737 ± 0.015	0.2 ± 1.9	0.987	0.828 ± 0.011	0.1 ± 0.2	0.996
Aspartic acid	0.766 ± 0.011	−5.8 ± 5.4	0.994	0.821 ± 0.012	0.4 ± 0.8	0.995
Methionine	0.851 ± 0.011	−0.1 ± 1.4	0.995	0.890 ± 0.010	0.1 ± 0.2	0.997
Threonine	0.700 ± 0.013	−9.9 ± 3.0	0.989	0.748 ± 0.017	0.2 ± 0.5	0.988
Serine	0.750 ± 0.015	−3.6 ± 4.3	0.989	0.804 ± 0.017	0.2 ± 0.7	0.990
Glutamic acid	0.864 ± 0.012	13.6 ± 17.4	0.994	0.885 ± 0.010	3.7 ± 1.8	0.997
Proline	0.829 ± 0.016	−5.2 ± 7.0	0.989	0.852 ± 0.010	0.9 ± 0.5	0.997
Glycine	0.744 ± 0.011	−3.4 ± 3.5	0.994	0.795 ± 0.017	0.4 ± 0.8	0.990
Alanine	0.766 ± 0.014	3.5 ± 4.4	0.991	0.820 ± 0.014	0.8 ± 0.6	0.994
Valine	0.805 ± 0.013	−7.9 ± 4.0	0.992	0.835 ± 0.015	0.4 ± 0.6	0.993
Isoleucine	0.809 ± 0.012	−1.6 ± 2.9	0.994	0.847 ± 0.013	0.2 ± 0.4	0.995
Leucine	0.775 ± 0.014	21.2 ± 7.2	0.991	0.864 ± 0.015	0.3 ± 1.0	0.994
Tyrosine	0.830 ± 0.014	−9.2 ± 2.4	0.991	0.844 ± 0.014	0.1 ± 0.3	0.994
Phenylalanine	0.822 ± 0.012	1.1 ± 3.7	0.993	0.862 ± 0.010	0.5 ± 0.4	0.997
Histidine	0.773 ± 0.014	−0.2 ± 2.3	0.991	0.829 ± 0.015	0.3 ± 0.3	0.993
Lysine	0.750 ± 0.011	−4.0 ± 2.4	0.993	0.799 ± 0.016	0.3 ± 0.5	0.991
Arginine	0.861 ± 0.009	−8.4 ± 3.7	0.997	0.892 ± 0.009	−0.0 ± 0.5	0.998

$n = 24$ for each regression line.

the intestinal contents were pushed out gently, homogenized, and stored in Eppendorf tubes at −20°C until further analysis.

2.3.1 Analysis and calculations

The proximate analysis of SFM and compound feeds was carried out with the official methods: dry matter (ISO 6496:2001), crude protein (ISO 5983-1:2005), crude fiber (ISO 6865:2001), crude fat (ISO 11085:2015), crude ash (ISO 5984:1992), and amino acids (ISO 13903:2005). Amino acid contents of feed and ileal samples were determined with an automatic amino acid analyzer (Ingos Amino Acid Analyzer AAA 400) after 24 h of acid hydrolysis with 6 M aqueous HCl at 110°C. To avoid the loss of methionine (MET) and cystine (CYS), before hydrolysis, samples were oxidized with formic acid. Tryptophan contents were not determined. The TiO_2 content was determined by a spectrophotometer (Jenway 6100) at 410 nm, according to the method of Short et al. (34).

The apparent amino acid digestibility of the diets was calculated from the amino acid and TiO_2 contents of feeds and ileal digesta using the following equation:

$$DC_{AA\,diet}: \left(\left(AA_{diet} - \left(AA_{digesta} \text{ x } Tid_{2\,Diet} / TiO_{2\,digesta}\right)\right) / AA_{diet}\right) \times 100$$

where:

$DC_{AA\,diet}$ = amino acid digestibility coefficient of the diets (%)
AA_{ddiet} = amino acid content of the diet (mg/g)
$AA_{digesta}$ = amino acid content of the ileal digesta (mg/g)
TiO_{2diet} = titanium dioxide content of the diet (%)
$TiO_{2digesta}$ = titanium dioxide content of the ileal digesta (%).

The ileal amino acid digestibility of sunflower meal was calculated by linear regression between the daily amino acid

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intake and the amount of the pre-cecally absorbed amino acids, as described by Rodehutscord et al. (26). The daily intake of the AAs (mg/day) was calculated by multiplying the feed intake (g/d) by the AA content of the diet (mg/g). The quantity of pre-cecally absorbed AAs was calculated as AA intake (mg/day) times the ileal amino acid digestibility of the diets ($DC_{AA\ Diet}$). The AA digestibility of SFM was the slope of the linear regression equation.

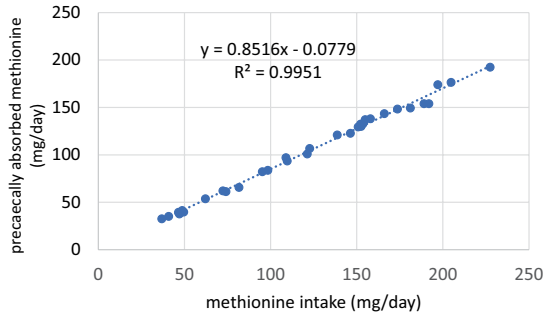

FIGURE 1
Relationship between the daily intake and ileal absorption of methionine, determined with pullets.

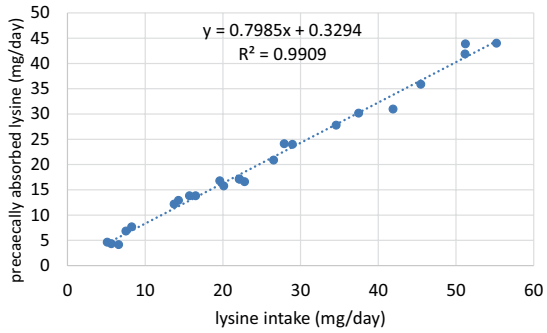

FIGURE 2
Relationship between the daily lysine intake and ileal absorption, determined with laying hens.

The measured AA digestibility of SFM was compared with those of the tables (35–37).

The AA digestibility of the diets was compared with one-way ANOVA, while the comparison of the measured AA digestibility values of SFM with those can be found in the tables was evaluated with multivariate ANOVA. The linear regression analysis was carried out using the following formula: $Y_i = \beta_0 + \beta_1 \times X_i$, where Y_i = dependent variable (ileal digested AA); β_0 = constant; β_1 = slope; X_i = independent variable (ingested AA). All the statistical analysis including the linear regression was carried out using the software package SPSS 24.0 for Windows (SPSS Inc., Chicago, IL, United States). The differences were considered significant at $p < 0.05$.

3 Results

The average daily feed intake of pullets in the C, SFM10, SFM20, and SFM30 groups were 53, 59, 58, and 58 g, respectively. Therefore, the birds consumed slightly more feed in the SFM-containing diets. In the case of pullets, the digestibility of the individual AAs of the four diets ranged between 58.6 and 88.9%, with the lowest and highest values being determined for threonine and glutamine, respectively (Table 4). Among the essential AAs, the digestion of MET was the highest (86.3%). Despite the higher fiber content of the SFM-containing diets, the absorption of certain amino acids was significantly increased. Among essential amino acids, the SFM significantly increased the digestibility of THR, VAL, LYS, and ARG. Leucine (LEU) was the only essential AA, of which digestibility was affected negatively. The digestibility of three non-essential amino acids, GLY, TYR, and ASP, also increased significantly.

In the laying hen trial, in contrast with the pullets, the average daily feed intake decreased with the increased proportion of SFM (control: 117 g, SFM10: 101 g, SFM20: 86 g, and SFM30: 77 g). The digestibility interval of the AAs was between 73.6 and 93.6% (Table 5). In this case, MET was the most highly digested amino acid (90.31%). In the trial with laying hens, feeding SFM did not modify the digestibility of AAs. The only significant difference was the impaired digestibility of ILE.

The details of the regression analyses are presented in Table 6. All the linear regression between the daily amino acid intake and the

TABLE 7 Comparison of the measured amino acid digestibility values of sunflower meal with table values.

	Table values			Measured values	
	Evonik (2017)	CVB (2017)	NRC (1994)	Pullet	Laying hen
Lysine	0.87	0.82	0.84	0.75	0.80
Methionine	0.92	0.92	0.93	0.85	0.89
Cystine	0.80	0.73	0.78	0.74	0.83
Threonine	0.82	0.76	0.85	0.70	0.75
Arginine	0.93	0.91	0.93	0.86	0.89
Isoleucine	0.89	0.85	0.90	0.81	0.85
Leucine	0.88	0.84	0.91	0.78	0.86
Valine	0.87	0.83	0.86	0.81	0.84
Histidine	0.88	0.77	0.87	0.77	0.83
Phenylalanine	0.90	0.87	0.93	0.82	0.86

TABLE 8 Paired comparisons of the amino acid digestibility values of sunflower meal.

		Evonik (2017)	CVB (2017)	NRC (1994)	Pullets	Hens
Evonik (2017)	*p*-value					
CVB (2017)	*p*-value	<0.001				
NRC (1994)	*p*-value	0.574	<0.001			
Pullets	*p*-value	<0.001	0.001	<0.001		
Hens	*p*-value	0.003	0.458	0.007	<0.001	

amount of pre-cecally absorbed amino acids were significant, with high r^2 values. It means that feeding SFM even at 30% did not cause a decrease in protein digestion. The table shows the slopes, the constants, and the coefficients of determination. In this methodology, the slopes mean the digestibility of SFM amino acids. As indicated, the slopes of the regression lines in the pullet trial ranged between 0.70 (THR) and 0.86 (ARG, GLU). In laying hens, the lowest slope belonged also to THR (0.74), while the highest belonged to MET and ARG (0.89). For all amino acids, higher slopes were obtained in hens than in pullets. The difference between the two animal groups was small for TYR (1.4%), GLU (2.0%), PRO (2.2%), and VAL (2.9) and high for CYS (9.1%) and LEU (8.8%). Two examples of the linear regression responses are shown in Figures 1, 2.

By comparing our results with some frequently used table values (35–37), it can be concluded that the digestibility coefficients of this trial are closer than those of CVB but show amino acid-dependent differences with the coefficients of NRC or EVONIK (Tables 7, 8). In Table 7, LYS and HIS showed the highest variance. The AA digestibility of SFM determined with pullets was below the table values in all cases except cystine. Comparing the measured and table values with multivariate ANOVA, the highest similarity was found between the EVONIK and NRC coefficients, without significant difference ($p = 0.574$). Regarding the measured coefficients, the hen digestibility values were close to those of CVB values ($p = 0.458$). The AA digestibility of pullets was significantly different from all the other groups.

4 Discussion

SFM is an alternative protein source for poultry (17). However, its high fiber and low energy content, in addition to the variation in its chemical composition are the main restricting factors to its use at higher incorporation rates (11). It has been previously hypothesized that higher proportions of dietary fiber in poultry diets have a diluting effect, which was believed to cause poor nutrient utilization (38). However, the poultry industry has recognized recently that certain types and amounts of fiber could be beneficial to gastrointestinal tract development, digestion, and gut health (39). The inclusion of additional dietary fiber could also be a strategy that supports multiple aspects of laying production (40). However, according to the available results, an increase in endogenous protein and amino acid losses is inevitable if high-fiber diets are fed (41). The age of birds can also modify the endogenous amino acid losses. Higher values have been recorded in early ages because of the incomplete development of the gastrointestinal tract and lower digestion (42, 43). It was reported that the inclusion of 8% cellulose in broiler diets resulted in higher crude protein and amino acid losses (i.e., GLU, ASP, and THR) compared to diets fed with 3% cellulose. These endogenous losses might not belong to the so-called diet-specific endogenous losses (13).

Since SFM is mainly a protein source, its effect on the amino acid digestibility of the compound diets is especially important. The digestibility of amino acids in birds can be determined by different methods. The so-called difference method is most common when the test material is incorporated into a basal diet and the amino acid digestibility of the test product is calculated from the AA digestibility of the basal and test product-containing diets. The disadvantage of this method is that, if the incorporation rate of the feedstuff is low, the inaccuracy of the measurement increases. Furthermore, in this case, it is not possible to evaluate the potential depressive incorporation rates. The advantage of the regression approach is that with this method, the endogenous AA losses can also be determined (44).

The amino acid digestibility of sunflower meal was investigated in only a few cases using regression analysis (45). In this trial, SFM was fed at 15 and 30% with unsexed Ross 308 broilers until day 21. According to the results of Alagawany et al. (46), the application of a higher amount of SFM will alter the amino acid profile and crude fiber and energy content of poultry diets. Based on their results, SFM could be an acceptable feed component of poultry rations and can be fed at 25% in broiler diets and 20% in layer diets. Green et al. (47) reported that the true digestibility of essential amino acids of SFM was lower than that of soybean meal. According to our results, sunflower meal did not have a depressive effect on the amino acid digestibility of the experimental diets, even at a 30% inclusion rate. Surprisingly, the digestibility of several essential amino acids improved significantly in pullets when the SFM-containing diets were fed. These amino acids were threonine, glycine, valine, lysine, arginine, tyrosine, and aspartic acid. The only exception was leucine, of which digestibility impaired in the SFM diets. Lysine is the first limiting amino acid of SFM protein, followed by methionine, cystine, and tyrosine (46). Although glycine has been categorized as a nonessential amino acid, it may also be limiting if low-protein diets are fed (48, 49). Therefore, the improvement of glycine digestibility could be a positive result since glycine supplementation in crystalline form is not permitted in the European Union. The improvement of amino acid digestibility is in line with the results of Yokhana et al. (50). In their experiment, the dietary insoluble fiber significantly improved the digestive tract weights and the trypsin activity in the small intestine of pullets, which may contribute to an improvement in feed utilization. During their experiment, 8-week-old pullets were also used, but in contrast to our experiment, only 1% structural fiber (Arbocell RC) supplementation was used. In our study, the range of crude fiber concentration of the experimental diets was 1.78–6.46%. Similar to other findings, in this range, the crude fiber could improve protein digestibility (51–53).

Our results suggest that pullets and laying hens have a high tolerance to dietary fiber, without negatively affecting their protein

digestion. This means that not only SFM but also probably other high-fiber-containing industrial by-products can be used at higher inclusion rates in the pullet and layer diets. The difference between the results of pullets and layers could be due to the digestive tract of the younger birds, similarly to broiler chickens, being more adaptive than that of the 50-week-old animals. It is known that the trypsin activity of the small intestine increases as the bird gets older (54). Very likely, the enzyme secretion of hens is higher than that of the restricted-fed pullets. Therefore, stimulating the gizzard motility by SFM and pancreatic enzyme secretion (55) was visible only with pullets. The reason for the impaired digestion of the two-branch chain amino acid is unknown. The investigations of the age effects on AA digestion are of specific interest because, in diet formulations, the same global digestibility values are used for all poultry species and age groups. Of course, this practice could cause inaccuracies.

Knowledge of the digestibility of amino acids is important in diet formulations because AA digestibility can vary greatly among different feedstuffs and among samples of the same ingredient (56). Currently, the use of ileal AA digestibility values is common in poultry and pig diet formulation. The so-called standardized ileal digestibility (SID) of amino acids means digestibility calculations based on the AA content of the ileum or terminal part of it. The standardization means the correction of the apparent digestibility with the basal endogenous amino acid losses (BEAAL) (25). Measuring the non-digested AAs from the ileum is more accurate since the AA content of the excreta is partly modified by the microbes in the ceca. The corrections with the endogenous amino acid losses (EAAL) are also important because the AA originated from the mucus, digestive enzymes, or other gut secretions also containing AAs (57). The advantage of the regression model used in this trial is that no additional measurement of EAAL is needed (26). This statement is, however, not entirely true since a part of the ileal EAAL does not belong to the BEAAL but is diet-specific. It is well known that the fiber content and the presence of anti-nutritive factors can also modify the amount of EAAL. This is the reason why, in the regression equations of this trial, the constants were not only positive. The most abundant amino acids in the ileal endogenous protein of poultry were glutamic acid, aspartic acid, threonine, proline, serine, and glycine. These amino acids are found in high concentrations in the intestinal and pancreatic secretions and mucoproteins, confirming that these are the major components of endogenous protein (57).

Comparing our results with the table's amino acid digestibility values, the largest differences were observed in the digestibility of lysine (75–88%), threonine (70–85%), and histidine (77–88%). The reason for these big differences is partly that the table values are based on different methodologies. The values of NRC originate from the so-called precision feeding method, using adult cecectomised roosters, calculating the digestion from the excreta, and using EAAL corrections with N-free diets (58, 59). The EVONIK and CVB data are based mainly on *ad libitum*-fed broiler chickens and ileal samplings. The AA digestibility of feedstuffs has been calculated in this case using the difference method after incorporating the test feedstuff into a basal diet. In these methods, the inclusion rate could contribute to inaccuracy, since a low percentage increases the standard deviation of the determination and a high inclusion rate can already be depressive. The differences are also due to the animals. Using laying hens or pullets in these trials is rare because of the high price of the birds. Of course, the digestion potential of adult roosters, broiler chickens, laying hens, and restricted-fed pullets is different (60–63).

5 Conclusion

Sunflower meal is a locally available potential alternative to soybean meal in several countries. According to the results of this experiment, poultry can tolerate the higher structural fiber of SFM. Feeding sunflower meal at even 30% does not have a negative effect on the amino acid digestibility of the compound feeds. In the case of young pullets, the digestibility of several amino acids was even increased as a response to SFM inclusion. This result attracts attention to the importance of having age and species-specific AA digestibility coefficients for the more fibrous feedstuffs. There is high variance in the AA digestibility between the measured and table values of SFM's amino acids. The main reason for this is the difference in the animal models of digestibility determinations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the Institutional Ethics Committee (Animal Welfare Committee, Georgikon Campus, Hungarian University of Agriculture and Life Sciences). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NS: Formal analysis, Investigation, Writing – original draft. ÁM: Investigation, Resources, Writing – original draft. KT: Investigation, Writing – review & editing. LP: Investigation, Writing – review & editing. BH: Investigation, Writing – review & editing. JP: Data curation, Writing – review & editing. KD: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing.

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The effect of combining green iron nanoparticles and algae on the sustainability of broiler production under heat stress conditions

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Background: Natural feed additives in broiler feed contribute to the overall health, productivity, and economic viability of broiler chickens while meeting consumer demands and preferences for natural products. The purpose of this research was to determine the effect of green iron nanoparticles (Nano-Fe) and *Halimeda opuntia* supplementation in broiler diets on performance, ammonia excretion in excreta, Fe retention in tissues and serum, carcass criteria, and meat quality under hot environmental conditions.

Methods: A total of 256 one-day-old male Ross 308 broiler chicks were randomly assigned to one of four feeding treatments for 42 days. Each treatment had eight replications, with eight chicks per replicate. The treatments were Negative control (CON), positive control (POS) supplemented with 1 g/kg *Halimeda opuntia* as a carrier, POS + 20 mg/kg Nano-Fe (NFH1), POS + 40 mg/kg Nano-Fe (NFH2).

Results: When compared to CON and POS, dietary Nano-Fe up to 40 mg/kg enhanced ($p < 0.001$) growth performance in terms of body weight (BW), body weight gain (BWG), and feed conversion ratio (FCR). Nano-Fe had the highest BWG and the most efficient FCR (linear, $p < 0.01$, and quadratic, $p < 0.01$) compared to POS. Without affecting internal organs, the addition of Nano-Fe and POS enhanced dressing and reduced ($p < 0.001$) abdominal fat compared to control (CON). Notably, the water-holding capacity of breast and leg meat was higher ($p < 0.001$), and cooking loss was lower in broilers given Nano-Fe and POS diets against CON. In comparison to POS, the ammonia content in excreta dropped linearly as green Nano-Fe levels increased. When compared to CON, increasing levels of Nano-Fe levels boosted Fe content in the breast, leg, liver, and serum. The birds fed on POS showed better performance than the birds fed on CON.

Conclusion: Green Nano-Fe up to 40 mg/kg fed to broiler diets using 1 g/kg *Halimeda opuntia* as a carrier or in single can be utilized as an efficient feed supplement for increasing broiler performance, Fe retentions, carcass characteristics, meat quality, and reducing ammonia excretions, under hot conditions.

KEYWORDS

algae, Ammonia, broilers, growth performance, Iron Fe nanoparticles, meat quality

1 Introduction

Food safety is a significant concern in hot climatic regions of the world, particularly for chicken meat, eggs, and other products consumed by humans. Heat stress frequently reduces feed intake, impairs growth rates, contributes to increased mortality rates, alters meat quality, causes economic losses, and affects physiological health, immunity, and respiratory distress in broiler chickens (1). The harmful effects of heat stress in chickens have been lessened by the use of several mitigating techniques. The rapidly developing field of nanotechnology has many potential uses in poultry feeding. Nanotechnology uses materials with new, distinctive properties between 1 and 100 nm in size (2). The physical and chemical characteristics of nanoparticles are different from those of their original, equivalent material, which can increase their bioavailability (3, 4). The nanoparticle's lower antagonism in the gut results in better absorption, less excretion into the environment, and higher feed efficiency (5).

Iron (Fe) is a necessary mineral that is frequently added to the diet of broilers. It plays a crucial position in a variety of enzymes and proteins that govern cell development and differentiation, transport oxygen, and preserve health (6, 7). It contributes significantly to the tricarboxylic acid cycle by supporting enzymes, which facilitates the removal of harmful metabolites by catalases and peroxidases with iron (8). Heat stress lowers the amounts of Fe in serum and tissue (9). A reduction in Fe causes the immune and antioxidant systems to malfunction, which is harmful to birds' health (10). This element is abundant in nature and is found in all components used in commercial poultry diets (11). Absorption and transport of dietary Fe across the intestinal mucosa occur in mechanisms that are strongly reliant on Fe status (12). Furthermore, Fe is mostly bound to phytate in cereals and oilseeds (13), which reduces its availability in poultry diets when phytase is not added (14). However, in animals, it is mostly present in myoglobin, cytochromes, hemoglobin (60–70%), ferritin, and hemosiderin (20–30%), as well as other Fe-containing enzymes (10%) (15). Because heme Fe has a preferred absorption pathway over inorganic Fe, animal byproducts containing muscle tissue and blood have higher Fe availability for poultry (16). Green nanotechnology refers to the use of environmentally friendly processes and materials in the synthesis of nanoparticles. This approach often involves using natural sources, such as plant extracts or microorganisms, to reduce and stabilize nanoparticles.

Green Nano-iron (Nano-Fe) formulations may address challenges related to the bioavailability of iron in conventional feed resources and mineral salts. The use of green nanotechnology is driven by the desire to minimize the environmental impact and potential toxicity associated with traditional nanoparticle synthesis methods (2). The use of green Nano-Fe in poultry nutrition offers several potential benefits, combining the advantages of nanotechnology with environmentally friendly and sustainable practices. Green Nano-Fe particles have more surface area than typical iron sources. This increased surface area can improve iron bioavailability, allowing for improved nutrient absorption in poultry's digestive system, which is crucial for broiler health and growth (5, 17, 18). Nanoparticles of Fe have been of interest in various fields, including agriculture and poultry production, due to their potential applications in areas such as nutrient delivery, disease treatment, and environmental remediation (17). Nano-Fe supplementation increased body weight in broiler meals without altering the composition of the liver, thigh, or breast (18).

Halimeda opuntia, commonly known as sea cactus, is a type of green algae that is found in marine environments. In addition, algae can be grown as ingredients and dietary supplements for poultry feed (19). According to Martins et al. (20), algae have a unique composition consisting of carbohydrates, proteins, lipids, vitamins, minerals, and bioactive substances including carotenoids. Microalgae are recommended as feed additives due to their high levels of macro- and micro-elements and ability to improve the growth performance, feed efficiency, and meat quality of broilers (21), which is primarily due to properties of polysaccharides that can increase the health and productivity of chickens.

However, there is no data on the effect of graded inclusion levels of green Nano-Fe and algae on broiler performance and meat quality under hot environmental conditions. We investigated the mechanism of the effects of green Nano-Fe supplementation on broiler productive performance under hot environmental conditions. We wanted to evaluate the effects of varied inclusion levels of green Nano-Fe in broiler diets using 1 g/kg algae as a carrier on growth performance, ammonia emission in excreta, Fe retention, carcass criteria, and meat quality under heat stress.

2 Materials and methods

2.1 Dietary treatments and experimental design

The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Alexandria, Egypt (AU08220810298). A total of 256 one-day-old male Ross 308 broiler chicks were randomly assigned to one of four feeding treatments until they reached 42 days old. Each treatment had eight replications, with eight chicks per replicate. Negative control (CON), positive control (POS) supplemented with 1 g/kg microalgae as a carrier, POS + 20 mg/kg Nano-Fe (NFH1), and POS + 40 mg/kg Nano-Fe (NFH2) were the treatments. The 42-day experiment was divided into two phases (0 to 21 days for the starter and 21 to 42 days for the grower). The experimental diets used in the present study contained around 20 mg and 40 mg of green Nano-Fe/kg, which is below the minimum recommended level of 85 mg Fe/kg (11). Furthermore, the levels of Nano-Fe were selected based on previous studies suggesting that chicken diets containing varying amounts of Fe (from 10 to 60 mg/kg in non-supplemented diets to about 160 mg in diets supplemented with 140 mg Fe-Gly or 100 mg Nano-Fe) (22, 23). The diets were formulated to meet Ross 308 broiler recommendations (Table 1). Chicks had full access to feed and water during the experimental period. The experiment was conducted at the Poultry Center, Faculty of Agriculture, South Valley University. The cage measurements for the chickens were 120 × 70 × 50 cm in length, breadth, and height, respectively. There were four nipple drinkers and hanging linear feeders in each pen. As the birds grew, so did the height of the nipple line. A 23-h continuous light scheme was implemented from the first day to 42 days of age. The ambient temperature was gradually reduced from 34.5°C (45 RH%) for days 1 to 21 to 28.5°C, 40 RH%, and 29.9 temperature-humidity index (THI) from 22 to 42 days of age.

THI = db°C- [(0.31–0.31RH; db°C-14.4)], where db is the dry bulb temperature in degrees Celsius and RH is the relative humidity

TABLE 1 The chemical composition of the basal diet (as-fed basis).

Ingredients, g/kg	Starter diet	Grower diet
Corn	276	300
Sorghum	276	300
Soybean (44% CP)	285	250
Corn gluten (60% CP)	95.0	60.0
Vit and Min. Premix[a]	3.00	3.00
Sunflower Oil	30.0	55.2
Dicalcium phosphate	20.0	18.0
Limestone	10.0	10.00
Salt	3.80	3.80
DL-methionine	0.40	---
L-lysine HCl	1.00	---
Total	1,000	1,000
Analyzed chemical composition, g/kg		
Dry matter	925	924
Crude protein	233	216
Ether extract	53.7	57.5
Crude fiber	25.8	37.8
Ash	67.4	61.8
Ca	13.22	12.84
P	7.05	7.21
Fe	0.024	0.026
GE, MJ/kg (Calculated)	18.55	19.18

[a]Supplied per kg diet: biotin (50 mg), pantothenic acid (10,000 mg), folic acid (1,000 mg), nicotinic acid (30,000 mg), vitamin A (1900 IU), K3 (1,000 mg), B1 (1,000 mg), B2 (5,000 mg), B6 (1,500 mg), and B12 (0.046 mg) in addition to D3 (1,300 IU), E (10,000 mg), and BHT (10,000 mg) and includes 60 mg of Mn, 50 mg of Zn, 0.1 mg of Se, 4 mg of Cu, 3 mg of I, and 0.1 mg of Co.

percentage/100. The calculated THI values were then classed as follows: 27.8 indicates no heat stress, 27.8–28.9 indicates moderate heat stress, 28.9–30.0 indicates severe heat stress, and > 30.0 indicates extremely severe heat stress (1).

2.2 Green synthesis of Fe nanoparticles

In accordance with the maceration technique outlined by Khalil et al. (24), green Fe oxide nanoparticles were produced from the leaf extract of *Ocimum basilicum*. To sum up, a hot-plate magnetic stirrer was used to heat 30 g of plant powder and 200 mL of distilled water to 80°C for 1 h. Solid residues were eliminated from the final solution by filtering it three times with the Whatman No. 1 filter paper. The filtered solution with a pH of 5.7 was heated for 2 h at 85°C and then 100 mL of Fe (III) chloride (6 g) was added as a precursor salt. The solution went from being brownish to violet in hue, and its pH was recorded. After allowing the mixture to reach room temperature, decantation was used to extract the Fe oxide nanoparticles. After three cycles of distilled water washing, the Fe oxide was allowed to dry at room temperature. These particles were characterized by transmission electron microscope (TEM; Figure 1).

2.3 Algae preparations

The macroalga *Halimeda opuntia* was collected by hand-picking from the Red Sea in Hurghada, Egypt. Healthy algae samples were cleaned from epiphytes, extraneous matter, and necrotic were removed. Samples were washed thoroughly with sea water then sterile distilled water, air dried, cut into small pieces, and then ground in a tissue grinder to pass through a 1 mm screen [IKA A 10, Germany] until reached a fine powder shape. The ground *Halimeda opuntia* was kept until used to mix with the experimental diets. One g/kg of *Halimeda opuntia* macroalgae was added to broiler diets, and this dose was chosen in compliance with previous studies suggesting that the ideal levels of macroalgae in broiler diets should range from 0.5 and 3 g/kg (19–21).

2.4 Broiler productive performance

From the first day of the experiment to the last, the body weight (BW) was recorded for each pen once a week. Furthermore, on the day that the birds were weighed, feed residue was measured in order to calculate the amount of feed that each pen consumed in between weigh-ins. The feed conversion ratio (FCR) was calculated by dividing the weight of feed consumed by the body weight gain (BWG) of each pen. This yielded the feed per gain. A correction for bird mortalities was applied to the magnitude of production variables, such as feed consumption and body weight.

2.5 Ammonia analysis

The excreta was collected daily at 21 to 42 days of age per pen for the determination of ammonia excretion according to the method proposed by Miles et al. (25). 200 g of freshly collected excrement was added to a 1,000 mL jar. A rubber stopper sealed the upper portion of the jar; the rubber plug featured an exhaust pipe and an intake pipe that linked to a U-shaped bubble absorption tube. The U-shaped bubble absorption tube (which was shielded from light) received around 10 mL of 2% boric acid. The U-shaped absorption tube's other end was linked to an inflating pump via a second buffering device, and the intake pipe was connected to a buffer device. In an acidic environment, the 2% boric acid absorption solution was used to repair the ammonia gas produced from chicken feces, creating a stable NH4+. By using the Kjeldahl nitrogen determination method, the nitrogen content in the absorption solution was ascertained. The nitrogen content was then translated into NH3 content in the unit mass of feces (fresh weight basis).

2.6 Carcass criteria and internal organs

At 42 days of age, 40 birds per treatment were selected at random (five birds per replicate pen), weighed, slaughtered according to the Halal method, and plucked. Weighing the remaining portion of the body after the head, neck, viscera, digestive tract, shanks, spleen, liver, heart, gizzard, and abdominal fat were removed allowed us to calculate the relative weight. The formula for calculating dressing percentage is dressed weight/live weight × 100. The percentage of abdominal fat, liver, heart, and empty gizzard were calculated based on live body weight.

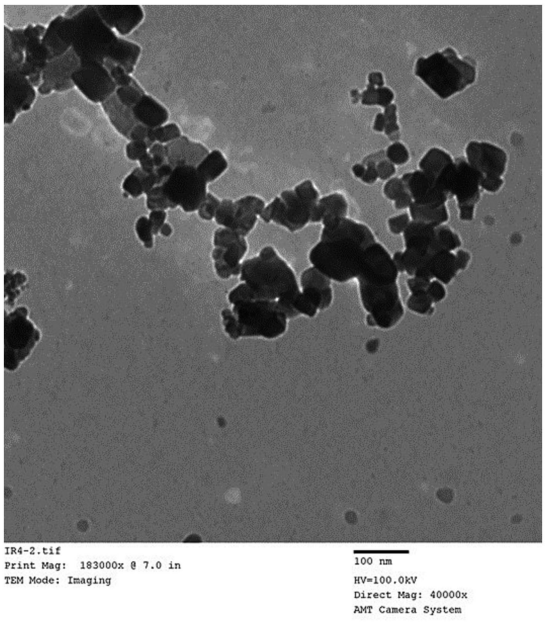

FIGURE 1
Transmission electron micrographs (TEM) of Nano-Fe.

2.7 Meat quality measurements

The water-holding capacity (WHC) and cooking loss were assessed from the left side of the breast muscle and the left leg in 40 birds per treatment which were randomly selected (5 birds per replicate). The low-speed centrifugation technique was utilized to quantify the WHC of breast muscles with minimal adjustments (26). 10 g of intact breast muscle was placed in a falcon tube with glass beads and centrifuged for 20 min at 10,000 g at 5°C. The precipitated meat was then removed immediately, dried with filter paper, and weighed once more. The WHC was calculated using the weight loss in muscle samples after centrifugation. Cooking loss was calculated, as previously stated (27). In summary, the muscle filets were placed separately in thin-walled thermotolerant polyethylene bags and cooked in a water bath until their core temperatures reached 70°C. Following that, they were refrigerated in crushed ice until they reached 5°C, and the cooking loss was calculated by reweighing them. Samples of the liver, breast, leg, and blood were taken and held at −20°C for the Fe chemical analysis.

2.8 Fe analysis

For each treatment, 40 were randomly selected and the birds' wing veins were utilized to extract blood (5 birds were used for each replicate), which was then placed in vacutainer tubes to collect serum. After 40 birds were killed, samples of their breast, leg, liver, and serum were taken, and they were promptly frozen at −20°C to be subjected to a Fe content study. The Fe concentrations were measured using an atomic absorption spectrophotometer (Perkin Elmer Analyst 800 model, Shelton, CT, United States).

2.9 Statistical analysis

The General Linear Models (GLM) technique for statistical analysis and SAS 9.2 software was used to examine the data of a completely randomized trial (SAS Institute) (28). The only constant in the model was the dosage of the supplements. The birds served as the experimental units for ammonia, carcass criteria, meat quality, and Fe retention, while the pen served as the experimental unit for growth performance. One-way ANOVA was utilized to examine the data, and Duncan multiple range tests were employed to compare means. The graphs were created using GraphPad Prism software, version 9 (GraphPad Software, La Jolla, CA, United States), along with a normal distribution test (Anderson—Darling test for normality). The linear and quadratic impacts of increasing Nano-Fe supplementations were calculated using orthogonal polynomial contrasts, with only POS (0 mg/kg Nano-Fe) taken into consideration as a control, and CON was not included in this analysis. A significance value of $p < 0.05$ was used. p values less than 0.001 were expressed as '<0.001' rather than the actual value.

3 Results

3.1 Productive performance

The effects of green Nano-Fe on the BW and BWG of broiler chickens during the starter phase (0 to 21 d) and grower phase (22 to 42 d) are shown in Table 2. When compared to CON, dietary Nano-Fe up to 40 mg/kg enhanced ($p < 0.001$) BW and BWG. Nano-Fe at 20 mg/kg and 40 mg/kg with 1 g/kg *Halimeda opuntia* as a carrier in broiler diets increased ($p < 0.05$) BW compared to CON and POS during 21 and 42 days of age. There was an increase ($p < 0.05$) in BWG in POS when compared with CON during 1–21, 22–42, and 1–42 days of age showing the positive effects of adding algae. Similarly, dietary treatments containing POS, Nano-Fe at 20 mg/kg, and Nano-Fe at 40 mg/kg increased BWG ($p < 0.001$) by 10.95, 10.30, and 14.50%, respectively, compared to control throughout the trial period (1–42 days). Considering the entire trial period, feed intake in the supplemented groups differed significantly, although the Nano-Fe at 40 mg/kg showed the highest feed intake compared to others (Table 3). The addition of Nano-Fe improved ($p < 0.05$) FCR when compared to CON and POS at 1–21, 22–42, and 1–42 days of age. The CON group performed the worst in terms of BW, BWG, and FCR when compared to the POS and Nano-Fe groups. POS, Nano-Fe at 20 mg/kg, and Nano-Fe at 40 mg/kg feeding treatments improved the FCR ($p < 0.006$) compared to the CON throughout the trial (1–42 days).

3.2 Ammonia contents

Figure 2 represents the effects of Nano-Fe supplementation on ammonia concentration at 21 and 42 days of age. Green Nano-Fe levels in broiler diets decreased ($p < 0.001$) excreta ammonia content when compared to CON and *Halimeda opuntia* alone at 21 and 42 days of age in heat stress. The POS group had the lowest ($p < 0.01$) level of excreta ammonia, followed by the Nano-Fe and CON groups. When POS was compared to green Nano-Fe levels in broiler diets at 21 and 42 days of age under heat stress, the excreta ammonia concentration reduced linearly ($p < 0.001$).

TABLE 2 Effects of green Nano-Fe and algae on body weight and body weight gain of broiler chickens.

Items	Body weight, g/bird			Body weight gain, g/bird		
	1 day	21 days	42 days	1–21 days	21–42 days	1–42 days
Treatments, mg/kg						
CON	42.50	709.5^{d}	1939.5^{c}	667.0^{d}	1230.0^{c}	1897.0^{c}
POS	42.75	741.6^{c}	2147.6^{b}	698.8^{c}	1406.0^{b}	2104.8^{b}
NFH1	42.38	772.0^{b}	2156.2^{b}	729.6^{b}	1384.2^{b}	2113.8^{b}
NFH2	42.25	800.6^{a}	2245.3^{a}	758.3^{a}	1445.7^{a}	2203.3^{a}
SEM	0.090	7.351	19.636	7.372	21.038	19.834
p value						
Treatment	0.240	<0.001	<0.001	<0.001	<0.001	<0.001
Linear	0.054	<0.001	0.002	<0.001	0.209	0.002
Quadratic	0.556	0.936	0.108	0.927	0.128	0.109

$^{a-d}$Means with different superscript in a column are significantly different ($p < 0.05$). CON: negative control. POS: positive control (1 g/kg *Halimeda opuntia*). NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe. NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe. SEM: Standard error of the means ($n = 8$).

TABLE 3 Effects of green Nano-Fe and algae on feed intake and feed conversion ratio of broiler chickens.

Items	Feed intake, g/bird			Feed conversion ratio		
	1–21 days	21–42 days	1–42 days	1–21 days	21–42 days	1–42 days
Treatment, mg/kg						
CON	901.6^{c}	2329.1^{a}	3230.7^{b}	1.352^{a}	1.896^{a}	1.703^{a}
POS	947.5^{b}	2340.6^{a}	3288.2^{b}	1.356^{a}	1.667^{b}	1.563^{b}
NFH1	907.9^{c}	2167.3^{b}	3075.2^{c}	1.244^{b}	1.566^{c}	1.455^{c}
NFH2	986.4^{a}	2469.3^{a}	3455.8^{a}	1.302^{b}	1.708^{b}	1.568^{b}
SEM	7.759	30.909	34.626	0.012	0.031	0.021
p value						
Treatment	<0.001	0.002	0.001	0.001	<0.001	<0.001
Linear	<0.001	0.040	0.008	0.020	0.312	0.835
Quadratic	<0.001	<0.001	<0.001	<0.001	0.002	<0.001

$^{a-d}$Means with different superscript in a column are significantly different ($p < 0.05$). CON: negative control. POS: positive control (1 g/kg *Halimeda opuntia*). NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe. NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe. SEM: Standard error of the means ($n = 8$).

3.3 Carcass criteria

According to the carcass criteria (Table 4), broilers fed diets containing POS and POS including Nano-Fe at 20 mg/kg and 40 mg/kg showed increases ($p < 0.05$) in dressing percentage and decreases in abdominal fat at the end of the experiment compared to CON. Supplementation of POS and POS with Nano-Fe had no effect ($p > 0.05$) on the percentages of liver, heart, spleen, and gizzard of broilers compared to CON in heat stress.

3.4 Physicochemical properties of meat

In terms of meat physicochemical criteria, POS and POS with Nano-Fe levels increased ($p < 0.05$) the WHC% of the breast muscles and leg muscles at 42 days of age in hot conditions (Figure 3). Supplementation of Nano-Fe to broiler diet linearly decreased WHC% in breast and leg meat compared to POS. Cook loss percentages of the breast and leg muscles at 42 days of age were reduced at POS, 20 mg/kg, and 40 mg/kg Nano-Fe levels compared to CON (Figure 4). When Nano-Fe was added to the broiler feed, cook loss in the leg and breast meat was linearly reduced in comparison to *Halimeda opuntia* (POS).

3.5 Iron retentions

Supplementation of algae and Nano-Fe to broiler diets improved (Linear, $p > 0.001$) the Fe contents in the breast and leg meat compared to POS at 42 days of age (Figure 5). The Fe content in the breast and leg was greater in the POS group than in the CON group. The Fe content in liver tissue was higher in the POS group compared to CON, however, there is no difference between POS and CON in Fe content in the serum of broiler chickens. The Fe content in liver tissues and serum was increased (linear, $p < 0.05$) with the increasing levels of Nano-Fe levels compared to POS (Figure 6).

4 Discussion

According to the current study, broiler hens fed diets containing *Halimeda opuntia* and green Nano-Fe enhanced production

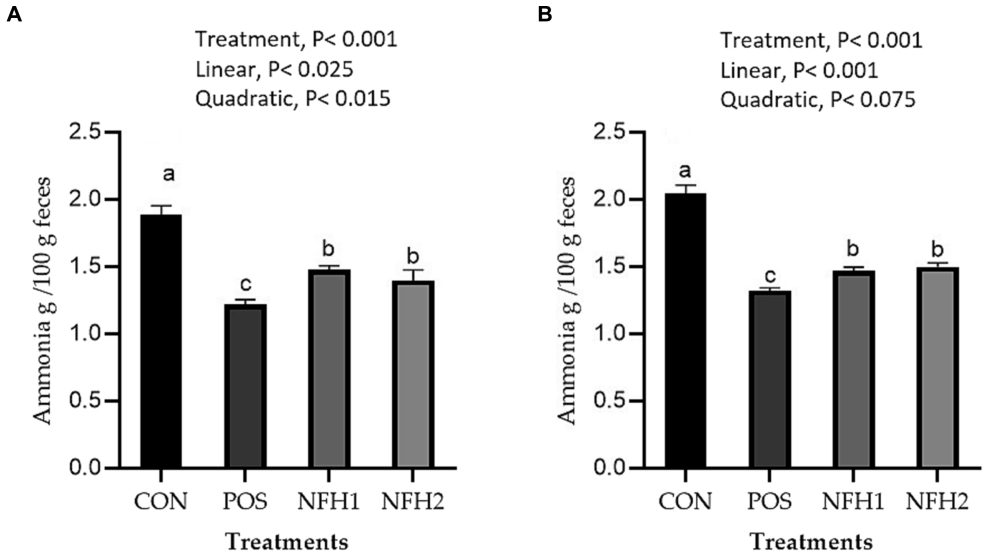

FIGURE 2
Effects of green Nano-Fe and algae on broiler chicken excreta ammonia contents at 21 **(A)** and 42 **(B)** days of age. Bars with different letters **(a–c)** are significantly different ($p < 0.05$). CON: negative control, POS: positive control (1 g/kg *Halimeda opuntia*), NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe, NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe, SEM: Standard error of the means ($n = 40$).

TABLE 4 **Effects of green Nano-Fe and algae on carcass characteristics at 42 days of age.**

Items	Dressing%	Abdominal fat%	Liver%	Heart%	Gizzard%	Spleen %
Treatment, mg/kg						
CON	75.18[b]	0.833[a]	1.880	0.424	1.264	0.116
POS	78.74[a]	0.619[b]	1.935	0.464	1.344	0.117
NFH1	78.80[a]	0.547[b]	1.966	0.445	1.358	0.114
NFH2	78.82[a]	0.513[b]	1.885	0.455	1.305	0.115
SEM	0.326	0.035	0.038	0.010	0.030	0.005
p value						
Treatment	0.028	0.001	0.859	0.106	0.711	0.054
Linear	0.002	0.001	0.675	0.730	0.668	0.142
Quadratic	0.485	0.057	0.590	0.222	0.674	0.062

[a–d]Means with different superscript in a column are significantly different ($p < 0.05$). CON: negative control. POS: positive control (1 g/kg *Halimeda opuntia*). NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe. NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe. SEM: Standard error of the means ($n = 8$).

performance and mitigated the detrimental effects of heat stress. Heat stress is widely recognized to impair feed intake, BWG, and production performance in chickens while also increasing mortality (29–32), resulting in a loss of earnings on poultry farms. Following these negative results, meat quality (33), animal welfare (30), and immunological function (28) gradually worsen. To date, numerous mitigation methods have been introduced to lessen the detrimental effects of heat stress in poultry. Nutritional remedies have been investigated as a viable way to mitigate the negative effects of heat stress (1). Green nanotechnology feeding has the most potential as a nutritional technique, and it deserves more exploration to improve thermotolerance in chickens. Fe is an essential nutrient for chickens, and nanoparticles can improve its bioavailability. Although nanoparticles have a higher surface area, they can be more easily absorbed in the digestive tract. This increased bioavailability may contribute to improved poultry health and growth. Considering Fe is a component of hemoglobin (34) and plays a crucial role in cellular and whole-body energy and protein metabolism (35), chickens are especially vulnerable to Fe deficiency. The use of Fe nanoparticles in poultry feed has shown promise in improving feed efficiency.

In the current investigation, dietary Nano-Fe at up to 40 mg/kg increased growth performance when compared to CON. likewise, dietary treatment with POS, NFH1, and NFH2 exhibited synergistic improvements in BWG ($p < 0.001$) by 10.95, 10.30, and 14.50% compared to CON throughout the experiment period (1–42 days). Furthermore, feeding treatments with POS, NFH1, and NFH2 increased FCR ($p < 0.006$) by 8.22, 14.56, and 7.93% compared to CON, respectively. Few studies have used green Nano-Fe combined with algae as a feed additive for poultry under heat stress. Similar findings were reported by Rehman et al. (36), who found that adding xylanase and Fe oxide nanoparticles to broiler feed enhanced the FCR values at 35 days of age and raised BW by 45% compared to the CON

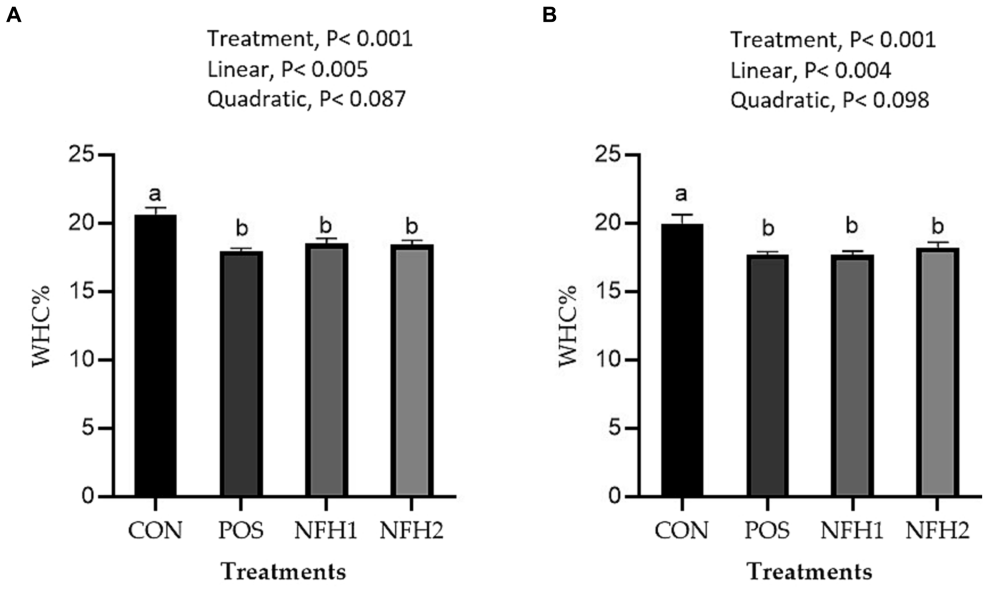

FIGURE 3
Effects of green Nano-Fe and algae on water holding capacity (WHC) of the breast **(A)** and leg **(B)** muscles in broilers at 42 days of age. Bars with different letters **(a,b)** are significantly different ($p < 0.05$). CON: negative control, POS: positive control (1 g/kg *Halimeda opuntia*), NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe, NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe, SEM: Standard error of the means ($n = 40$).

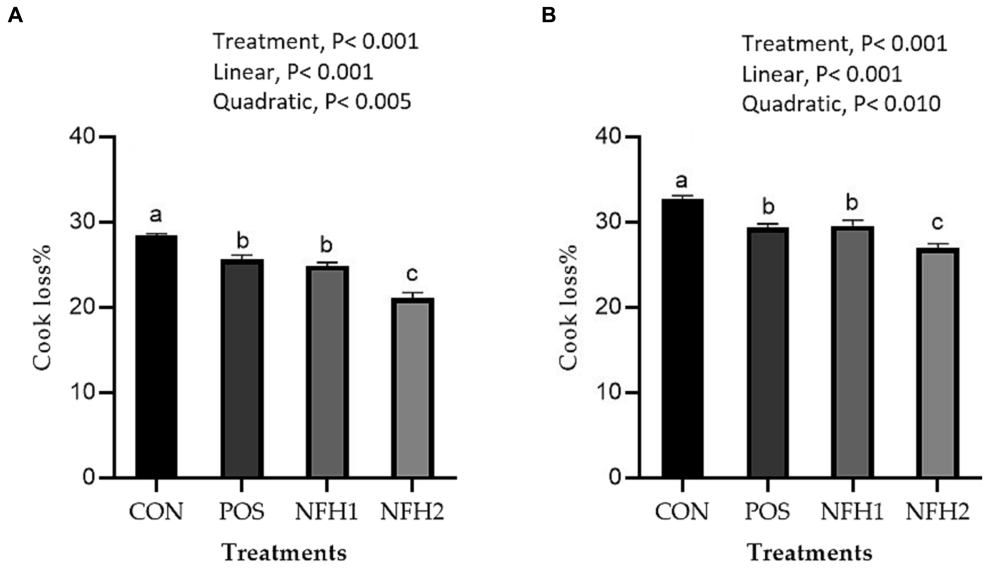

FIGURE 4
Effects of green Nano-Fe and algae on cooking loss of the breast **(A)** and leg **(B)** muscles in broilers at 42 days of age. Bars with different letters **(a–c)** are significantly different ($p < 0.05$). CON: negative control, POS: positive control (1 g/kg *Halimeda opuntia*), NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe, NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe, SEM: Standard error of the means ($n = 40$).

group. The addition of 40–160 mg Fe/kg from Fe-Gly elicited higher responses; broiler chicks fed 100 mg Fe/kg exhibited the highest FCR and BWG (22). Fe oxide nanoparticles added to the diet of broiler chicks increased BW and BWG without posing any negative health risk (8). Sarlak et al. (37) observed improvements in performance indicators such as feed intake and FCR when dietary Fe was added to chicken diets compared to the CON treatment. When broiler chicks were fed Nano-Fe, their BW increased and the FCR improved (8). Compared to a CON diet without Nano-Fe, broiler diets supplemented with Nano-Fe significantly boosted BWG by 8% (38). Algae is recommended as feed additives due to their high levels of macro- and micro-elements and ability to improve the growth performance and feed efficiency of broilers (21), which is primarily due to the properties of seaweed polysaccharides that can increase the health and productivity of chickens. Furthermore, broilers' diets containing 1 and 3.0% macroalgae *Ulva lactuca* from days 12 to 33

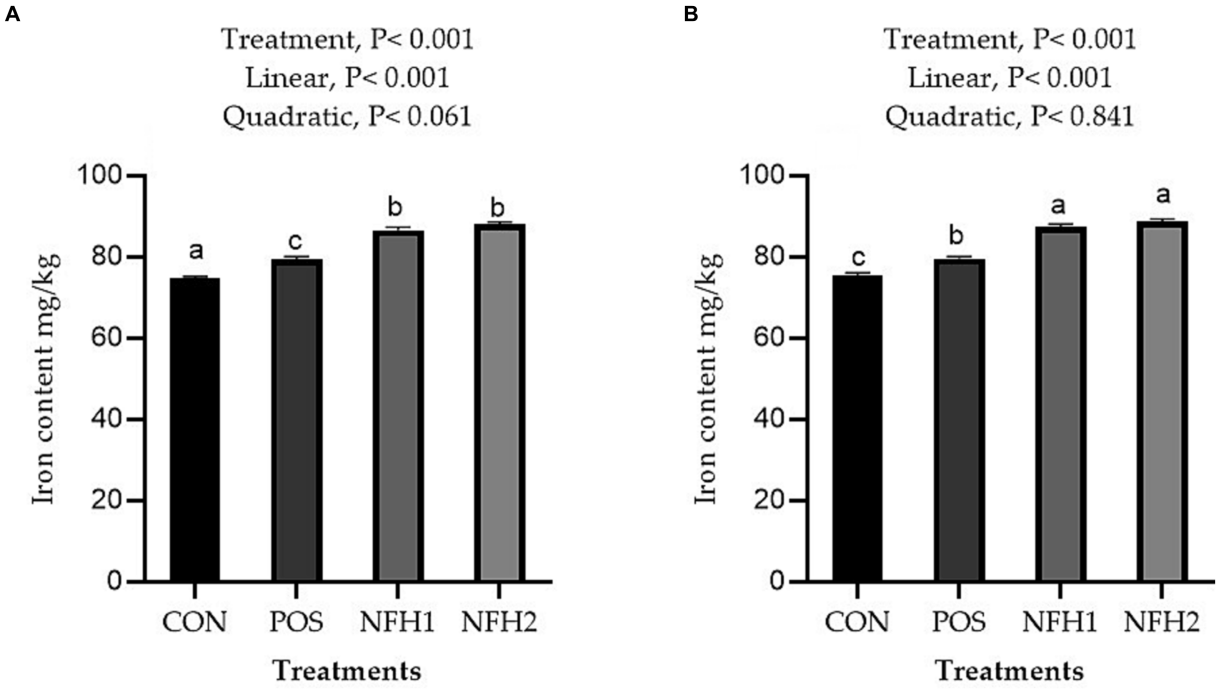

FIGURE 5
Effects of green Nano-Fe and algae on Fe content in the breast **(A)** and leg **(B)** muscles of broilers at 42 days of age. Bars with different letters **(a–c)** are significantly different ($p < 0.05$). CON: negative control, POS: positive control (1 g/kg *Halimeda opuntia*), NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe, NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe, SEM: Standard error of the means ($n = 40$).

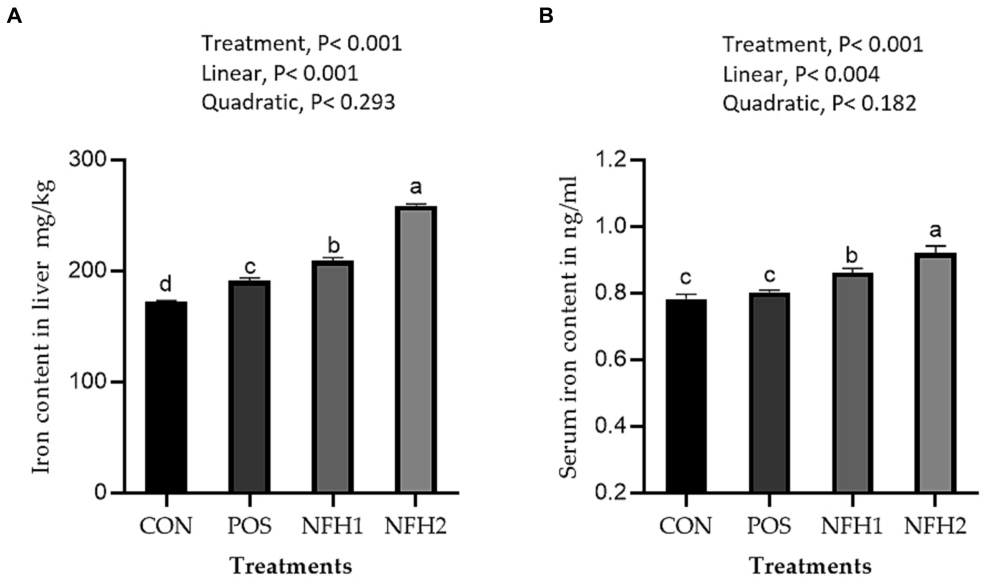

FIGURE 6
Effects of green Nano-Fe and algae on Fe content of the liver muscles **(A)** and serum **(B)** in broilers at 42 days of age. Bars with different letters **(a–c)** are significantly different ($p < 0.05$). CON: negative control, POS: positive control (1 g/kg *Halimeda opuntia*), NFH1: 1 g/kg *Halimeda opuntia* with 20 mg/kg Nano-Fe, NFH2: 1 g/kg *Halimeda opuntia* with 40 mg/kg Nano-Fe, SEM: Standard error of the means ($n = 40$).

post-hatch revealed a significant improvement in BWG and FCR compared to CON (39). When added seaweed to broiler diets at a level of 0.5%, improved BWG, FCR, and decreased mortality rate when compared to a CON diet (40). These findings could be due to broiler immune systems and antioxidant metabolism might be strengthened by active components found in macroalgae (41),

which would increase broiler productivity (42). The FCR and BW increase of broiler chicks given additional algal astaxanthin at doses of 2.3 and 4.6 mg/kg of diet showed marginal benefits (43). Additionally, broiler FCR was enhanced, and BWG was significantly boosted when 1% or 2% DHA-rich algae were added to the diet (44). The evidence of increased growth performance connected with algae-derived compounds may be inconsistent.

Furthermore, in the current study, the ammonia content of excreta was decreased with increasing green Nano-Fe levels with macroalgae compared to POS. Nano-Fe might help to upregulate the functional nitrogen metabolism pathway in intestinal bacteria, boosting the utilization of nitrogenous compounds in the host intestine and lowering ammonia elimination through excreta. Also, macroalgae may contribute to improved nutrient utilization by the broilers, leading to reduced ammonia excretion in the feces. Ammonia is produced by the microbial fermentation of uric acid and urea in feces, which causes respiratory diseases and chronic stress in livestock and poultry (45–47). Ammonia is created through the deamination of amino acids and the hydrolysis of urea. Changes in ammonia content, as well as ammonia uptake through epithelial cells, have an impact on the microbiota (48). Furthermore, as the ammonia level in the gut dropped, the compensatory effect of ammonia on intestinal cells was reduced, resulting in improvements in the intestinal barrier and histomorphology of the host intestine (49). The potential for macroalgae and microalgae to reduce fecal ammonia in broilers is an area of ongoing research and interest in the field of poultry nutrition. Ammonia is a common byproduct of the breakdown of nitrogen-containing compounds in manure, and high levels of ammonia in poultry housing can have negative effects on bird health and welfare. Seaweed contains bioactive compounds, such as certain polysaccharides and secondary metabolites, which may have the ability to influence microbial populations in the gut and reduce ammonia production (50).

In the current study, supplementations of Nano-Fe to broiler diets improved ($p < 0.05$) Fe in the breast, leg, liver, and blood. Fe is abundantly stored in the body, especially in the liver and bone marrow reticuloendothelial cells (34). Depending on the body's Fe state, dietary demands for Fe can be regulated to enhance or decrease its rate of absorption via different recognized mechanisms (16). These mechanisms are connected to receptors on the surface of enterocytes, such as the heme carrier protein 1, which is responsible for heme-Fe absorption in the colon (51), and the divalent metal transporter 1, which may accept inorganic Fe^{+2} and immediately release it into the cytoplasm (52). Because Fe absorbs more rapidly than inorganic Fe, animal wastes including muscle tissue and blood provide greater Fe to poultry (16). Ma et al. (53) investigated the dietary Fe needs of broilers aged 1 to 21 days and discovered that 97 to 136 mg Fe/kg was necessary to sustain their complete expression in various tissues. In addition, serum ferritin levels were considerably higher in diets supplemented with 75, 150, or 300 mg/kg Fe, but not in diets supplemented with 600 mg/kg Fe (54). The Fe content of chick serum increased progressively as the Fe level in the diet increased (37). A large dose of ferrous methionine dramatically raised the hepatic Fe concentration in Ross broilers, according to research by Seo et al. (55). The Fe content of broiler liver, according to Ma (56), declined gradually when dietary Fe levels rose over 120 mg/kg. This could be because the liver was able to maintain the proper balance of Fe, preventing excessive deposition of Fe that could harm the body. The inconsistencies in the results in previous studies could be attributed to the broiler variety and their specific feed.

The effects of algae on the Fe content in broiler meat tissue are not extensively studied, and the available literature on this specific topic might be limited. However, algae, including certain types of seaweed, are known to contain various minerals, including Fe, which can be transferred to the animals consuming them (57). The bioavailability of Fe in the diet is crucial. The type of Fe present in the algae and its bioavailability could impact its transfer to the broiler meat (58). The ability of the broilers to absorb and incorporate dietary Fe into their tissues can vary based on factors such as age, health status, and genetics.

In the current investigation, supplementation of green Nano-Fe to broiler diets improved percentages of carcass dressing and reduced abdominal fat without any side effects on internal organs at 42 days of age. The results are consistent with Rehman et al. (37) showed that Fe oxide nanoparticles have a lot of potential for usage in chicken feed for large-scale meat production without any negative toxicological effects. Our results are consistent with Lin et al. (59) who found that varied amounts of Fe at 50, 70, 90, 110, 130, and 150 mg/kg did not affect the weight indices of Fabricius' liver, kidneys, spleen, thymus, and bursa. Concerning carcass criteria, algae is recommended as feed additives due to their high levels of macro- and micro-elements and ability to improve broiler meat criteria (3). Male broilers' diets containing 3.0% macroalgae *Ulva lactuca* revealed a significant improvement in breast muscle yield and dressing percentage compared to control (39). The improvements in carcass criterion and abdominal fat can be attributed to the favorable effects of DHA in the green alga *Ulva* on lipid utilization in serum (60). Fe, as an essential component of Fe-containing critical enzymes in broilers, plays a vital function in Fe metabolism and meat quality.

The current study found that Nano-Fe improved meat quality including WHC and cooking loss under heat stress conditions, which could be attributed to the Fe improving antioxidant activation. A recent study (61) found that Fe supplementation improved enzymatic antioxidant protection in chicken serum. While Kurtoglu et al. (62) showed that Fe-deficiency anemia lowered plasma antioxidant activities. As far as we are aware, there are no published articles on the effect of green Nano-Fe on broiler chick carcass criteria and meat quality. Fe is a required component of hemoglobin in erythrocytes and is needed by hemoglobin and myoglobin (63, 64) for oxygen delivery, storage, and usage in muscles (65). The greatest noticeable indicator of meat quality, color, is mostly determined by hemoglobin and myoglobin (66). Furthermore, Sun et al. (67) reported that adding astaxanthin-rich *Haematococcus pluvialis* to a broiler diet increased the pH of the breast muscle and lowered the WHC of the breast muscle compared to the control. The application of green Nano-Fe with algae as a carrier in broiler diets holds promise for improving production traits and meat quality as well as Fe retention. With the growing global demand for sustainable and affordable poultry nutrition alternatives, the role of green nanotechnology is gaining popularity (68–70). The use of green nanotechnology aligns with the broader goal of promoting sustainability in poultry production, optimizing resource use, minimizing environmental impact, and enhancing the overall efficiency and health of animals and poultry (71, 72). However, thorough research, including dose, safety assessments, and regulatory considerations, is necessary to ensure the

responsible and effective implementation of this technology in the poultry industry.

5 Conclusion

Overall, the present study reveals that broiler chickens fed diets containing Nano-Fe and *Halimeda opuntia* showed synergistic enhancements growth performance, meat quality, Fe absorption, and decreased abdominal fat, but had no significant effects on internal organs. Future research ought to inquire into the impact of green Nano-Fe on immune status, microbiome, and gene expression related to immunity and heat stress.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of the University of Alexandria, Egypt (AU08220810298). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YA: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. AE: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing. EE: Conceptualization, Investigation, Methodology, Writing – review & editing. AAAA-W: Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. JL: Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of different dietary threonine and glycine supplies in broilers fed low-protein diets

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The reduction of crude protein (CP) content of broiler diets with balanced amino acid supply can increase the nitrogen (N) utilization efficiency and reduce ammonia emission, the risk of many health problems in birds. Feeding low protein (LP) diets without the impairment of performance traits needs the optimized dietary levels of threonine (Thr) and the non-essential amino acid (AA) glycine (Gly) and serine (Ser). However, the required concentrations and interactions of Thr and Gly + Ser, expressed as Gly equivalent (Gly_{equi}), in LP diets are not fully understood. Therefore, the aim of this study was to investigate the effects of three LP (LP1–3) grower (11–24 days) and finisher (25–35 days) diets with 2% CP reduction compared to the control (C), differing in standardized ileal digestible (SID) Thr to lysine (Lys) ratio (C, LP1, LP3: 63%, LP2: 72%) and Gly_{equi} levels (C: 15.65 g/kg, LP1: 13.74 g/kg, LP2: 13.70 g/kg, LP3: 15.77). The LP treatments did not impair the performance traits of broilers. The LP2 treatment with increased SID Thr-to-Lys ratio (+9.0%) resulted in significantly higher body weight gain and a more advantageous feed conversion ratio in the whole fattening compared to the control treatment with normal CP level ($p < 0.05$). The LP3 treatment containing swine meat meal with similar Gly_{equi} levels compared to the normal CP treatment led to the most advantageous feed conversion ratio in the finisher phase and the highest nitrogen retention efficiency ($p < 0.05$). However, the LP3 treatment with a high starch-to-CP ratio negatively influenced the relative carcass weight and the ratio of abdominal fat of broilers ($p < 0.05$).

KEYWORDS

broiler, low protein diet, threonine, glycine, nitrogen

1 Introduction

Feeding low-protein (LP) diets to broilers has been the most effective method to lower nitrogen (N) excretion and increase N utilization efficiency, reducing the risk of wet litter problems and incidence of dysbiosis (1–3). Our current knowledge allows the reduction of up to 2% dietary crude protein (CP) in maize/soybean meal-based broiler diets in each feeding phase without performance loss and deterioration of product quality (4). This level of CP reduction can be successfully achieved by a precise adjustment of the essential amino acid (AA) supply of birds using the 'ideal protein concept' on a standardized ileal digestible (SID) amino acid basis (5). A wide range of free crystalline AA as feed supplements are available and can be used for this purpose.

Threonine (Thr) has been usually the third limiting AA in broiler diets, and feed grade L-Thr has been commercially available since the 1980s (6). Thr mainly serves as a substrate

for the synthesis of proteins, mucin, and immunoglobulins and plays a crucial part in stress response and maintenance of gut epithelium integrity (7). The ideal ratios of essential AA should be considered even more carefully in LP diets. The ideal digestible Thr-to-lysine (Lys) ratio has been increased up to higher than 65% in diets for modern broilers, which can be further adjusted according to the target performance trait, stress level, and the challenges of the immune system (8, 9). Thr can be metabolized by either Thr aldolase or Thr dehydrogenase to glycine (Gly) in poultry, which is the precursor AA of uric acid synthesis (10). In addition, Gly can be metabolized from serine (Ser), and this reaction can be reversed (11, 12). The metabolic interconversion of Gly and Ser is continuous and unlimited, and Ser has the same effects as Gly on an equimolar basis (11). Therefore, the calculation of a dietary Gly equivalent (Gly_{equi}) has been recommended using the molar equivalent of Ser being 0.7143 (13). Dietary Gly + Ser levels largely decrease when CP is reduced in vegetarian diets, and these AAs become the first growth-limiting non-essential AA when the CP content of diets is below 19% during 7 to 21 days of age and below 17% during 21 to 35 days of age (14, 15). The interrelationship of dietary Thr and Gly_{equi} is quite complex and has been reported by many studies (16–21). Dietary Thr levels higher than recommended may reduce the requirement of dietary Gly_{equi} to achieve certain response levels of performance traits (22). This so-called sparing or replacement effect can be partly attributed to the conversion of Thr to Gly (23). Furthermore, the increasing dietary levels of Thr may reduce the catabolism of other AAs, thereby reducing the need for Gly_{equi} for uric acid formation (23). On the other hand, Gly supplementation decreases the activity of the enzymes Thr aldolase and Thr dehydrogenase, which can lead to a decreased degradation of Thr and an increased availability of Thr for physiological needs (19).

The interaction between Thr and Gly concerning broiler performance has been studied mostly during the first 3 weeks of broiler life. The interactions between Thr and Gly_{equi} from 7 to 21 days were quantified by Siegert et al. (22). They found that the increasing dietary Thr reduced the Gly_{equi} required to achieve certain BWG and FCR responses. The effects of an additional 0.2% or 0.4% Gly were dependent on the CP level of the diet fed from day 0 to day 21 in the study by Waldroup et al. (16). When Gly was added to diets with 16% or 18% CP (1.30 or 1.62% Gly + Ser), the BW of birds at 21 days of age was significantly improved. However, the addition of Gly to diets with 20, 22%, or 24% CP (1.86, 2.08, and 2.28% Gly + Ser) showed no or little benefit. The authors did not observe any performance improvement of an additional 0.2% or 0.4% Thr (0.80–0.98% Thr in basal diets) or interaction between Gly and Thr. Ospina-Rojas et al. (18) investigated an LP diet with 19% CP fed from day 0 to day 21 with two Thr levels (0.93 and 1.07% Thr) and four concentrations of Gly + Ser (1.84 to 2.26%). The increasing supplemental Gly + Ser improved the FCR of birds in a quadratic manner at 0.93% Thr level, but it had no significant effect on the FCR at 1.07% Thr concentration. The research focusing on the interaction between Thr and Gly in the grower-finisher phase of fattening is more limited and needs further precise quantification. The FCR of broilers from 21 to 35 days showed a stronger response to Gly when the digestible Thr-to-Lys ratio of diets was 65% compared to the 72% digestible Thr-to-Lys ratio (19). Broilers fed a diet with a lower digestible (Gly + Ser)-to-Lys level showed a stronger response of FCR to dietary Thr level (135% vs. 149%) (19). Corzo et al. (17) also observed similar interactions for BWG of birds from 21 to 42 days of age. In the study by Star et al. (21), the BWG of broilers from 7 to 28 days of age only responded when both dietary Gly and Thr levels were very low. In addition to their respective concentrations in diets, the effects of dietary Thr and Gly are also related to the dietary level of Met+Cys, choline, Arg, guanidino acetic acid, and creatine, as reviewed by Siegert and Rodehutscord (23).

Most of the studies focusing on the Thr-Gly interactions applied only one LP diet and crystalline Gly and Thr supplementation (17–19). The objective of this study was to use practically formulated diets with both normal and low CP content without crystalline Gly, which is only allowed as a flavoring agent in the EU. To increase the Gly concentration of LP diets, swine meat meal rich in Gly was used in our experiment. Furthermore, the present study focuses not only on the Thr and Gly interactions concerning broiler performance but on the carcass characteristics, meat quality, efficiency of nitrogen retention, and nitrogen forms of excreta using practically formulated LP diets suitable in the EU.

2 Materials and methods

2.1 Experimental animals and treatments

A floor pen trial was carried out at the experimental farm of the Institute of Physiology and Nutrition, Georgikon Campus, Hungarian University of Agriculture and Life Sciences (Keszthely, Hungary). A total of 576 1-day-old male broiler chickens (Ross 308) were purchased from a local hatchery (Gallus Ltd., Devecser, Hungary) and divided randomly into 24 floor pens at a stocking rate of 24 birds per pen (14 bird/m^2). Animals were vaccinated against infectious bronchitis (CEVAC BRON), Newcastle disease (CEVAC VITAPEST), and infectious bursal disease (CEVAC TRANSMUNE) in the hatchery using vaccines produced by Ceva (Ceva Santé Animale, France). Chopped wheat straw was used as litter material. The animals were provided *ad libitum* water and feed during the entire duration of the experiment. The climatic conditions and light program, based on the breeder's guidelines, were computer-controlled and identical for all pens. The room temperature was set to 34°C on day 0 and reduced gradually to 24°C at 18 days of age. The light intensity was 30 lux in the first week and 10 lux thereafter, with a constant day length of 23 h from day 0 to day 7 and 20 h light and 4 h dark period thereafter.

Three dietary phases were used during the 35-day-long experiment: starter (from 0 to 10 days), grower (from 11 to 24 days), and finisher (from 25 to 35 days). All birds were fed the same starter phase diet, and 4 dietary treatments consisting of 6 replicates with 24 birds in each were established and experimental diets were fed in the grower and finisher phases in the pelleted form. The design of the experiment is described in Table 1. Diets of the control C treatment were formulated in line with the breeder's recommendations for Ross 308 (Aviagen, Newbridge, United Kingdom) and adequate levels of CP and SID essential AA. Low protein (LP) diets, LP1, LP2, and LP3, contained 2.0% less crude protein than diet C with control CP level in each dietary phase. Dietary treatments were different in SID Thr level and SID Thr-to-Lys ratio as well as SID (Gly + Ser)-to-Lys ratio and

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TABLE 1 Experimental design.

Treatments[a]	Grower diets (11–24 days)						Finisher diets (25–35 days)					
	CP (%)	SID Thr (%)	SID Thr-to-Lys ratio (%)	Gly + Ser (%)	SID (Gly + Ser) to Lys ratio (%)	Gly_{equi} (g/kg)	CP (%)	SID Th (%)	SID Thr-to-Lys ratio (%)	Gly + Ser (%)	SID (Gly + Ser) to Lys ratio (%)	Gly_{equi} (g/kg)
C	21.00	0.74	63	1.85	137	15.65	19.00	0.65	64	1.68	143	14.19
LP1	19.00	0.73	63	1.62	121	13.74	17.00	0.65	64	1.45	124	12.30
LP2	19.00	0.84	72	1.62	121	13.70	17.00	0.74	73	1.45	124	12.26
LP3	19.00	0.73	63	1.81	130	15.77	17.00	0.65	64	1.57	130	13.55

[a]C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal-based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as protein source; CP – crude protein; Gly_{equi} - Gly equivalent (g/kg feed) = glycine (g/kg) + [0.7143 x serine (g/kg)].

the Gly_{equi}. The composition of experimental diets is shown in Table 2, while the calculated and measured nutrient content of experimental diets can be seen in Table 3. The experimental LP diets were isocaloric with diet C. The increased Gly_{equi} in the LP3 diet was achieved by the partial replacement of soybean meal with swine meat meal rich in Gly. Diets were formulated based on standardized ileal digestible (SID) AAs in accordance with the ideal protein concept. LP diets were supplemented with six feed-grade crystalline essential AAs (Lys, Met, Val, Thr, Arg, and Ile) to meet the calculated concentrations of SID AAs in the C diets except for the SID Thr level of the LP2 diet. All diets contained phytase and xylanase enzymes, but no amino acid-releasing impact of these enzymes was considered in feed formulations.

2.2 Measurements

The body weight (BW) of broilers was measured individually at the start of the trial and the end of each dietary phase, and the mean BW was calculated for each pen. Feed intake (FI) of broilers was recorded per pen at the end of each dietary phase. Body weight gain (BWG) and feed conversion ratio (FCR) were calculated per pen at the end of each phase and for the whole trial period. Mortality and the weight of dead birds were registered daily during the whole trial. At day 35, two chickens with average BW from each pen (12 birds per treatment) were randomly selected and transferred to balance cages, where chickens consumed the same finisher diets but supplemented with 0.5% TiO_2 as an indigestible internal marker. After 5 days adaptation period, representative excreta samples were collected from each bird daily for 2\u00B0consecutive days (days 41 and 42). The samples of 12 birds per treatment were pooled, mixed thoroughly, frozen, and stored at −20°C until further analyses. Before the analyses, excreta was homogenized properly, and then the dry matter content, total-N, ammonium-N (NH_4^+-N), and uric acid-N contents were determined. The dry matter content of excreta samples was measured in an exicator (100°C for 24 h). The total N of excreta was determined according to the Kjeldahl method with Foss-Kjeltec 8,400 Analyzer Unit (Nils Foss Allé 1, DK-3400 Hilleroed, Denmark), the ammonium-N by the method of Peters (24), and the uric acid-N as described by Marquardt (25). All N parameters were adjusted on a dry matter basis. The sum of NH_4^+-N and uric acid-N was considered as urinary N content (26). Feed samples were analyzed for dry matter (ISO 6496), crude protein (ISO 5983-1:2005), phosphorus (ISO 6491), calcium (ISO 6896) content, and amino acid composition (ISO 13903:2005) using methods of International Organization for Standardization (ISO). The TiO_2\u00B0concentration of experimental diets and excreta samples was determined using a UV-spectroscopy assay (27). Nitrogen retention was calculated using the following equation (28): Apparent nitrogen retention = 1 – [([TiO_2] diet/[TiO_2] excreta) × ([nitrogen] excreta/[nitrogen] diet)]. At the end of the experiment, two birds per pen (12 birds per treatment) representing the average BW of the pen were selected to be slaughtered by cervical dislocation. After evisceration, carcass composition (% of carcass weight, % of breast meat, % of thigh weight, % of abdominal fat) and breast meat quality were determined. The pH of the breast muscle, *Pectoralis major* (*P. major*), was measured immediately after slaughtering (pH_{0h}) and after 24 h storage at 4°C (pH_u) with a portable pH meter (Testo 205; Testo Ltd., Hungary) by inserting a glass electrode directly in the thickest part of the muscle. The water-holding

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TABLE 2 Composition of experimental diets (%).

Ingredients	Starter (0–10 days)	Grower (11–24 days)				Finisher (25–35 days)			
		C	LP1	LP2	LP3	C	LP1	LP2	LP3
Maize	39.13	42.48	49.30	49.30	57.03	47.95	54.97	54.98	60.34
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Soybean meal extr.	40.70	37.40	30.50	30.39	20.50	32.10	25.20	25.10	18.20
Swine meat meal	0.00	0.00	0.00	0.00	6.00	0.00	0.00	0.00	4.00
Sunflower oil	5.10	6.00	5.30	5.30	3.10	6.40	5.40	5.40	4.00
Limestone	1.65	1.39	1.39	1.39	0.60	1.20	1.23	1.23	0.69
MCP	1.32	1.10	1.10	1.10	0.05	0.89	0.90	0.90	0.20
L-Lysine (Biolys)	0.41	0.27	0.56	0.56	0.69	0.21	0.50	0.50	0.61
DL-Methionine	0.40	0.32	0.37	0.37	0.40	0.29	0.34	0.34	0.37
L-Valine	0.10	0.06	0.16	0.16	0.21	0.06	0.17	0.17	0.21
L-Threonine	0.14	0.08	0.16	0.27	0.21	0.06	0.15	0.24	0.18
L-Arginine	0.03	0.00	0.15	0.15	0.21	0.00	0.19	0.19	0.25
L-Isoleucine	0.03	0.01	0.12	0.12	0.21	0.01	0.12	0.12	0.19
Salt	0.30	0.30	0.30	0.30	0.20	0.30	0.30	0.30	0.23
Sodium bicarbonate	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Premix[a]	0.50	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Phytase[b]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
NSP enzyme[c]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Coccidiostat[d]	0.06	0.06	0.06	0.06	0.06	0.00	0.00	0.00	0.00

C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal-based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as a protein source.

[a]Premix was supplied by UBM Ltd. (Pilisvörösvár, Hungary). The active ingredients contained in the premix were as follows (per kg of diet): Starter and grower premixes - retinyl acetate - 5.0 mg, cholecalciferol - 130 g, dl-alpha-tocopherol-acetate - 91 mg, menadione - 2.2 mg, thiamine - 4.5 mg, riboflavin - 10.5 mg, pyridoxin HCl - 7.5 mg, cyanocobalamin - 80 g, niacin - 41.5 mg, pantothenic acid - 15 mg, folic acid - 1.3 mg, biotin - 150 g, betaine - 670 mg, monensin-Na - 110 mg (only grower), narasin - 50 mg (only starter), nicarbazin - 50 mg (only starter), antioxidant - 25 mg, Zn (as $ZnSO_4H_2O$) - 125 mg, Cu (as $CuSO_45H_2O$) - 20 mg, Fe (as $FeSO_4H_2O$) - 75 mg, Mn (as MnO) - 125 mg, I (as KI) - 1.35 mg, Se (as Na_2SeO_3) - 270 g; Finisher premix - retinyl acetate - 3.4 mg, cholecalciferol - 97 g, dl-alpha-tocopherol-acetate - 45.5 mg, menadione - 2.7 mg, thiamin - 1.9 mg, riboflavin - 5.0 mg, pyridoxin HCl - 3.2 mg, cyanoco-balamin - 19 g, niacin - 28.5 mg, pantothenic acid - 10 mg, folic acid - 1.3 mg, biotin - 140 g, L-ascorbic acid - 40 mg, betaine - 193 mg, antioxidant - 25 mg, Zn (as $ZnSO_4H_2O$) - 96 mg, Cu - 9.6 mg, Fe (as $FeSO_4H_2O$) - 29 mg, Mn (as MnO) - 29 mg, I (as KI) - 1.2 mg, Se (as Na_2SeO_3) - 350 g.

[b]Axtra® Phy 5,000 TPT phytase 500 FTU (Danisco Animal Nutrition & Health, USA).

[c]Danisco Xylanase 8,000 G (Danisco Animal Nutrition & Health, USA).

[d]Maxiban® G160 premix (Elanco Animal Health, Australia).

capacity of meat was estimated by measuring drip loss of the raw meat: the *P. major* muscle was weighed immediately after slaughter and placed in a plastic bag, hung from a hook, and stored at 4°C for 24 h. After hanging, the sample was wiped with an absorbent paper and weighed again. The difference in weight corresponding to the drip loss was expressed as the percentage of the initial muscle weight (29).

2.3 Statistical analysis

The averages of examined parameters were analyzed in a completely randomized design using a one-way analysis of variance (ANOVA) with dietary treatments as the main effects. For performance results (BW, BWG, FI, and FCR), the pen was the experimental unit, whereas for other variables, the individual bird was the experimental unit. When the F-test revealed a significant treatment effect, the significant differences between groups were tested by the Tukey HSD test. All statistical analyses were carried out using the software package SPSS 22.0 for Windows (IBM Corp., Armonk, NY, United States). Statistical significance has been declared at $p < 0.05$.

3 Results

There were no remarkable differences between measured and calculated values of dietary total AA (Table 3). The effect of dietary treatments on the total intake of the balanced SID essential AA without Thr and the intake of SID Thr and Gly + Ser of broilers in the grower and finisher phases is presented in Table 4. The total intake of the balanced SID essential AA without Thr was not significantly different among treatment groups. In addition, the results show that the calculated differences between SID Thr and Gly + Ser levels in the dietary treatments resulted in significant differences in SID Thr and Gly + Ser intake of broilers.

The results of production parameters are shown in Table 5. The performance parameters were not significantly influenced by the same starter diet fed in all pens when the feeding of experimental diets started on day 10. There were no significant differences between the BW of the treatment groups at the end of the grower phase, while in the finisher phase, the broilers fed the LP2 diet showed a significantly higher BW than the broilers consuming the C diet ($p < 0.05$). The BWG of birds in the grower, finisher, and whole trial period was

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TABLE 3 Calculated and measured the nutrient content of the experimental diets (%).

Calculated nutrients	Starter (0–10 days)	Grower (11–24 days)				Finisher (25–35 days)			
		C	LP1	LP2	LP3	C	LP1	LP2	LP3
Crude protein	22.50	21.00	19.00	19.00	19.00	19.00	17.00	17.00	17.00
AMEn (MJ/kg)	12.55	13.05	13.08	13.07	13.12	13.46	13.41	13.40	13.46
Starch	33.16	35.02	38.80	38.80	42.94	38.06	41.97	41.97	44.84
Crude fat	7.29	8.24	7.64	7.60	6.12	8.72	7.83	7.83	6.89
SID Lysine	1.30	1.15	1.16	1.16	1.15	1.01	1.01	1.01	1.01
SID Methionine	0.70	0.60	0.62	0.62	0.65	0.55	0.57	0.57	0.60
SID Met+Cys	0.98	0.88	0.87	0.87	0.87	0.81	0.80	0.80	0.81
SID Arginine	1.40	1.29	1.24	1.24	1.23	1.14	1.14	1.14	1.15
SID Threonine	0.83	0.74	0.73	0.84	0.73	0.65	0.65	0.74	0.65
SID Valine	0.97	0.88	0.87	0.87	0.87	0.80	0.80	0.80	0.81
SID Isoleucine	0.85	0.79	0.78	0.78	0.78	0.70	0.70	0.70	0.71
SID Glycine	0.76	0.72	0.63	0.63	0.80	0.65	0.56	0.56	0.67
SID Serine	0.92	0.87	0.77	0.76	0.70	0.79	0.69	0.69	0.64
Ca	1.06	0.92	0.91	0.91	0.92	0.80	0.80	0.80	0.80
$P_{available}$	0.51	0.46	0.45	0.45	0.46	0.40	0.40	0.40	0.40
Gly + Ser	1.96	1.85	1.62	1.62	1.81	1.68	1.45	1.45	1.57
SID Thr-to-Lys ratio	0.64	0.63	0.63	0.72	0.63	0.64	0.64	0.73	0.64
Gly_{equi} (g/kg)[a]	16.56	15.65	13.74	13.70	15.77	14.19	12.30	12.26	13.55
AMEn-to-CP ratio[b]	0.56	0.62	0.69	0.68	0.69	0.71	0.78	0.78	0.79
Starch-to-CP ratio[c]	1.47	1.67	2.03	2.03	2.25	2.00	2.45	2.44	2.63
Measured nutrients									
Dry matter	89.07	89.16	89.37	89.26	89.30	89.72	89.64	89.59	89.69
Crude protein	22.27	21.25	19.20	19.17	19.08	19.23	17.14	17.18	17.05
Lysine	1.43	1.27	1.26	1.26	1.27	1.11	1.09	1.09	1.13
Methionine	0.72	0.63	0.65	0.66	0.68	0.58	0.59	0.60	0.62
Met+Cys	1.07	0.96	0.94	0.93	0.95	0.88	0.87	0.89	0.88
Arginine	1.53	1.40	1.35	1.36	1.37	1.24	1.25	1.26	1.27
Glycine	0.88	0.83	0.72	0.74	1.05	0.74	0.65	0.63	0.85
Serine	1.07	1.01	0.88	0.87	0.82	0.91	0.76	0.77	0.73
Gly + Ser	1.95	1.84	1.59	1.61	1.87	1.65	1.41	1.40	1.58
Threonine	0.97	0.86	0.84	0.94	0.85	0.76	0.73	0.75	0.76
Valine	1.08	0.99	0.96	0.97	0.98	0.89	0.88	0.87	0.89
Isoleucine	0.97	0.89	0.87	0.86	0.87	0.79	0.77	0.78	0.79
Ca	1.06	0.92	0.90	0.94	0.89	0.85	0.84	0.82	0.85
P	0.61	0.64	0.58	0.58	0.58	0.54	0.52	0.52	0.52

C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal-based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as protein source.

[a]Gly equivalent (g/kg feed) = glycine (g/kg) + [0.7143 x serine (g/kg)].

[b]Ratio of dietary AMEn and crude protein concentration.

[c]Ratio of dietary starch and crude protein concentration.

significantly affected by the experimental diets ($p < 0.05$). In the grower phase, the feeding of LP diets did not lead to significantly different BWG compared to the C treatment. However, there was a significant difference between the LP2 and LP3 groups: the increased SID Thr-to-Lys level of the LP2 diet resulted in a higher BWG of birds than the LP3 diet with higher Gly_{equi} ($p < 0.05$). The BWG of broilers in the LP1 and LP3 groups was significantly higher than that in the C group in the finisher phase ($p < 0.05$). As for the whole experiment, only the broilers fed the LP2 diet achieved a significantly higher BWG than the birds in the C treatment ($p < 0.05$). In contrast to BW and BWG data, no significant differences were found in the FI of experimental animals among the treatments in any phases of the

TABLE 4 Intake of SID EAA[1], SID Thr, and Gly + Ser of broilers during the feeding phases (g/bird, means of 6 pens per treatment; $n = 6$).

Treatment[3]	SID EAA			SID Thr			Gly + Ser		
	Grower	Finisher	G + F[2]	Grower	Finisher	G + F	Grower	Finisher	G + F
C	63.55	82.42	145.98	9.43[b]	12.01[b]	21.44[b]	23.43[a]	30.49[a]	53.93[a]
LP1	63.81	85.27	149.08	9.47[b]	12.46[b]	21.92[b]	20.62[b]	27.02[b]	47.64[b]
LP2	64.26	83.18	147.44	10.97[a]	13.83[a]	24.80[a]	21.02[b]	26.17[b]	47.20[b]
LP3	62.71	84.53	147.24	9.34[b]	12.24[b]	21.58[b]	23.94[a]	29.75[a]	53.68[a]
Pooled SEM	0.52	0.74	1.07	0.16	0.18	0.32	0.35	0.45	0.66
p-value	NS[4]	NS	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Total intake of balanced SID essential amino acids without Thr: Lys + Met+Cys + Arg + Val + Ile; [2]Grower+Finisher; [3]C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as a protein source; [4]NS – non-significant ($p > 0.05$); [a,b]Means with different superscripts in the same column are significantly different ($p < 0.05$).

TABLE 5 Performance parameters of birds in the starter, grower, and finisher phases and in the whole experiment (mean ± SEM; $n = 6$ pens per treatment).

	Treatment[1]	0 d	10 d	24 d	35 d
Body weight (g/bird)	C	47.9 ± 0.1	269.9 ± 8.0	1247.8 ± 14.5	2479.9 ± 25.6[b]
	LP1	47.9 ± 0.1	271.4 ± 9.2	1266.3 ± 22.9	2617.1 ± 46.2[ab]
	LP2	48.2 ± 0.2	273.2 ± 7.2	1305.2 ± 29.1	2633.3 ± 37.9[a]
	LP3	48.3 ± 0.1	289.5 ± 3.7	1226.7 ± 25.4	2579.2 ± 30.4[ab]
	p-value	NS[2]	NS	NS	0.029
		Starter (0–10days)	Grower (11–24days)	Finisher (25–35days)	Whole trial (0–35days)
Body weight gain (g/bird)	C	222.0 ± 8.0	977.9 ± 10.3[ab]	1232.1 ± 26.8[b]	2432.0 ± 25.6[b]
	LP1	223.5 ± 9.3	994.9 ± 19.2[ab]	1350.8 ± 35.8[a]	2569.2 ± 46.1[ab]
	LP2	225.0 ± 7.1	1031.9 ± 23.9[a]	1328.1 ± 22.4[ab]	2585.0 ± 37.9[a]
	LP3	244.7 ± 1.2	937.1 ± 24.2[b]	1352.5 ± 23.8[a]	2530.9 ± 30.3[ab]
	p-value	NS	0.027	0.018	0.030
Feed intake (g/bird)	C	270.8 ± 3.7	1273.6 ± 24.6	1848.1 ± 31.4	3351.0 ± 16.8
	LP1	276.7 ± 4.3	1297.1 ± 18.0	1916.1 ± 48.6	3489.9 ± 59.7
	LP2	272.9 ± 5.5	1306.1 ± 21.4	1869.2 ± 20.5	3419.1 ± 9.6
	LP3	283.3 ± 0.6	1279.9 ± 23.7	1882.6 ± 29.3	3447.7 ± 50.5
	p-value	NS	NS	NS	NS
Feed conversion ratio (kg/kg)	C	1.23 ± 0.03	1.30 ± 0.02[ab]	1.50 ± 0.02[b]	1.40 ± 0.01[a]
	LP1	1.25 ± 0.05	1.30 ± 0.01[ab]	1.42 ± 0.03[ab]	1.36 ± 0.01[ab]
	LP2	1.22 ± 0.03	1.27 ± 0.01[a]	1.43 ± 0.01[ab]	1.33 ± 0.01[b]
	LP3	1.16 ± 0.01	1.37 ± 0.03[b]	1.39 ± 0.02[a]	1.36 ± 0.01[ab]
	p-value	NS	0.016	0.013	0.026

[1]C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal-based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as a protein source.; [2]NS – non-significant ($p > 0.05$); [a,b]Means with different superscripts in the same column are significantly different ($p < 0.05$).

experiment. The FCR of broilers was significantly influenced by dietary treatments in both the grower and finisher phases as well as during the whole fattening ($p < 0.05$). In the grower phase, the LP diets did not lead to significantly different FCR values than the C diet. As for FCR in LP treatments, the same significant difference was seen between LP2 and LP3 as it was observed in the case of BWG ($p < 0.05$). Experimental animals fed the LP3 diet showed better FCR than the control birds consuming the C diet in the finisher phase ($p < 0.05$). The FCR value calculated for the whole study in the LP2 group exceeded the FCR in the C group but did not differ significantly from the two other LP treatments.

The dietary treatments significantly affected the relative carcass weight and abdominal fad pad ($p < 0.05$), while the relative breast meat yield and thigh weight were not significantly influenced by experimental feeding (Table 6). The relative carcass weight of broilers in the LP1 and LP2 groups was not different from the C group. However, this trait in the LP3 treatment was significantly lower than in the C and LP2 treatments. Furthermore, the feeding of the LP3 diet resulted in a higher relative abdominal fat pad compared to the C diet ($p < 0.05$). The dietary treatments did not significantly influence the drip loss of breast meat or the pH of the breast meat fillet measured either immediately after slaughter or after 24 h ($p > 0.05$; Table 7).

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TABLE 6 Carcass weight and composition[1] (%, mean ± SEM; n = 12 broilers per treatment).

Treatment[2]	Carcass weight	Breast meat yield	Thigh weight	Abdominal fat
C	65.67 ± 0.45[a]	22.37 ± 0.35	19.21 ± 0.29	0.68 ± 0.09[b]
LP1	65.18 ± 0.42[ab]	21.20 ± 0.50	19.00 ± 0.20	0.98 ± 0.09[ab]
LP2	66.25 ± 0.25[a]	22.25 ± 0.34	19.15 ± 0.20	0.87 ± 0.08[ab]
LP3	64.06 ± 0.32[b]	20.92 ± 0.45	18.92 ± 0.19	1.04 ± 0.09[a]
p-value	<0.001	NS[3]	NS	0.032

[1]Values expressed as a percentage of live BW; [2]C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal-based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as a protein source; [3]NS – non-significant ($p > 0.05$); [a,b]Means with different superscripts in the same column are significantly different ($p < 0.05$).

TABLE 7 Breast meat quality parameters (mean ± SEM; *n* = 12 broilers per treatment).

Treatment[1]	pH_{0h}[2]	pH_u[3]	Drip loss (%)
C	6.49 ± 0.06	5.76 ± 0.02	1.08 ± 0.06
LP1	6.47 ± 0.04	5.82 ± 0.02	1.07 ± 0.09
LP2	6.56 ± 0.05	5.85 ± 0.03	1.04 ± 0.05
LP3	6.59 ± 0.04	5.79 ± 0.02	1.10 ± 0.06
p-value	NS[4]	NS	NS

[1]C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal-based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as a protein source; [2]pH_{0h} = pH measured immediately after slaughter; [3]pH_u = pH measured after 24 h storage at 4°C; [4]NS – non-significant ($p > 0.05$).

The effect of dietary treatments on the nitrogen retention efficiency of broilers was significant (66.4, 72.3, 69.3, and 73.5% in the C, LP1, LP2, and LP3 groups, respectively; Figure 1; $p < 0.05$). The experimental animals of treatment LP3 achieved significantly higher nitrogen retention efficiency than the birds in the C group ($p < 0.05$). The mean dry matter content of excreta was 21.0, 23.9, 24.4, and 22.4% in the C, LP1, LP2, and LP3 treatment groups, respectively, and showed only the tendency of increase in the LP groups compared to the C treatment ($p = 0.109$). The dietary treatments did not significantly influence the concentration of fecal-N, uric acid-N, NH_4^+-N, urinary-N, and total-N in the excreta of broiler chickens. The ratio of urinary-N within the total-N in excreta was 43.1, 42.7, 42.3, and 42.7% in the C, LP1, LP2, and LP3 groups, respectively, and these values were not affected by dietary treatments (see Table 8).

4 Discussion

In our study, the dietary LP treatments were applied only from the growing phase. A common starter diet was fed because an early CP reduction would have had minimal impact on the total CP and soybean meal reduction, nitrogen emission, and final performance. In most trials, the reduction in dietary CP by 2% could be achieved without impaired production traits when at least three-phase feeding was used, pelleted diets were fed, and the diets were balanced in at least six limiting AAs (15, 30, 31). In our study, all performance traits of broilers fed LP diets met or exceeded the performance parameters of control birds fed the C diet. The essential AA contents of diets were set according to the ideal protein concept, and the formulation was based on both total and SID AA requirements. Regarding the crystalline essential AA supplementation, not only the four first limiting amino acids (Lys, Met, Thr, and Val) but also L-Arg and L-Ile were used. Broilers fed LP diets had a generally low performance in some studies, which can be explained by the fact that crystalline L-Val, L-Arg, and L-Ile were not used (32, 33). In some studies, when LP diets provided an imbalanced essential AA supply, birds regulated their imbalanced AA intake via hyperphagia, and a significantly higher FI was observed (34, 35). The feeding of LP diets in our study did not lead to a significant increase in FI compared to the C treatment. Furthermore, the intended same SID AA intake in the case of six main essential AAs and the targeted differences of SID Thr and Gly + Ser intake of broilers between the LP and C groups were realized.

The performance of broilers in the LP1 group was similar or even higher (BWG in the finisher phase) compared to those in the C group both in the grower and finisher phases. It means that the extent of decreased Gly supply (Gly_{equi} of 15.65 vs. 13.74 and 14.19 vs. 12.30 in the grower and finisher phases, respectively) while meeting Thr requirements of birds did not impair broiler performance in a significant manner. The explanation for the higher BWG of birds in the finisher phase is not clear, but it can be associated with the increased digestibility of AA in the LP1 diet compared to the C diet. Liu et al. (4) showed that reduced CP feeding generally increases the AA digestibility in the distal jejunum, probably due to the larger ratio of essential crystalline AA found in the reduced protein diets. In the finisher phase, the FCR improvement of broilers in the LP3 group compared to the C group was even better than those in the LP1 group. This result suggests that an increase in the Gly_{equi} of finisher LP diets from 12.3 to 13.55 g/kg is advisable concerning the FCR of broilers. Similar to our results, previous studies have shown that the increased supply of Gly had the most impact on improving FCR (36, 37), possibly by improving nutrient utilization and protein synthesis by enterocyte development and mucin secretion (38). According to the existing literature, the requirement for Gly_{equi} from 0 to 21-day-old broiler chickens is estimated to vary between 11 and 20 g/kg depending on the uric acid formation, Thr supply, Met-to-(Met+Cys) ratio, and choline level (22, 23). Other studies have found an optimum Gly + Ser ranging from 1.8 to 2.3% from 0 to 35 days of age (16, 18, 19). The dietary Gly + Ser level necessary to optimize FCR from 21 to 35 days in LP diets was estimated to be 1.54% at the digestible Thr level of 0.77% (19). The 1.57% calculated Gly + Ser concentration in our finisher LP3 diet, together with the 0.65% SID Thr level, seems to be low, and a further effective increase might be possible. The minimum SID (Gly + Ser)-to-Lys ratios in grower 2 (21–31 days) and finisher phases (31–41 days) are 1.42 and 1.40, respectively, as suggested by Rostagno et al. (39) and confirmed by Mansilla et al. (40). The positive effects of the increased SID (Gly + Ser)-to-Lys ratio in our finisher LP3 diet compared to the LP1 diet (1.30 vs. 1.24) on the FCR of broilers supports a further increase toward the suggested SID (Gly + Ser)-to-Lys ratios.

The relationship between dietary Gly_{equi} and Thr concerning performance traits has been demonstrated (17, 18) and quantified for the phase of 7 to 21 days (22, 23). The decreasing Gly_{equi} impairs the BWG and FCR of broilers in a linear and non-linear manner, respectively, and the negative effect is more pronounced at a lower

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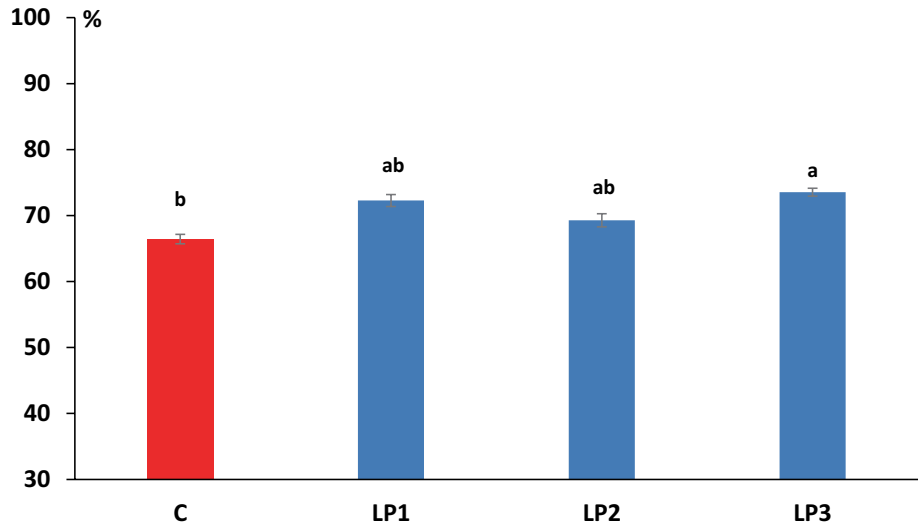

FIGURE 1
Effect of dietary treatments on the efficiency of dietary N retention (mean + SEM; $n = 12$ broilers per treatment). [a,b]Means with different superscripts are significantly different; C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (–2%); LP2 – soybean meal-based diet with reduced crude protein levels (–2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (–2%) in which soybean meal partially replaced with swine meat meal as protein source.

TABLE 8 The concentration of N-forms in broiler excreta (mean ± SEM; $n = 12$ broilers per treatment).

Treatment[1]	Fecal-N	NH_4^+-N	Uric acid-N	Urinary-N[2]	Total-N
	mg/g dry matter				
C	21.87 ± 0.71	3.91 ± 0.25	12.64 ± 0.43	16.55 ± 0.61	38.42 ± 1.21
LP1	20.37 ± 1.19	3.39 ± 0.18	11.69 ± 0.60	15.08 ± 0.72	35.47 ± 1.79
LP2	23.06 ± 1.23	3.98 ± 0.14	12.86 ± 0.69	16.84 ± 0.80	39.90 ± 1.98
LP3	21.32 ± 1.12	4.01 ± 0.23	11.89 ± 0.69	15.91 ± 0.87	37.23 ± 1.83
p-value	NS[3]	NS	NS	NS	NS

[1]C - control diet; LP1 – soybean meal-based diet with reduced crude protein levels (−2%); LP2 – soybean meal-based diet with reduced crude protein levels (−2%) and higher crystalline L-Threonine supplementation; LP3 – diet with reduced crude protein levels (−2%) in which soybean meal partially replaced with swine meat meal as a protein source; [2]The sum of NH_4^+-N and uric acid-N was considered as urinary-N content; [3]NS – non-significant ($p > 0.05$).

dietary level of Thr. In the case of FCR, the positive response of birds to the same increase of dietary Thr level is higher at the lower level of Gly_{equi} concentration. Furthermore, the positive effect of a 1 g/kg increase in dietary Thr level on the FCR is higher than the effect of the same increase of Gly_{equi} concentration in the feed. According to the relationship, it is possible to improve FCR by increasing Thr supply while the Gly_{equi} is decreasing in the diet. The treatment LP2 meant similar changes in dietary Thr and Gly_{equi} supplies in comparison with the C treatment in our study, and positive effects on the performance of birds were observed. This positive effect was still a tendency in the grower phase, but it became significant by the end of fattening. The performance improvement of LP2 treatment means that the increase of SID Thr concentration by 0.10% (0.74% vs. 0.84% in the grower and 0.65% vs. 0.74% in the finisher phase) and the increase of SID Thr-to-Lys ratio by 9.0% (63 vs.72% in the grower and 64% vs. 73% in the finisher phase) compared to a control treatment can be beneficial when dietary CP is reduced by 2% and Gly_{equi} by 2 g/kg. The increased supply of birds with Thr in the LP2 treatment group could support the primary functions of Thr for protein synthesis. The enzymatic conversion of Thr to Gly, the so-called replacement effect, may also explain these results (23). In addition, the reduced catabolism of AA other than Thr during increased Thr supply may reduce the need for Gly_{equi} in uric acid formation, which could serve as another Gly-sparing effect (23). Unfortunately, the relationship between dietary Gly_{equi} and Thr concerning performance traits for the whole fattening from hatch to 35 or 42 days of age is not so precisely quantified as it was made for the phase from 7 to 21 days (22). However, similar interactions between Thr and Gly_{equi} may exist until the end of the finisher phase based on our results. Similarly, previous studies focusing on the second half of the fattening confirmed the above-mentioned Thr-Gly interaction, in which the positive performance response of birds to the same increase of dietary Thr level is higher at the lower level of Gly_{equi} concentration. Response of FCR to dietary Thr level was stronger at the lower digestible (Gly + Ser)-to-Lys level (135 vs. 149%) (19). BWG of broilers from 21 to 42 days responded more for Thr when they were fed at 143% compared to 153% digestible (Gly + Ser)-to-Lys ratio level (17). Further studies are needed to quantify the effects of Thr-Gly interaction on broiler performance concerning the whole fattening period.

In the present study, the results of birds in the LP1 treatment are in line with similar previous studies showing no effect of essential

AA-supplemented LP diets up to 2% CP reduction on the yield of carcass and valuable carcass parts (30, 40–42). In the study by Mansilla et al. (40), the reductions of SID (Gly + Ser)-to-Lys ratio parallel with the 2% CP reduction compared to the control diet were 5.0, 9.0, and 12.0% in the grower1, grower2 and finisher diets, respectively. The reductions of the SID (Gly + Ser)-to-Lys ratio were higher (15.0% in the grower and 19% in the finisher) in the present experiment, but the carcass weight results were similar in both studies. Only a few results have been published on the effects of Thr––Gly interaction concerning product quality of broilers fed LP diets. Similar to our results observed in the LP2 group, the increase of the digestible Thr concentration from 0.57 to 0.65% while decreasing the Gly + Ser level from 1.65 to 1.55% did not influence the relative carcass weight and breast fillet weight of birds fed an LP diet with 18.2% CP from 21 to 42 days of age (17). An increase of dietary Thr from 9.3 to 10.7 g/kg can even decrease the relative breast weight, which may be due to the toxic effect of increased plasma uric acid and ammonia concentrations (18). In the same study, there was a positive significant linear effect of increasing Gly + Ser concentrations from 18.4 to 22.6 g/kg on the relative breast weight of broilers fed an LP diet containing 19% CP from 0 to 21 days (18).

Our results suggest that the significant negative effects of LP3 treatment on carcass weight and composition could be associated with the starch-to-CP ratio of experimental diets. The content of starch as the main energy provider nutrient typically increases when dietary CP is reduced in isocaloric LP diets (4). In contrast, dietary lipid level usually decreases with the protein level, which was the case in this experiment as well. The starch-to-CP ratio increased in the LP treatments compared to the C treatment, and it was the highest in the LP3 diet containing swine meat meal. The higher starch-to-CP ratio deteriorated the FCR value in the starter phase of our previous experiment in a quadratic manner (43). A similar quadratic relationship was observed between these two factors from 7 to 35 days in two studies (44, 45). In the present study, the higher starch-to-CP ratio of LP diets did not impair the FCR of birds compared to the C diet. In our opinion, however, the highest starch-to-CP ratio in the LP3 diets (2.25 and 2.63 in the grower and finisher phases, respectively) resulted in a significantly lower relative carcass weight in comparison with those in the C diets (1.67 and 2.0 in the grower and finisher phases, respectively). This effect could be seen as a strong tendency in the case of breast meat yield. Based on the digestive dynamics of dietary starch, the absorption of its glucose content has been shown to compete with AA absorption, which may affect the availability of AA for tissue protein accretion (46, 47). However, Hilliar et al. (48, 49) found that the addition of crystalline Gly in an LP diet reduced breast meat yield compared to the control without additional Gly. In this case, the theoretical starch effect as an explanation can be excluded, but the cause is unclear. In contrast to the results of many previous trials (47, 50, 51), feeding the isoenergetic LP1 and LP2 diet did not increase the abdominal fat pad significantly compared to the C diet. However, a slight negative tendency was observed in these two treatments as well, which had a significant negative effect in the LP3 treatment group. The dietary AMEn-to-CP ratio increases while maintaining the dietary AMEn of LP diets constant, and the surplus energy can increase abdominal fat (50, 51). In other studies, the reduction in dietary energy and CP while maintaining the same AMEn-to-CP ratio successfully prevented the accumulation of abdominal fat, but the growth performance of broilers was suppressed (52, 53). The AMEn-to-CP ratio of LP diets was nearly the same, but only the LP3 treatment resulted in a significant increase in the abdominal fat pad ratio. The use of synthetic Gly supplementation can reduce the fatness of broilers fed LP diets (37, 54, 55). According to studies with poultry, rats, and swine, increasing dietary Gly or betaine (trimethylglycine) has been demonstrated to stimulate lipid oxidation and reduce plasma concentrations of triglycerides and fat deposition (56–58). In contrast, the use of swine meat meals to increase the Gly + Ser concentration of LP3 diets led to opposite results in the present study. We assume that the higher starch-to-CP ratio in the LP3 diet compared to the LP1 and LP2 diets was associated with the significantly increased abdominal fat ratio. If the tissue protein accretion was decreased in the LP3 group due to the high starch-to-CP ratio, as assumed based on the carcass weight result, the surplus energy formed could lead to an accumulation of abdominal fat pad in a significant manner. Another possible explanation is the elevated hepatic acetyl-CoA concentration derived from relatively high dietary starch levels in birds offered LP diets (4). Acetyl-CoA can serve as a precursor for fatty acid synthesis and influence the activity of numerous enzymes (4). These negative consequences of LP diets supplemented with 6 and 4% swine meat meal on carcass weight and abdominal fat pad need more focused investigations. In addition to the dietary balance of AA, the digestive dynamics of main nutrients, especially starch, lipid, and protein, should be considered in the further development of LP diets.

As we know, the effects of dietary Thr-Gly interaction on the meat quality of broilers fed LP diets have not been investigated. The pH and drip loss of breast meat in the present experiment did not show significant changes due to dietary LP treatments with different Thr and Gly_{equi} supplies. The drip loss is one of the parameters that is associated with the water-holding capacity of meat and influences its sensory and technological quality. The negative relationship between drip loss and ultimate pH in poultry meat is well known (52, 59). The feeding of LP diets can result in higher ultimate pH and decreased drip loss (43, 60). The higher drip loss of the meat proved to be more acidic with a higher level of glucose, glycogen, and glycolytic potentials (52, 53, 59). The post-mortem breakdown of the glycogen accumulated in the muscle tissues is responsible for the proper acidity of the meat after slaughter. If an adequate amount of glycogen is not available, the pH of the meat becomes less acidic, and the water-holding capacity of meat is higher. The drip loss of meat could be associated with excess AAs (60). After the deamination of the not utilized AAs, the carbon chain is used by the muscle tissue for the synthesis of various carbohydrates, such as glycogen. This suggests that our experimental diets provided a similar balanced AA profile without or with a similar amount of excess AA.

The dietary N retention efficiency of broilers measured in our experiment was improved by 3–7% with LP diets compared to the C diet, and this improvement is in line with the previously published results (2–13%) of LP diets providing an adequate essential AA supply (40, 61, 62). However, the N retention efficiency of broilers has not been reported by the dose–response studies with Thr-Gly supplemented LP diets (17–19, 21). The mean increase of the efficiency of N retention was +2.63%/CP percentage point, which is a little lower than the corresponding value of +3.2% published by Belloir et al. (15). The improvements were strong tendencies in the LP1 and LP2 groups compared to the C group, and it was a significant difference between LP3 and C treatments. According to our result, the increased level of Gly_{equi} in LP diets with 2% CP reduction can be recommended to increase N retention efficiency, as it was advisable to increase the FCR of finishing birds as well. Extremely advantageous efficiency values

higher than 70% were measured in the LP1 and LP3 treatments, which have also been reported by other research groups (14, 15). The increasing N utilization efficiency decreases the dietary Gly_{equi} needed for uric acid synthesis based on model calculations (63, 64). The estimation shows that an N utilization efficiency higher than 70% requires only less than 10 g/kg Gly_{equi} for uric acid production. Most of the ammonia released from poultry manure originates from the breakdown of uric acid (65). The feeding of LP diets can decrease the uric acid level of blood plasma (21) and the concentrations of total and urinary N and uric acid in broiler excreta (15, 31, 43). However, the results of the present experiment failed to confirm the advantageous results of previous studies associated with N emission. The uric acid concentration of excreta in broilers fed the control C diet was already quite low (12.54 mg/g dry matter), and maybe a further decrease was not possible by feeding LP diets. In our previous two experiments, the LP diets reduced the uric acid levels in the excreta when higher uric acid levels than in the present trial were measured in the excreta of broilers fed normal CP diets (17.65 and 15.30 mg/g dry matter) (31, 43).

In addition to the performance traits, the economic effectiveness of LP diets depends on the actual prices of raw materials, especially soybean meal and crystalline AA supplements. According to the present Hungarian prices, the cost of the experimental diets based on the ingredients only were 364, 362, 363, and 352 EUR per ton in the case of the grower, and 346, 343, 344, and 339 EUR per ton in the case of finisher diets of C, LP1, LP2 and LP3 treatments, respectively. All the LP diets had lower prices than the C diet, and assuming similar performance of birds fed C and LP diets, they can contribute to higher profitability of broiler production.

5 Conclusion

According to the results of this experiment, the increased SID Thr-to-Lys ratio and Gly_{equi} of LP diets with 2% lower CP content than adequate may have positive effects on broiler performance and nitrogen retention efficiency. The dietary SID Thr-to-Lys ratio of LP diets higher than recommended can improve the final BW, BWG, and FCR of birds. Swine meat meal as a source of Gly can be used to increase Gly_{equi} in practical LP diets in the EU, which can lead to a more advantageous FCR in the finisher phase and higher N retention efficiency. However, the LP diets containing swine meat meal may have a high starch-to-CP ratio, which can contribute to a decreased relative carcass weight and an increased abdominal fat pad ratio of broilers at market age.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Welfare Committee, Hungarian University of Agriculture and Life Sciences, Georgikon Campus, under the license number MÁB-3/2022. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PS: Data curation, Formal analysis, Methodology, Project administration, Writing – original draft. BH: Data curation, Formal analysis, Methodology, Project administration, Writing – original draft. NS: Data curation, Formal analysis, Methodology, Project administration, Writing – original draft. KD: Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. LP: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of adding bile acids to dietary storage japonica brown rice on growth performance, meat quality, and intestinal microbiota of growing–finishing Min pigs

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Introduction: This study investigated the effects of storage japonica brown rice (SJBR) and bile acids (BA) on the growth performance, meat quality, and intestinal microbiota of growing–finishing Min pigs.

Methods: A total of 24 healthy Min pigs with a similar body weight of 42.25 ± 2.13 kg were randomly divided into three groups with eight replicates of one pig each. The groups were as follows: CON (50% corn), SJBR (25% corn +25% SJBR), and SJBR + BA (25% corn +25% SJBR +0.025% hyodeoxycholic acid). The experimental period lasted from day 90 (the end of the nursery phase) to day 210 (the end of the finishing phase).

Results: The results showed the following: (1) Compared with the CON group, there was no significant difference in the average daily gain (ADG) and average daily feed intake (ADFI) of the SJBR and SJBR + BA groups, and the feed conversion ratio (FCR) was significantly decreased ($p < 0.05$). (2) Compared with the CON group, the total protein (TP) content in the serum was significantly increased, and the blood urea nitrogen (BUN) content was significantly decreased ($p < 0.05$) in the SJBR and SJBR + BA groups; moreover, HDL-C was significantly higher by 35% ($p < 0.05$) in the SJBR + BA group. (3) There were no significant differences in carcass weight, carcass length, pH, drip loss, cooking loss, and shear force among the groups; the eye muscle area was significantly increased in the SJBR group compared with the CON group ($p < 0.05$); back fat thickness was significantly decreased in the SJBR + BA group compared with the SJBR group ($p < 0.05$); and the addition of SJBR significantly increased the mRNA expression of MyHC I in the *longissimus dorsi* (LD) muscle of growing–finishing Min pigs ($p < 0.05$). (4) The cecal bacteria were detected using 16S rDNA, and the proportion of *Lactobacillus* was increased gradually at the genus level, but there was no significant difference among the different groups.

Conclusion: In conclusion, 25% SJBR can improve the growth performance and increase the abundance of intestinal beneficial bacteria, and based on this, adding bile acids can reduce the back fat thickness of growing–finishing Min pigs.

KEYWORDS

storage japonica brown rice, growth performance, intestinal microbiota, meat quality, Min pigs

1 Introduction

In recent years, with the continued growth of animal production, feed deficiency has become the key constraint affecting modern animal husbandry development in China (1). Additionally, the contradiction between humans and animals competing for food is becoming increasingly prominent, and the development of new feed raw materials has become a research hotspot (2). Corn is the main energy feed ingredient worldwide, and the nutritional value of brown rice is equivalent to that of corn (3). Brown rice is obtained from hulled rice, which has similar effective energy value, essential amino acids, crude protein, mineral, and vitamin content to corn. Thus, it can potentially replace feed corn (4). Brown rice contains various phenolic acids, which have antioxidant activity that protects cells from oxidative damage and is one of the most common antioxidants in the diet (5, 6). Studies have shown that applying germinated brown rice extract to obese mice induced by a high-fat diet significantly reduces serum triglyceride and total cholesterol levels by downregulating genes involved in lipid synthesis, thereby improving lipid distribution in mice (7). Most importantly, studies have reported that partially or completely replacing corn with brown rice can achieve the same feeding effect as corn for livestock and poultry (8, 9).

Compared to fresh brown rice, storage brown rice stored for 3 years has almost no difference in most nutritional parameters. Nevertheless, the decomposition of crude fat in storage brown rice produces a large amount of free fatty acids, which can oxidize and produce an unpleasant odor (10). In addition, a prolonged storage time can significantly reduce the activities of rice amylase, peroxidase, and polyphenol oxidase (11). Storage brown rice can be used as a high-quality energy raw material to replace corn with proper processing to eliminate the presence of fungal toxins and anti-nutritional factors (12).

Bile acids are biotransformed in the intestine mainly by anaerobic interactions of *Bacteroides*, *Eubacterium*, *Clostridium*, and *Lactobacillus* and are restored to free bile acids after binding to taurine and glycine via bile salt hydrolase-catalyzed uncoupling of conjugated bile acids (13). Bile acids reduce the production of short-chain fatty acids (SCFA), a metabolite of the intestinal microbiota, and have bacteriostatic activity, effectively inhibiting the growth and proliferation of intestinal pathogenic bacteria, maintaining the balance of intestinal micro-ecology, protecting the intestinal mucosa from bacterial invasion, and regulating the immune function of the body (14, 15). Bile acids promote the digestion and absorption of fats while stimulating bile secretion, helping to improve immunity and regulate the gut microbiota (16, 17). In addition to regulating glycolipid metabolism, bile acids also regulate intestinal cell proliferation and resist intestinal oxidative damage (18). After implanting a catheter in the duodenum, enteral malnourished piglets were treated with 30 mg/kg body weight of chenodeoxycholic acid, which could significantly increase their intestinal mass and ileal villus height/eye socket depth ratio and promote their intestinal development (19). Therefore, using storage brown rice as an energy feed substitute for corn and improving its utilization rate through additives may alleviate various problems such as corn supply–demand contradiction and pressure to reduce rice inventory. This study was devoted to investigating the feasibility of partially replacing corn with storage brown rice and the effect of bile acids as additives in improving the performance of Min pigs.

Min pig, one of the excellent local pig breeds in China, has cold resistance, rough feeding resistance, strong disease resistance, and good meat quality (20). Although there have been studies on the application of dietary brown rice in poultry and swine, the extent to which brown rice replacing part corn affects the meat quality and gut microbiota distribution of Chinese local pig breeders is still unclear. This study assumes that storage brown rice is a good substitute for corn and does not affect the normal growth of Min pigs during the growing–finishing period. Therefore, this study aimed to investigate how replacing some corn in the diet with storage brown rice and adding bile acids affected the growth performance, carcass traits, and meat quality of growing–finishing Min pigs.

2 Materials and methods

2.1 Experimental design and animals

The bile acid (hyodeoxycholic acid, purity >30%) used in this study was obtained from Shandong Longchang Animal Health Products Co., Ltd. (Jinan, China). A total of 24 healthy 83-day-old Min pigs (half male and half female) with an average body weight of 42.25 ± 2.13 kg were randomly allotted to three experimental groups with eight duplicates and one pig per replicate. The dietary treatments included the following: (1) CON (50% corn), (2) SJBR (25% corn +25% storage japonica brown rice), and (3) SJBR + BA (25% corn +25% storage japonica brown rice +0.025% bile acids). Each treatment had eight replicates, with one pig per replicate. The Min pigs were fed *ad libitum* and had free access to water with a temperature of 16–22°C and a relative humidity of 60–70% throughout the trial. The pigs were adaptively reared for 7 days. The experimental period lasted from day 90 (the end of the nursery phase) to day 210 (the end of the finishing phase). The experimental diet was formulated based on the Nutrient Requirements of Swine in China (GB/T 39235–2020) for growing–finishing pigs (21). The compositions and nutrition levels of the experimental diets are listed in Table 1. The initial body weight and final body weight were measured on the first and last days of the formal experimental period, and the daily feed intake was recorded to calculate ADG, ADFI, and FCR.

2.2 Sample collection

At the end of the feeding experiment, all the pigs in each treatment were transported to a modern slaughterhouse and slaughtered for sample collection. After arriving at the slaughterhouse, all pigs were allowed to rest for 4 h. Then, the jugular vein blood of selected pigs was bled, which complies with the current regulations applicable to the slaughterhouse. The carcasses were scalded, eviscerated, and vertically separated along the midline. The carcass weight was recorded to calculate the slaughter rate. The back fat thickness was measured by a three-point method: the back fat depth at the left carcass of the first rib, the last rib, and the last lumbar spine. The right carcass at the 10th rib was used for the loin-eye area (height × width/0.7 cm^2), meat color, shearing force, drip loss, and cooking loss determination. The *longissimus dorsi* (LD) muscle samples (the 10th rib of the right carcass) were separated and frozen at −80°C until further analysis.

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TABLE 1 The composition and nutrition levels of experimental diets (as-fed basis).

Items	Treatment[1]		
Ingredients (%)	CON	SJBR	SJBR + BA
Corn	50	25	25
Storage japonica brown rice	0	25	25
Peanut meal	2	2	2
DDGS	9	9	9
Rice bran	10	10	10
Wheat middling	12	12	12
Rice bran meal	13	13	13
Limestone	1.25	1.25	1.25
$CaHPO_4$	0.25	0.25	0.25
NaCl	0.4	0.4	0.4
Phytase	0.02	0.02	0.02
Lysine	0.34	0.34	0.34
Threonine	0.07	0.07	0.07
Methionine	0.03	0.03	0.03
Tryptophan	0.03	0.03	0.03
Lysine residue	0.8	0.8	0.8
t-BHQ	0.01	0.01	0.01
Bile acids	—	—	0.025
Zeolite powder	0.07	0.07	0.07
Choline bitartrate	0.23	0.23	0.205
Premix[2]	0.5	0.5	0.5
Total	100	100	100
Nutrition levels (%)[3]			
Metabolic energy (MJ/kg)	12.16	12.20	12.20
Crude protein	13.30	13.30	13.30
Ether extract	4.49	4.12	4.12
Crude fiber	4.44	3.91	3.91
Ash	4.75	4.84	4.84
Ca	0.58	0.58	0.58

[1]CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group.

[2]The premix supplied the following (per kg diet): 20 mg cupric carbonate, 0.3 mg potassium iodate, 80 mg ferric citrate, 20 mg manganese carbonate, 0.3 mg sodium selenate, 70 mg zinc carbonate, 5,000 IU vitamin A, 4,500 IU vitamin D3, 90 mg vitamin E, 8 mg vitamin K3, 4 mg vitamin B1, 30 μg vitamin B12, 0.4 mg biotin, 0.6 mg folic acid, 14 mg pantothenic acid, 30 mg nicotinic acid.

[3]Metabolic energy was calculated value, and other nutrient levels were measured values.

2.3 Chemical analysis of plasma

The blood samples (10 mL) from each pig were collected using heparin tubes and centrifuged at 4,000 rpm for 10 min after slaughter immediately. Then, the plasma samples were separated and stored in 1.5 mL Eppendorf tubes frozen at −20°C until further analysis. The concentrations of total protein (TP, Cat# A045-4-2), alkaline phosphatase (ALP, Cat# A059-2-2), albumin (ALB, Cat# A028-2-1), glutamic-pyruvic transaminase (ALT, Cat# C009-2-1), glutamic-oxalacetic transaminase (AST, Cat# C010-2-1), blood urea nitrogen (BUN, Cat# C013-2-1), creatinine (CREA, Cat# C011-2-1), uric acid (UA, Cat# C012-2-1), total cholesterol (T-CHO, Cat# A111-1-1), high-density lipoprotein cholesterol (HDL-C, Cat# A112-1-1), low-density lipoprotein cholesterol (LDL-C, Cat# A113-1-1), and triglycerides (TG, Cat# A110-1-1) were measured by a Unicel DxC 800 Synchron (Clinical System, Beckman Coulter, Fullerton, CA, United States), following the kit instructions from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.4 Meat quality measurements

The LD muscle samples (the 10th rib of the right carcass) were selected for meat quality measurement. The meat color was measured at 45 min and 24 h after slaughter with a hand-held colorimeter (CR-410, Konica Minolta Sensing Inc., Osaka, Japan), respectively. Similarly, at 45 min and 24 h after slaughter, the pH probe (Matthäus pH Star, Germany) was inserted into the LD muscle to measure the pH value. The LD muscle samples were taken, and the initial weight was recorded. Then, the sample was hung and placed in a sealed fresh-keeping box, suspended at 4°C for 24 h. Then, it was reweighed to calculate the dripping loss. The meat samples were boiled in the bag with 75°C water in a water bath until the internal temperature of the meat reached 70°C, and then dried and cooled at room temperature. Cooking loss was determined by calculating the weight loss during cooking.

2.5 Quantitative real-time PCR for target genes

Total RNA from the LD muscle and intestinal tissue was isolated using TRIzol reagent (Thermo Fisher Scientific Co., MA, USA). The concentration and purity of total RNA were measured by a nanophotometer (Thermo Fisher Scientific Co., MA, USA), followed by measuring the absorbance at 260/280 nm. Then, the extracted RNA (1,000 ng) was reverse-transcribed into cDNA using the Bio-DL Life ECO Gradient Qualitative PCR Gene Amplifier (Hangzhou, China). Subsequently, the cDNA was used as a template for RT-qPCR using a reverse transcription reagent kit (TransGen Biotech, Beijing, China) through an ABI PRISM 7500 SDS thermal cycler apparatus (Applied Biosystems, Foster City, CA, United States). The primer sequences (Table 2) were designed through NCBI and synthesized by Sangon Biotech (Shanghai, China). The expression levels of mRNA related to myosin heavy chain of muscle fiber and intestinal tight junction protein were calculated by the $2^{-\triangle\triangle Ct}$ method as described in the previous study (22). The expression level of GAPDH was determined as a reference gene, and the transcription levels of target genes were normalized to GAPDH mRNA in each sample.

2.6 The 16S sequencing and data analyses

The total DNA from cecal bacteria was extracted according to the manufacturer's instructions. (QIAGEN QIAamp PowerFecal DNA Kit, Germany). The total DNA was eluted in 50 μL of elution buffer and stored at −80°C until measurement in the PCR. Universal primers

TABLE 2 Primer sequences and the PCR product-amplified fragments used in RT-qPCR.

Genes	Accession no.	Product size (bp)	Sequences (5′ → 3′)
MyHC I	NM_213855.2	133	F: AGTGCAGGCGGAACAAGACAATC
			R: AGCATTCATCTCCTCCTCGTCCTC
MyHC IIx	NM_001104951.2	127	F: ACTGAGGAAGACCGCAAGAACATTC
			R: ACTTGGAGAGGTTGACGTTGGATTG
Myoglobin	NM_214236.1	115	F: CTCATCAGGCTCTTTAAGGGTCACC
			R: TGTTGCCGTGCTTCTTCAGGTC
MyHC IIa	NM_214136.1	153	F: ACAGTGAAGACGGAAGCAGG
			R: TGCGTAACGCTCTTTGAGGT
GAPDH	NM_001206359.1	104	F: ATCCTGGGCTACACTGAGGAC
			R: AAGTGGTCGTTGAGGGCAATG

MyHC I, myosin heavy chain I; MyHC IIx, myosin heavy chain IIx; MyHC IIa, myosin heavy chain IIa.

341F and 805R were used for PCR amplification of the V3–V4 hypervariable regions of 16S rDNA genes (341F, 5′-CCTACGGGNGGCWGCAG-3′; 805R, 5′-GACTACHVGGGTATCTAATCC-3′). The 5′ ends of the primers were tagged with specific barcodes per sample and sequenced with universal primers. The PCR conditions to amplify the prokaryotic 16S fragments consisted of an initial denaturation at 98°C for 30 s, 32 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, extension at 72°C for 45 s, and then final extension at 72°C for 10 min. The PCR products were confirmed with 2% agarose gel electrophoresis. Throughout the DNA extraction process, ultrapure water, instead of a sample solution, was used as a negative control to exclude the possibility of false-positive PCR results. The PCR products were purified by AMPure XP Beads (Beckman Coulter Genomics, Danvers, MA, United States) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent, United States) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, United States), respectively.

Samples were sequenced on an Illumina NovaSeq platform according to the manufacturer's recommendations provided by LC-Bio (Hangzhou, China). Quality filtering on the raw reads was performed under specific filtering conditions to obtain high-quality clean tags according to fqtrim (v0.94). Chimeric sequences were filtered using VSEARCH software (v2.3.4). Alpha diversity was applied in analyzing the complexity of species diversity for a sample through five indices, namely, Chao1, observed species, goods coverage, Shannon, and Simpson, and all the indices in our samples were calculated using QIIME2. Beta diversity was calculated by QIIME2; the graphs were plotted using the R package. BLAST was used for sequence alignment, and the feature sequences were annotated with the SILVA database for each representative sequence.

2.7 Statistical analysis

The experimental data were analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL, United States) with a one-way ANOVA model, followed by Duncan's multiple range tests. The data in figures and tables were shown as the mean ± standard error of means (SEM). In this study, differences were shown to be significant at $p < 0.05$, and trends toward significance were at $0.05 < p < 0.10$.

TABLE 3 Effects of adding bile acids to dietary storage japonica brown rice on the growth performance of growing–finishing Min pigs.

Items	Treatments			*p*-value
	CON	SJBR	SJBR + BA	
IBW, kg	41.12 ± 2.31	41.81 ± 1.96	40.71 ± 2.91	0.95
FBW, kg	102.13 ± 6.16	99.31 ± 5.83	94.00 ± 7.06	0.58
ADG, kg	0.38 ± 0.03	0.43 ± 0.02	0.43 ± 0.06	0.67
ADFI, kg	2.25 ± 0.13	2.29 ± 0.15	2.22 ± 0.24	0.96
FCR	5.97 ± 0.15^{a}	5.38 ± 0.13^{b}	5.27 ± 0.19^{b}	0.02

In the same row, values with no letter or the same small letter superscripts mean no significant difference ($p > 0.05$), whereas values with different small letter superscripts mean significant differences ($p < 0.05$).
IBW, initial body weight; FBW, finial body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio = ADFI (kg)/ADG (kg).
CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group. Data are expressed as the mean ± SEM ($n = 8$).

3 Results

3.1 Growth performance

The growth performance of growing–finishing Min pigs with different treatments is shown in Table 3. In this study, the SJBR and BA supplementation significantly reduced the ratio of ADFI-to-ADG (F/G) compared to the CON treatment ($p < 0.05$). Concurrently, the average daily gain (ADG) and average daily feed intake (ADFI) were not affected by the dietary replacement of corn with SJBR and bile acids.

3.2 Plasma biochemical index

As shown in Table 4, there is no significant difference in the contents of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, uric acid, total cholesterol, triglyceride, and low-density lipoprotein cholesterol between groups. Compared

TABLE 4 The effects of adding bile acids to dietary storage japonica brown rice on the plasma biochemical indices of growing–finishing Min pigs.

Items	Treatments			*p*-value
	CON	SJBR	SJBR + BA	
ALT, U/L	55.30 ± 3.53	54.13 ± 2.21	51.25 ± 2.06	0.56
AST, U/L	65.80 ± 2.19	62.53 ± 2.71	59.88 ± 5.03	0.51
ALP, U/L	6.30 ± 0.67	5.95 ± 0.66	5.72 ± 0.61	0.82
TP, g/L	83.28 ± 0.78^{b}	91.07 ± 1.03^{a}	88.23 ± 1.92^{a}	0.003
ALB, g/L	38.02 ± 0.80	38.73 ± 1.55	41.08 ± 0.76	0.16
BUN, mmol/L	5.96 ± 0.20^{a}	5.00 ± 0.20^{b}	4.79 ± 0.33^{b}	0.01
UA, μmol/L	15.75 ± 0.42	16.65 ± 0.32	16.30 ± 0.29	0.21
TCHO, mmol/L	1.98 ± 0.12	1.98 ± 0.04	2.08 ± 0.04	0.61
TG, mmol/L	0.37 ± 0.04	0.35 ± 0.03	0.32 ± 0.05	0.63
HDL-C, mmol/L	0.40 ± 0.03^{b}	0.46 ± 0.02^{ab}	0.54 ± 0.03^{a}	0.02
LDL-C, mmol/L	0.75 ± 0.07	0.65 ± 0.03	0.60 ± 0.06	0.14

ALT, glutamic-pyruvic transaminase; AST, glutamic-oxalacetic transaminase; ALP, alkaline phosphatase; TP, total protein; ALB, albumin; CREA, creatinine; BUN, blood urea nitrogen; UA, uric acid; TG, triglyceride; T-CH, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
In the same row, values with no letter or the same small letter superscripts mean no significant difference ($p > 0.05$), whereas values with different small letter superscripts mean significant differences ($p < 0.05$).
CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group. Data are expressed as the mean ± SEM ($n = 8$).

TABLE 5 The effects of adding bile acids to dietary storage japonica brown rice on the carcass traits and meat quality of growing–finishing Min pigs.

Items	Treatments			*p*-value
	CON	SJBR	SJBR + BA	
Carcass weight, kg	64.70 ± 1.57	67.10 ± 1.14	67.17 ± 1.59	0.41
Length of carcass, cm	71.90 ± 0.96	71.33 ± 0.92	75.87 ± 1.28	0.11
Eye muscle area, cm^2	29.24 ± 0.90^{b}	32.77 ± 1.01^{a}	30.79 ± 0.57^{ab}	0.03
Backfat thickness, mm	50.74 ± 0.50^{a}	49.87 ± 0.86^{a}	46.25 ± 1.31^{b}	0.01
$pH_{45\ min}$	6.52 ± 0.11	6.48 ± 0.17	6.57 ± 0.15	0.92
$pH_{24\ h}$	5.70 ± 0.19	5.74 ± 0.15	5.73 ± 0.19	0.99
Drip loss	3.37 ± 0.07	3.31 ± 0.10	3.28 ± 0.11	0.81
Cooking loss	22.61 ± 0.52	22.17 ± 0.63	22.98 ± 0.49	0.59
shear force, N	49.40 ± 0.99	50.45 ± 1.18	49.31 ± 1.18	0.73

In the same row, values with no letter or the same small letter superscripts mean no significant difference ($p > 0.05$), whereas values with different small letter superscripts mean a significant difference ($p < 0.05$).
CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group. Data are expressed as the mean ± SEM ($n = 8$).

with the CON group, the total protein content in the SJBR group and SJBR + BA group significantly increased ($p < 0.05$), while the urea nitrogen content significantly decreased ($p < 0.05$). Meanwhile, the content of high-density lipoprotein cholesterol in the SJBR + BA group was significantly increased by 35% ($p < 0.05$).

3.3 Carcass traits and meat quality

As shown in Table 5, compared with the CON group, the eye muscle area of the SJBR group significantly increased ($p < 0.05$). In addition, the SJBR + BA group significantly reduced back fat thickness compared with the SJBR group ($p < 0.05$). However, there were no significant differences in carcass weight, carcass length, $pH_{45\ min}$, $pH_{24\ h}$, drip loss, cooking loss, or shear force among the groups. We detected the relative mRNA expression levels of myosin heavy chain (MyHC) subtypes (I, IIa, IIx, and IIb) of four different muscle fiber types (slow oxidative type—I, fast oxidative type—IIa, intermediate type—IIx, and fast glycolytic type—IIb) in the LD muscle. The results showed that the expression level of the MyHC I gene in the LD muscle fibers of the SJBR + BA group was significantly increased ($p < 0.05$), and there was no significant difference in the expression levels of MyHC IIa, MyHC IIx, and MyHC IIb genes between the groups (Figure 1).

3.4 Fecal bacterial community structure

To evaluate the impact of SJBR diets on the microbial composition of feces, a total of 1,232,084 V3–V4 16S rRNA effective sequences from the 18 samples, with an average of 68,449 sequences per sample, were used for subsequent analysis (Table 6). The goods coverage showed that the sampling in each group provided sufficient OTU coverage (Figure 2A). Overall, 1,898, 1,795, and 1,555 ASVs were recorded in the CON, SJBR, and SJBR + BA groups, respectively; of which, 667 OTUs were shared among the three groups (Figure 2B).

3.5 Alpha diversity of the fecal microbiome

To analyze the abundance and diversity of intestinal microbiota, the alpha diversity of samples, including the Chao1 index, observed OTUs, Shannon index, and Simpson index, was performed. As shown in Figure 3, there was no significant difference in the Principal Component Analysis (PCA), Chao1 index, observed OTUs, or Simpson index among different treatment groups, while the Shannon index of the SJBR group significantly decreased ($p < 0.05$) (Figure 4).

3.6 Relative abundance of species structure of fecal microbiota

At the phylum and genus levels, the relative abundance of species structure in the fecal microbiota in each group of pigs was analyzed. As shown in Figure 5A, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteriota* were predominant in the three dietary treatments at the phylum level. In comparison to CON, the profiling of microbial phyla in SJBR + BA was characterized by a high proportion of *Proteobacteria* (11.83% vs. 5.44%) and a low proportion of *Bacteroidetes* (7.26 vs. 9.09%), and the *Firmicutes* to *Bacteroidetes* ratio was higher in the SJBR + BA group than the SJBR and CON groups. At the genus level (Figure 5B), the proportion of *Lactobacillus* gradually increased (CON 15.93%, SJBR 23.44%, and SJBR + BA 27.42%), with no significant difference. In order to conduct a more detailed study of microorganisms with significant differences between

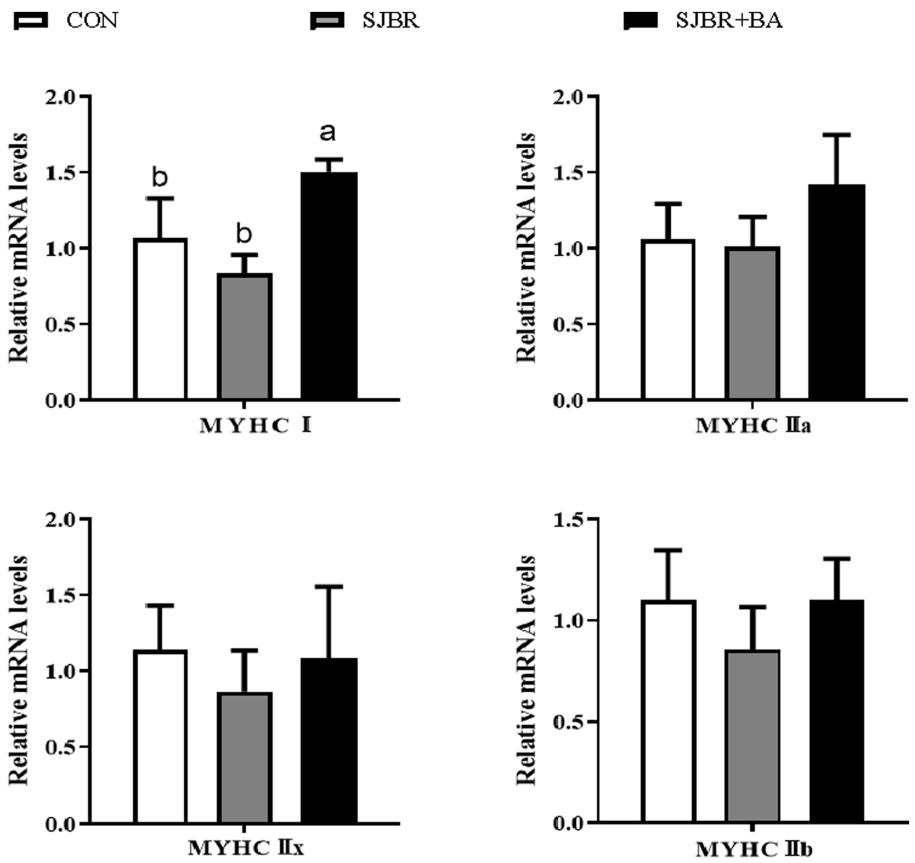

FIGURE 1
The effects of adding bile acids to dietary storage japonica brown rice on mRNA expression of myosin heavy chain (MyHC) isoforms in the longissimus dorsi (LD) muscle of growing–finishing Min pigs. CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group. Data in the column chart were expressed as the mean ± SEM ($n = 6$). ^{a,b}Bars with different letters were declared significant at $p < 0.05$.

different varieties, we selected the top 15 at the genus level, which showed significant differences in the Kruskal–Wallis test.

4 Discussion

The growth performance directly affects the meat production capacity of growing–finishing pigs and the economic benefits of animal husbandry enterprises. The results of this study showed that, compared with the CON group, there were no significant differences in the ADG and ADFI of the SJBR and SJBR + BA groups after replacing half of the corn with storage japonica brown rice, indicating that there is no potential harm to the growth performance of Min pigs. Similar to the current results, there were no significant differences in ADG, ADFI, and FCR between the brown rice replacement group (50, 75, and 100%), and the control group was fed a corn–soybean meal basal diet (3). Zhang et al. also found that the ADG of experimental pigs fed brown rice increased slightly, and the ADFI-to-ADG ratio was reduced, even though there was no significant difference between the brown rice group and the corn group. However, it had a good effect on cost reduction and efficiency in animal production (23). Nevertheless, studies have shown that feeding weaned piglets with brown rice as a 50% substitute for corn resulted in an increase of 2.43 and 2.88% in apparent ileal digestibility of dry matter and energy compared to the control group, respectively (24). The reasons for the different results may be attributed to the different growth stages of the experimental animals selected by researchers, as well as the differences in the storage time of brown rice. Additionally, the substitution of brown rice for corn in swine diets at all feeding stages did not affect or even improve growth performance, most likely due to the slightly higher nutritional value of brown rice compared to corn and its low content of anti-nutritional factors.

There is a certain degree of positive relationship between the total protein content of serum and the protein synthesis of body tissues; as the total protein content of serum increases, the effect of tissue protein synthesis will become stronger, and its effect on promoting the growth of tissue organs will also become stronger (25). The addition of brown rice from storage japonica in this assay produced a significant increase in the total protein content in the serum of the pigs used, which may be explained by the higher crude protein content of brown rice from storage japonica than from maize. Total protein can reflect both the level of nutrient metabolism of the animal body, the body's immune function, and the health of the liver (26) because the liver is the primary site where blood protein is produced, and if total serum protein is significantly reduced, it indicates liver damage (27). This indicates that adding brown rice from storage japonica has no adverse effect on pig health among those in the fattening stage. The serum urea nitrogen level is an indicator that can

TABLE 6 Valid data statistics table.

Sample	Raw_Tags	Raw_ Bases	Valid_Tags	Valid_ Bases	Valid%	Q20%	Q30%	GC%
CON1	85,977	42.99 M	69,627	28.67 M	80.98	95.99	89.54	52.78
CON2	87,604	43.80 M	71,808	29.45 M	81.97	96.56	90.99	53.02
CON3	84,821	42.41 M	68,494	28.29 M	80.75	96.42	90.73	52.70
CON4	82,855	41.43 M	67,559	27.87 M	81.54	96.54	90.98	52.66
CON5	84,226	42.11 M	70,694	29.17 M	83.93	96.41	90.64	52.65
CON6	84,988	42.49 M	69,508	28.67 M	81.79	95.76	88.92	52.79
SJBR_2	80,570	40.28 M	65,759	27.22 M	81.62	96.78	91.50	52.92
SJBR_3	86,097	43.05 M	70,832	29.37 M	82.27	96.40	90.68	52.59
SJBR_4	85,839	42.92 M	65,138	26.97 M	75.88	91.99	81.31	53.15
SJBR_5	81,354	40.68 M	65,738	27.10 M	80.80	96.74	91.38	52.80
SJBR_6	83,744	41.87 M	67,734	28.39 M	80.88	96.44	90.75	51.79
SJBR_1	80,545	40.27 M	65,962	27.33 M	81.89	96.63	91.19	52.99
SJBR_BA_1	82,311	41.16 M	69,187	28.89 M	84.06	96.60	91.08	52.13
SJBR_BA_2	81,378	40.69 M	65,507	27.13 M	80.50	94.45	86.15	53.16
SJBR_BA_3	87,173	43.59 M	68,647	28.44 M	78.75	96.69	91.23	52.72
SJBR_BA_4	86,440	43.22 M	71,108	29.56 M	82.26	96.62	91.17	52.22
SJBR_BA_5	87,103	43.55 M	70,683	29.25 M	81.15	94.08	85.56	52.90
SJBR_BA_6	81,445	40.72 M	68,099	28.48 M	83.61	95.50	88.43	52.34

CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group.

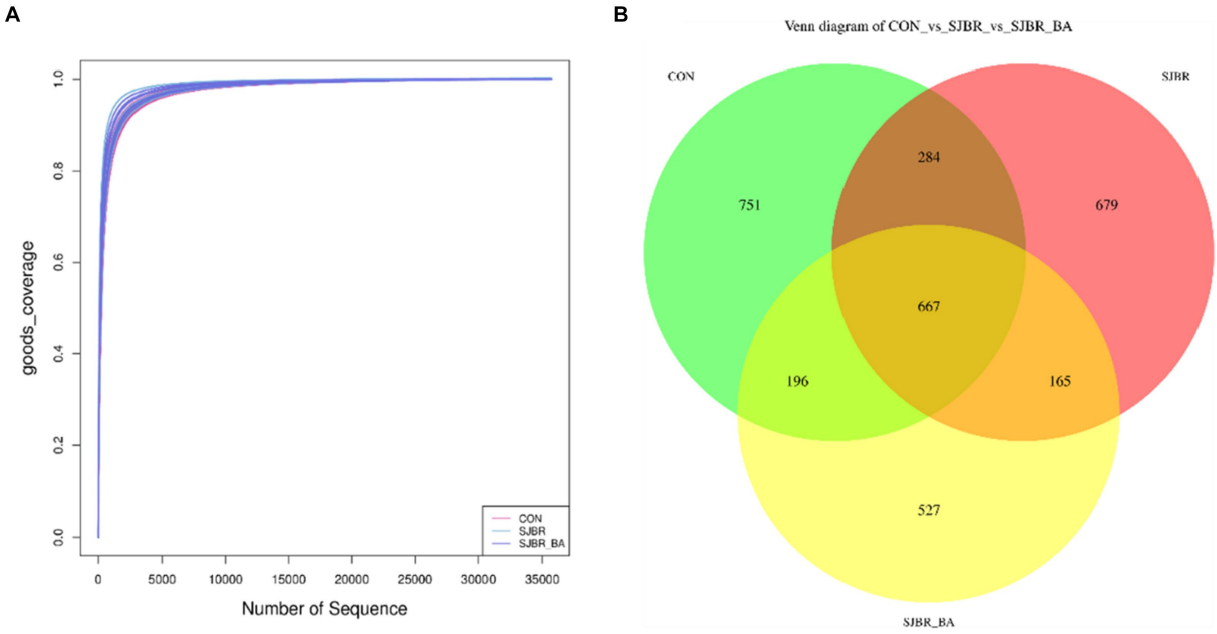

FIGURE 2
(A) Rarefaction curve of three dietary treatments. **(B)** ASV Venn of three dietary treatments. CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group.

reflect the body's protein metabolism and nutritional status (28). The decrease in urea nitrogen content in serum indicates a weakened decomposition of amino acids in animals, an increase in nitrogen storage, and a strengthened protein synthesis effect (29). When the metabolic status of proteins and amino acids changes, the concentration of serum urea nitrogen also changes, and the imbalance of amino acids leads to the degradation of excess amino acids through oxidation, increasing the rate of urea synthesis and resulting in an

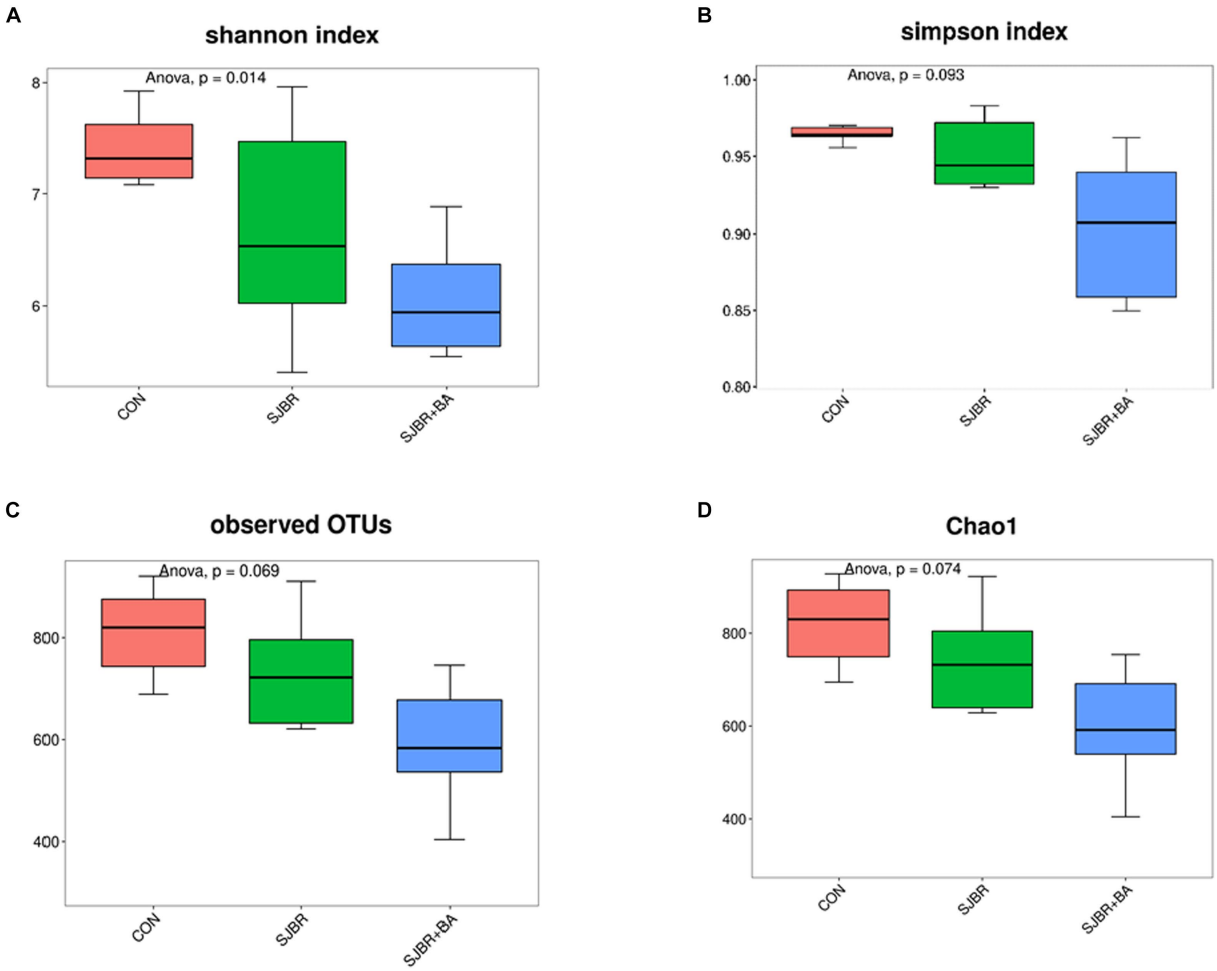

FIGURE 3
Measurements of fecal microbiome alpha and beta diversity at the amplicon sequence variant (ASV) level. Measurement of alpha diversity at the ASV level using the **(A)** Shannon, **(B)** Simpson, **(C)** observed OTUs, and **(D)** Chao1 among three dietary treatments. Data are expressed as the mean ± SEM ($n = 6$). CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group.

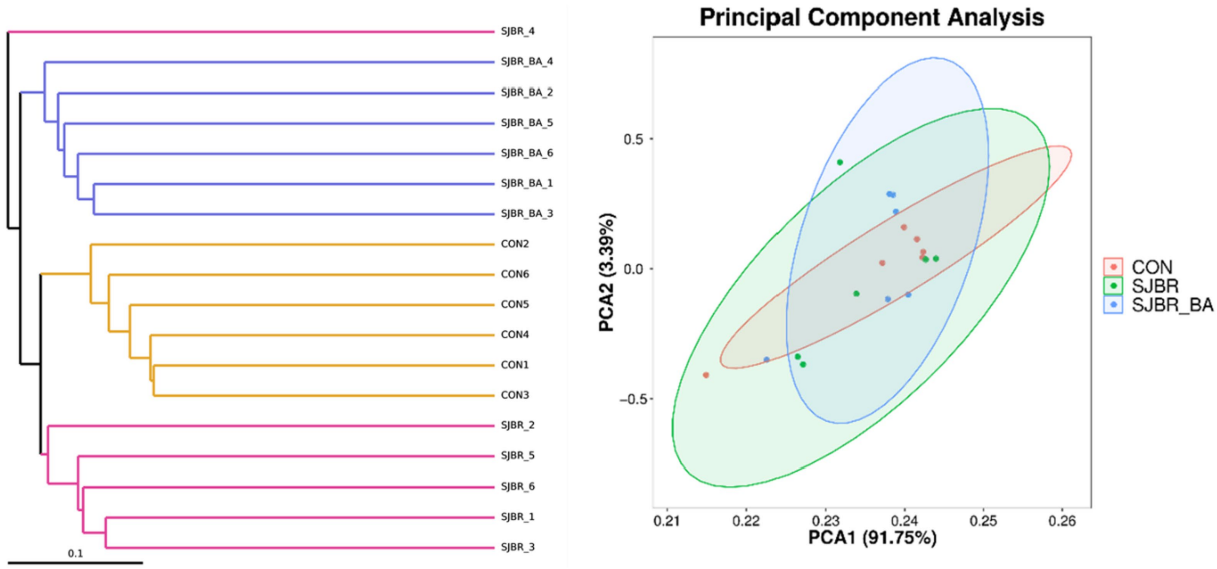

FIGURE 4
Principal component analysis (PCA) based on the Bray–Curtis distance of all the samples among the three dietary treatments. Data are expressed as the mean ± SEM ($n = 6$). CON, control group; SJBR, storage japonica brown rice group; SJBR + BA, storage japonica brown rice + bile acid group.

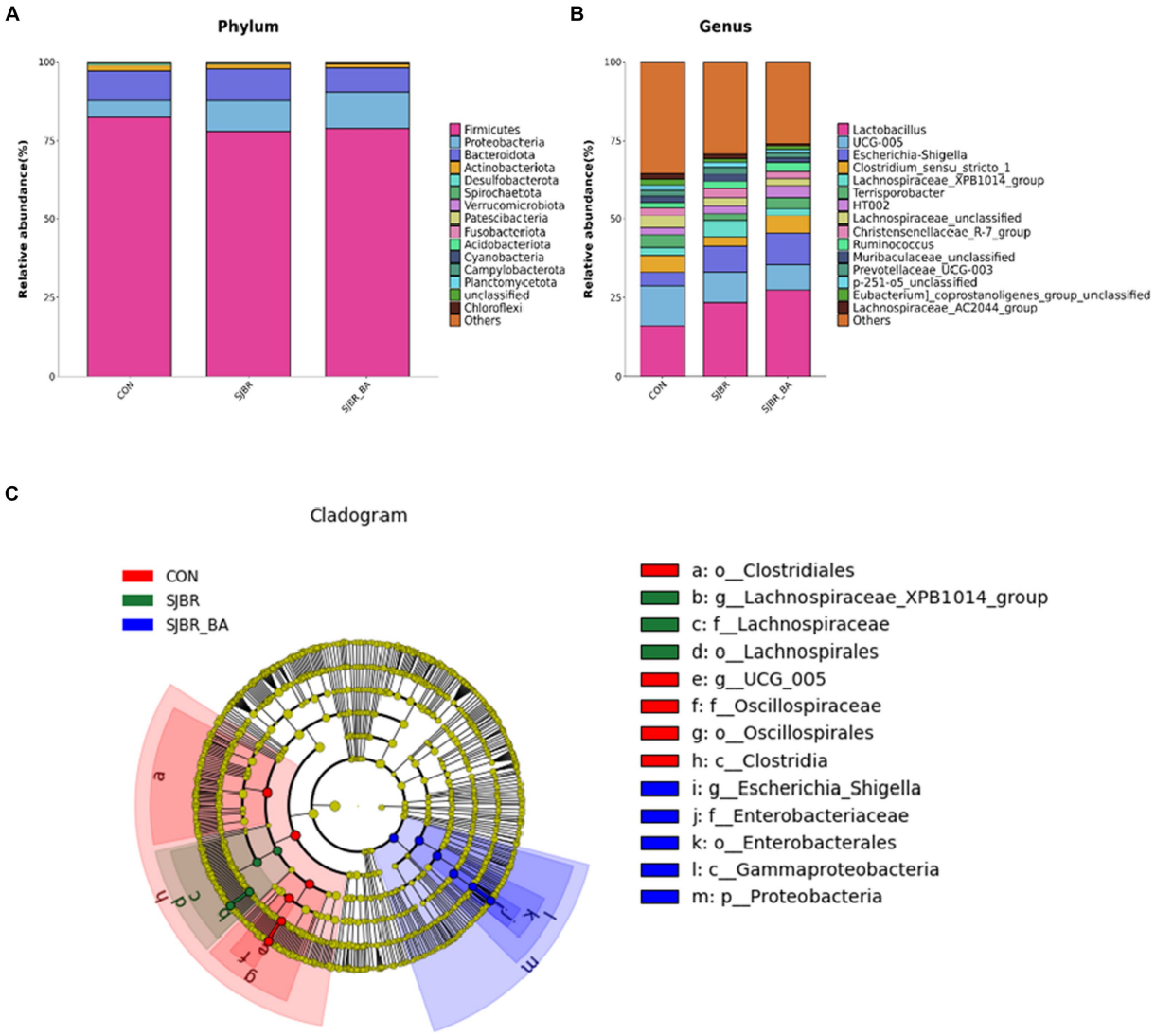

FIGURE 5
The effects of SJBR and BA on the composition of fecal microbiota from phylum to genus in growing–finishing Min pigs ($n = 6$). Comparison of the major microbes at the **(A)** phylum and **(B)** genus levels among three dietary treatments, respectively. The abundances of the top 15 microbes are expressed as proportions. **(C)** Linear discriminant analysis effect size (LEfSe) analysis based on amplicon sequence variants (ASVs) characterized the microbiomes among three treatments of growing–finishing Min pigs.

increase in plasma urea nitrogen levels. Generally, the lower the amino acid utilization rate, the higher the serum urea nitrogen concentration. Similarly, studies have found a significant negative correlation between the growth of lean tissue and the concentration of serum urea nitrogen (30). Therefore, the urea nitrogen content in serum can reflect the animal's utilization of protein and amino acids in feed. The results of this experiment showed that compared with the CON group, the addition of storage japonica rice brown rice significantly reduced the serum urea nitrogen content of Min pigs, indicating that during the fattening period, Min pigs improved the utilization of amino acids in storage japonica rice brown rice feed and enhanced protein synthesis. Bile acids can directly or indirectly regulate the gut microbiota composition by activating innate immune genes in the intestine (31). Studies have found that some gut microbiota involve the conversion of primary to secondary bile acids and the production of short-chain fatty acids (32).

The present study showed that SJBA did not change carcass characteristics such as carcass weight and length, pH, drip loss, cooking loss, or shear force compared to those in the corn group but increased the eye muscle area of the SJBR group. Similar to the results of this study, pig diets supplemented with brown rice did not alter carcass characteristics compared to pigs without brown rice but showed some changes in the meat composition of fattening pigs (33). Additionally, the present study also found that bile acids reduced the back fat thickness of Min pigs, although it did not completely improve the carcass characteristics. Bile acids can affect the host's digestion, absorption, and metabolism of lipids by altering the composition and structure of the gut microbiota (34), which may be due to the effects of metabolites of the gut microbiota (such as SCFAs, secondary bile acids, and trimethylamine) and bacterial factors (such as lipopolysaccharides) (35). Ge et al. found that adding bile acids to AA broiler feed significantly reduced the abdominal fat rate (18). Adding

different concentrations of bile acids (0, 75, 150, and 300 mg/kg) to the high-fat diet of juvenile fish can improve their digestion and utilization ability, reduce fat deposition, and improve their meat quality (36). The addition of bile acids to the diet significantly reduced the fat content in the whole body, muscles, and the liver of tilapia, promoting its lipid metabolism (37).

Muscle fibers are the basic unit of muscle tissue, and their type and composition play a decisive role in the growth and development of livestock and poultry, as well as meat quality after slaughter (38). According to different enzyme catalysis (adenosine triphosphate enzyme and succinate dehydrogenase), it can be divided into type I oxidized muscle fibers and type II enzymolysis muscle fibers. According to the diversity of myosin heavy chain (MyHC) isomers, they can be divided into the slow oxidation type (type I), rapid oxidation type (type IIa), intermediate type (type IIx), and rapid glycation type (type IIb) (39). The conversion between mature MyHC isomers follows a certain pattern, and the four types are generally classified as follows: I↔IIa↔IIx↔IIb (40). There are reports that the content of type I, IIa, and IIx muscle fibers in livestock muscles is positively correlated with muscle tenderness, color, and fat content, while the content of type IIb muscle fibers is negatively correlated with meat quality traits (41). The results of this experiment showed that there was no significant effect on the mRNA expression of MyHC IIa, MyHC IIx, and MyHC IIb genes among the groups. After adding bile acid, the mRNA expression level of the MyHC I gene in the LD muscle of growing and fattening pigs significantly increased, indicating that bile acid has a certain positive effect on improving pork quality.

The intestinal microbiota has an important impact on the health of the host. In studies of the gut microbiota related to the growth performance of pigs, the feed is an important influence in modifying the structure of the gut microbiota (42). The level of dietary protein, fiber, and fat can all affect the composition of the pig's intestinal microbiota, thereby affecting the growth, feed utilization, fat deposition, and inflammatory immunity in pigs (43). It has been shown that feeding brown rice flour completely instead of corn to weaned piglets contributes to an increase in beneficial intestinal bacteria (e.g., *Bifidobacteria* and *Lactobacillus*) and a decrease in serum TGF-β1 concentration (44), suggesting that replacing corn with brown rice can improve intestinal health and enhance immune function. In this study, the Shannon diversity index decreased significantly in the SJBR + BA group, while there was no significant difference in the Chao1 and Simpson index among the three treatments, indicating supplementation of bile acids in dietary SJBR rather reduced the microbial richness compared to individual SJBR. This result may seem incomprehensible; however, a previous study reported that the reduced Shannon index may be related to the expected inhibition of dietary lipid absorption (45). The experimental subjects in this study were Chinese fat-type pigs, whose fat deposition is distinctly different from that of lean-type pigs, which may be the reason for the opposing effects. It needs to be recognized that the mechanism of action needs to be further explored and studied in depth. During the growth period, there were no significant differences in fecal microbial composition at the phylum level between treatment groups; during the fattening period, the relative abundance of phylum *Bacteroidetes* and *Firmicutes* was higher, and genera *Lactobacillus* and *Streptococcus* were lower in the brown rice complete replacement diet group compared to the control group (3), suggesting that the long-term feeding of brown rice affects the intestinal microflora of pigs. Both *Bacteroidea* and *Firmicutes* are obesity-related bacteria (46). In fatter pigs, *Firmicutes* has a higher relative abundance, while *Bacteroidea* has a lower relative abundance (47, 48). This indicates that the pigs in this experiment are overweight, possibly related to insufficient exercise within the limit bar. It is worth noting that after adding storage japonica brown rice, the abundance of *Firmicutes* decreased slightly, but the difference was insignificant. Some studies have reported the correlation between gut microbiota composition and carcass characteristics in pigs and found that the abundance of *Lactobacilli* in the gut microbiota of high-quality pork is higher than that of low-quality pork (49, 50).

Intestinal health depends on the three major barriers of the intestine, namely, the intestinal mucosal barrier, the intestinal immune barrier, and the intestinal biological barrier (51). The effect of *Lactobacillus* on the intestinal mucosal barrier function may be mediated by different bacterial structures and chemical components (52). Soluble proteins isolated from *Lactobacillus rhamnosus* can improve *in vitro* intestinal epithelial barrier disruption by inducing the redistribution of Occludin and ZO-1 proteins (53). *Lactobacillus* is the core microorganism in the pig intestine. It was found that *Lactobacillus* stimulates the expression of cellular immune factors, such as CD4 and TNF-α, and inhibits the NF-κB pathway through metabolically produced active substances, thus promoting the development and maturation of the intestinal mucosal immune system and balancing the intestinal immune response (54). Some studies have shown that 100% replacement of corn with brown rice noodles can promote the increase in beneficial bacteria (such as *Bifidobacteria* and *Lactobacilli*) in the intestine of piglets and can also reduce TGF in the serum-β1 concentration, indicating that replacing corn with brown rice can promote intestinal health and enhance the body's immunity (44).

Compared with the CON group, adding storage japonica brown rice in this experiment increased the relative abundance of *Lactobacilli*, indicating that SJBR may have a certain positive effect on pork quality and intestinal health during the fattening period. Together with *Spirillum*, it changes primary bile acid into secondary bile acid and SCFAs. Research has shown that the *Ruminococcaceae* genus is an important fiber-degrading bacterium that can degrade fiber substances (55). In this experiment, the addition of SJBR significantly reduced the proportion of *UCG-005* genera in the intestines of pigs, which may be related to the lower crude fiber content of SJBR. The large amount of lactic acid it produces makes it a powerful broad-spectrum bactericide, killing viruses, and acting as an immune modulator. It can inhibit the growth of pathogenic bacteria and can be used as a probiotic to restore the structure of the bacterial community (56). In this experiment, the addition of SJBR increased the proportion of *Lactobacilli* in the intestines of pigs compared to the CON group, which indicated that storage japonica brown rice is beneficial for maintaining intestinal homeostasis in pigs. In contrast, bile acid cannot significantly increase the proportion of probiotics in the intestines of pigs.

5 Conclusion

In summary, this study revealed that supplementation of 25% storage japonica brown rice in the feed during the fattening period might have a certain positive effect on the growth performance, meat quality, and intestinal health of growing–finishing Min pigs.

Meanwhile, this study found that adding 0.025% bile acid (hyodeoxycholic acid) to 25% storage japonica brown rice feed can significantly reduce back fat thickness and improve meat quality. The current study indicates that replacing part corn with storage japonica brown rice is feasible, but how to further improve the growth efficiency of pigs with the storage brown rice diet is still a key challenge that needs to be studied.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/bioproject/; PRJNA1076370.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of Jilin University (SY202209301). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CW: Writing – original draft, Formal analysis. KZ: Writing – original draft, Formal analysis. DW: Writing – original draft, Methodology. HY: Writing – original draft, Data curation. YZ: Writing – review & editing, Resources, Data curation. HF: Writing – review & editing, Investigation. JZ: Writing – review & editing, Conceptualization, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of enzyme supplementation on growth performance, digestibility of phosphorus, femur parameters and fecal microbiota in growing pigs fed different types of diets

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A 42-days study was conducted to evaluate the effects of different dietary types (corn-or wheat-soybean meal-based diet) and phytase (Phy) or a multi-carbohydrase and phytase complex (MCPC) supplementation on growth performance, digestibility of phosphorus (P), intestinal transporter gene expression, plasma indexes, bone parameters, and fecal microbiota in growing pigs. Seventy-two barrows (average initial body weight of 24.70 ± 0.09 kg) with a 2 × 3 factorial arrangement of treatments and main effects of diet type (corn- or wheat-soybean meal-based-diets) and enzyme supplementation (without, with Phy or with MCPC). Each group was designed with 6 replicate pens. The MCPC increased ($p < 0.05$) average daily gain (ADG) and final body weight (BW). A significant interaction ($p = 0.01$) was observed between diet type and enzyme supplementation on apparent total tract digestibility (ATTD) of P. The ATTD of P was higher ($p < 0.05$) in wheat soybean meal-based diets compared to corn-soybean meal-based diets. Compared with the corn-soybean meal-based diet, the relative expression of SLC34A2 and VDR genes in the ileum and SLC34A3 in jejunum of growing pigs fed the wheat-soybean meal based diet was lower ($p < 0.05$). The MCPC significantly reduced ($p < 0.05$) the relative expression of TRPV5 and CALB1 genes in the ileum and increased the expression of CALB1 in the duodenum compared to control diet. The phytase increased ($p < 0.05$) the relative expression of SLC34A1 gene in the duodenum in comparison to control diet and MCPC-supplemented diet. The Ca and P contents in plasma from pigs fed corn-soybean meal-based diet were higher ($p < 0.05$) than those from pigs fed wheat-soybean meal-based diet, and the parathyroid hormone (PTH) and calcitonin (CT) concentrations were lower ($p < 0.05$) than those fed wheat-soybean meal-based diet. The content of Ca and P in the femur and the bone strength of pigs in the corn-soybean meal group were significantly higher ($p < 0.05$) than those in the wheat-soybean meal groups. The phytase increased ($p < 0.05$) the Ca and P content and bone strength of the femur. Additionally, diet type and both enzymes significantly improved fecal microbial diversity and composition. Taken together, diet type and exogenous enzymes supplementation could differently influence the growth performance, utilization of phosphorus, intestinal transporter gene expression, bone mineralization and microbial diversity and composition in growing pigs.

KEYWORDS
phytase, multi-carbohydrase and phytase complex, pigs, mineralization, microbiota

1 Introduction

Corn and wheat are often used as energy feed sources for monogastric animals. However, these feed ingredients contain non-starch polysaccharides (NSP), including arabinoxylan, glucan, cellulose and mannans, which can reduce feed efficiency and nutrient digestibility (1). Arabinoxylan is the most common NSP found in cereals grains such as wheat and corn which can exert an antinutrient effect in monogastric animals since they do not have endogenous enzymes able to digest arabinoxylan (2). Hence, a multi-carbohydrase containing xylanase, beta-glucanase and arabinofuranosidase in the diets can produce oligosaccharides from arabinoxylan degradation by random hydrolysis of β-1,4-glycosidic bonds (1). Additionally, in these plant-based feed ingredients, up to 80% of the total phosphorus is in the form of phytate generally known as an antinutrient (3, 4). Due to the negligeable endogenous enzyme activity within the gut, the phytate could not be degraded in the intestinal tract to improve the availability of P (5). Therefore, phytase is commonly used in the feed for monogastric animals. The supplementation of phytase in swine diets can improve not only the digestibility of calcium and phosphorus but also that of amino acids and energy and reduce the excretion of phosphorus in animal manure (6).

Moreover, phosphorus and calcium are two closely related minerals in animals because they are regulated by the same hormones, including vitamin D, parathyroid hormone, fibroblast growth factor 23 and calcitonin (7–9). These hormones play key roles in the absorption, accumulation, resorption, and excretion pathways of P and Ca (10). In addition to hormones, we assumed that diet type and enzyme supplementation with phytase alone or in combination with a multi-carbohydrase can influence the gene expression related to Ca and P absorption along the intestinal tract of growing pigs.

Compared with corn-based diets, previous study reported that in pigs, the wheat-based diets can significantly change the composition of microbiota and the concentration of microbial metabolites in the gut probably due that wheat-based diets contain more NSP than diets based on corn which may affect the hindgut microbes (11). Some other authors have found that the addition of xylanase in the diet significantly affects the abundance of microorganisms in the intestinal tract of pigs (12). Thus, the effects of phytase and enzyme mixture supplementation in the corn and wheat-based diets on growth performance, bone mineralization and gut microbiota in growing pigs remained elusive. Therefore, this study aimed to investigate the effects of different dietary types (corn- or wheat-soybean meal-based diet) and phytase (Phy) or a multi-carbohydrase and phytase complex (MCPC) supplementation on growth performance, digestibility of P, intestinal transporter gene expression, plasma indexes, bone parameters and fecal microbiota composition and diversity in growing pigs.

2 Materials and methods

2.1 Experimental design and diets

Seventy-two healthy DLY [Duroc × (Landrace × Yorkshire)] barrows (70-d of age) with body weight (BW) of 24.70 ± 0.09 kg were used in a 42-d trial after a 5-d adaptation. Barrows were assigned into 6 treatment groups in a randomized complete block design involving a 2 diet types × 3 enzymes supplementation factorial arrangement of treatments using body weight as the blocking factor. Two diet types include corn-soybean meal-based diet or wheat-soybean meal-based diet. Enzyme supplementation includes without any supplementation, with a 6-phytase expressed in *Buttiauxella* spp., Phy or with a multi-carbohydrase and phytase complex (MCPC) which is a mixture of xylanase, *β*-glucanase and *α*-arabinofuranosidase and a 6-phytase at optimal dose (Rovabio Advance Phy, Adisseo France SAS). At day 36 of the experimental period, 0.5% chromium dioxide was added to the feed as an indigestible marker for digestibility determination. Each treatment consisted of 6 replicate pens with 2 pigs per pen. The ambient temperature is controlled at 26 ± 2°C, and the relative humidity is controlled at 60% ± 5%.

Two types of diets were formulated according to the recommended nutrient requirements for growing pigs (NRC 2012). Experimental diets included a corn-soybean meal-based diet or a wheat-soybean meal-based diet. The phytase was added to provide at least 1,000 FTU/kg diet, and the MCPC was supplemented to supply at least 1,800 U of xylanase, 1,244 U of β-glucanase, and 1,000 FTU of phytase/kg diet. The Composition and calculated nutrient levels of the experimental diets were presented in Table 1. All diets were antibiotics-free and mashed. Experimental diets and water were offered *ad libitum* throughout the experimental period.

2.2 Sample collection

On day 39 of the experimental period, fecal samples were collected from each replicate pen and during 4 consecutive days, at least 100 g of fresh fecal samples in each pen were collected with disposable gloves during defecation to avoid falling to the ground and put them into the sample bag immediately, then 10% of dilute sulfuric acid and 4–5 drops of toluene for nitrogen fixation and preservation of the fresh stool in a 1:10 ratio (13). After mixing, put it in −20°C for refrigeration in time. On day 43 and after 12 h of fasting, 6 pigs per treatment (1 pig per pen) were selected according to the average weight for blood Research Topic. Blood samples were centrifuged at 3,500 × *g* for 10 min at 4°C. Then, plasma samples were stored at −20°C for further analysis. After blood sampling, all pigs were euthanized. Then the abdominal cavity of the pig was opened, and the duodenum, jejunum, and ileum were quickly separated according to the anatomical structure. The duodenum, ileum, and approximately 10 cm jejunum (the same part for each pig) were cut longitudinally, and gently rinsed with 0.9% pre-cooled normal

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TABLE 1 Diet composition and analyzed nutrient content of the experimental diets (as-fed basis).

	Corn-soybean meal			Wheat-soybean meal		
	Without	Phy	MCPC	Without	Phy	MCPC
Ingredient, %						
Corn	67.73	67.73	67.73	–	–	–
Wheat	–	–	–	81.00	81.00	81.00
Soybean meal 48%	25.33	25.33	25.33	11.83	11.83	11.83
Soybean oil	3.00	3.00	3.00	3.09	3.09	3.09
Chloride choline	0.10	0.10	0.10	0.10	0.10	0.10
Monocalcium phosphate	0.90	0.90	0.90	0.45	0.45	0.45
Limestone	1.40	1.40	1.40	1.38	1.38	1.38
NaCl	0.30	0.30	0.30	0.30	0.30	0.30
MCPC	–	–	0.01	–	–	0.01
L-lysine HCl 95%	0.53	0.53	0.53	0.84	0.84	0.84
DL-methionine 98.5%	0.04	0.04	0.04	0.05	0.05	0.05
L-threonine 98.5%	0.07	0.07	0.07	0.20	0.20	0.20
L-isoleucine 99%	–	–	–	0.07	0.07	0.07
L-valine 98%	–	–	–	0.09	0.09	0.09
Premix[1]	0.10	0.10	0.10	0.10	0.10	0.10
Chromium oxide	0.50	0.50	0.50	0.50	0.50	0.50
Nutrient contents[2], %						
NE, kcal/kg	2,531	2,531	2,531	2,534	2,534	2,534
Crude protein	17.01	16.96	17.03	17.00	16.88	16.91
Ca	0.71	0.72	0.71	0.73	0.75	0.74
Total P	0.55	0.55	0.56	0.49	0.49	0.53
Digestible P	0.30	0.30	0.30	0.30	0.30	0.30
SID lysine	1.26	1.26	1.26	1.26	1.26	1.26
SID Met + Cys	0.61	0.61	0.61	0.61	0.61	0.61
SID threonine	0.70	0.70	0.70	0.71	0.71	0.71
SID tryptophan	0.19	0.19	0.19	0.19	0.19	0.19
SID isoleucine	0.67	0.67	0.67	0.68	0.68	0.68
SID valine	0.79	0.79	0.79	0.79	0.79	0.79

[1]Premix, per kilogram of diet, 8,800 IU of vitamin A, 880 IU of vitamin D, 64 IU of vitamin E, 4 mg of vitamin K (menadione sodium bisulfite), 70 μg of vitamin B12, 14 mg of riboflavin, 60 mg of d-pantothenic acid, 30 mg of niacin, 6 mg of vitamin B6, 200 μg of biotin, 1.2 mg of folic acid, 120 mg of Fe (as iron carbonate), 25 mg of Mn (as manganese oxide), 17 mg of Cu (as copper chloride), 0.3 mg of I (as ethylenediamine dihydroiodide), 0.2 mg of Se (as sodium selenite), and 120 mg of Zn (as zinc oxide). [2]The values of crude protein, Ca and total P were actually measured, the others were calculated. Phy: phytase; MCPC: a multi-carbohydrase and phytase complex.

saline. Then, a sterile microscope slide was used to gently scrape the intestinal mucosa into a sterile frozen storage tube (each segment of the intestine requires a new fragment, and the entire operation is operated on ice), and then stored it in −80°C to facilitate the further determination. At the end of experiment, rectal digesta were collected with sterile swab and tubes directly from the anus before stored at −80°C. Femur samples were collected and weighed, then quickly stored at −20°C for subsequent analysis.

2.3 Growth performance

After fasting for 12 h, pigs in each pen were weighed at the beginning of the study and at 42d using platform scale. Feed intake was measured by recording the added and remained feed in the trough, and the average daily gain (ADG) and feed-to-gain (F:G) ratio based on the feed intake were calculated.

2.4 Measurements in plasma samples

The content of calcium and phosphorus in plasma and the activity of alkaline phosphatase (ALP) were determined using commercial kits (CAT# A059-1-1, Nanjing Jiancheng Bioengineering Institute, Jiangsu, China); vitamin D3 (CAT# 1593), parathyroid hormone (PTH, CAT# 12481), and calcitonin (CT, CAT# 5225) contents were determined by spectrophotometer (Pharmacia, Cambridge, United Kingdom) with the corresponding enzyme-linked

immunosorbent assay kits (Jiangsu Meimian Industrial, Inc., Nanjing, China).

2.5 Diets, feces and femurs analyses

The femurs were analyzed for bone breaking strength at Wuhan Pinjian Testing Technology Co., Ltd. (Wuhan, China). Then, the cortical cross-sectional diameter (internal and external) was measured with a digital caliper, and the geometric characteristics of the femur were determined according to previously reported method (14). Fecal samples were dried in an oven at 65°C, then diets and fecal samples were crushed and passed through a 0.5 mm screen. Femur samples were dried at 105°C for 24 h to determine the dry matter. The ash content was determined by burning samples in a muffle furnace 550°C for 24 h followed the method 942.05, AOAC, 2006. The calcium and phosphorus was analyzed by a spectrophotometer (Pharmacia, Cambridge, United Kingdom) after washing at 600°C followed method 965.17; AOAC, 2006 (P) and method 968.08; AOAC, 2006 (Ca).

2.6 Relative quantitative real-time PCR

The frozen small intestinal mucosa sample (about 0.1 ~ 0.2 g) was crushed into powder (liquid nitrogen was constantly added to keep the temperature low during the grinding process), and then added to a sterile centrifuge tube containing 1 mL RNAiso Plus reagent (Takara, Dalian, China), and centrifuged at 12,000 *g* for 15 min at 4°C (ThermoMicro17R, Thermo Fisher Scientific Inc., Waltham, United States). Then, the total RNA was extracted from mucosa samples collected from the duodenum, jejunum, and ileum. For each sample, a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, United States) was used to verify the concentration and quality of total RNA at 260 nm and 280 nm. The optical density ratio (260 nm/280 nm) was between 1.8 and 2.0. The integrity of RNA was checked by formaldehyde agarose gel electrophoresis. Reverse transcription of each sample was performed using the Prime Script RNART kit (Dakara Biotechnology Company, Dalian, China).

QuantStudio 5 real-time PCR detection system and SYBR reagent (Takara, Dalian, China) were used to quantitatively detect SLC34A1(Na^+-Pi cotransporter 1); SLC34A2 (Na^+-Pi cotransporter 2); SLC34A3(Na^+ -Pi cotransporter 3); TRPV5 (transient receptor potential vanilloid 5); TRPV6 (transient receptor potential vanilloid 6); CALB1 (calbindin); PMCA1b (plasma membrane Ca^{2+} adenosintriphosphatase); VDR (vitamin D receptor); and FGF23 (fibroblast growth factor 23) mRNA expression levels using GAPDH as a housekeeping. These specific primers were synthesized commercially and purchased from Sanguang Biotechnology Co., Ltd. (Shanghai, China) and were listed in Supplementary Table S1. The 10 μL qRT-PCR system consists of 5 μL SYBR (Dalian Takara), 0.5 μL forward primer, 0.5 μL reverse primer, 3 μL ribozyme H_2O, and 1 μL cDNA template. The reaction was carried out at 95°c for the 30s, denatured at 95°c for 40 times for 5 s, annealing for 30s, and finally extended at 72°c for 5 min. Through melting curve analysis, the correctness of PCR amplification was confirmed. Use the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression rate of the target gene relative to the reference gene (15).

2.7 Fecal 16S rRNA

The fecal microbial flora structure was analyzed by 16S rRNA amplicon sequencing, and the V3–V4 region of the bacterial 16S rRNA hypervariable region was sequenced and analyzed. First, fecal microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, GmbH Hilden, Germany) according to the provided manual, and 1% agarose gel electrophoresis was used to detect the quality of DNA, and 2% agarose gel electrophoresis was used to detect the size of PCR product bands. All this process was completed by Beijing Nuohe Zhiyuan Biological Company (Beijing, China). Briefly, the raw data were preprocessed to eliminate adapter pollution and low quality to obtain clean reads. The paired-end clean reads with overlaps were merged to tags by Connecting Overlapped Pair-End (COPE) software. Bacterial tags were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity by scripts of UCHIME software. Sequence analysis was performed by Uparse software (Uparse v7.0. 1,001). Sequences with ≥97% similarity were assigned to the same OTUs. Then, the Silva Database was used based on the Mothur algorithm to annotate taxonomic information for each representative sequence. Subsequent analyses of alpha diversity and beta diversity were performed based on this output normalized data. PCoA (principal coordinates analysis) analysis was performed using the WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). A heatmap was visualized using R software, and log 10 transformation was applied to the bacterial relative abundance data matrix (16).

2.8 Statistical analysis

The PROC MIXED procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, United States) was used to analyze all data except the growth performance in the trial. The model included the fixed effects of diet type (corn-soybean meal diet and wheat-soybean meal diet), enzyme effect (without enzyme, Phy addition, and MCPC addition), and their interaction, and the random effect of block. For the data of the growth performance, the above model additionally included the initial body weight as covariant. The pen was used as the experimental unit. Statistical significance was declared at $p < 0.05$, and $0.05 \leq p < 0.10$ was considered as statistical trend.

3 Results

3.1 Growth performance

The effect of diet type and enzyme supplementation on growth performance of growing pigs is shown in Table 2. No significant interaction was observed between diet type and enzyme supplementation on all performance parameters. No significant difference between pigs fed corn- or wheat-soybean meal-based diet on growth performance. Compared with pigs fed the diets without enzyme supplementation, phytase addition tended ($p = 0.08$) to improve final BW (+4.8%) and ADG (+8.2%), while MCPC addition improved ($p = 0.02$) final BW and ADG by 6.3 and 10.6%, respectively.

TABLE 2 Effects of diet type and enzyme supplementation on growth performance of growing pigs.

Item	Diet type (DT)		Enzyme (Enz)			SEM[1]	*p*-value		
	Corn-SBM	Wheat-SBM	Without	Phy	MCPC		BD	Enz	BD × Enz
Initial BW[2], kg	24.70	24.71	24.70	24.70	24.71	0.09	0.95	0.99	0.99
Final BW, kg	62.97	62.86	60.66^{b}	63.58^{ab}	64.50^{a}	1.23	0.85	0.02	0.23
ADG[3], kg/pig/d	0.91	0.91	0.86^{b}	0.93^{ab}	0.95^{a}	0.03	0.84	0.02	0.23
ADFI[4], kg/pig/d	1.92	1.88	1.82	1.91	1.96	0.06	0.41	0.12	0.35
F:*G*[5]	2.11	2.07	2.13	2.07	2.07	0.04	0.23	0.24	0.82

Means ($n = 6$ replicate pen per treatment) with different superscripts (a, b) in the same row differ ($P < 0.05$). [1]SEM, standard error of the mean. [2]BW, body weight. [3]ADG, average daily body weight gain. [4]ADFI, average daily feed intake. [5]*F*:*G*, the ratio of average daily feed intake: average daily gain. Phy, phytase; MCPC, a multi-carbohydrase and phytase complex.

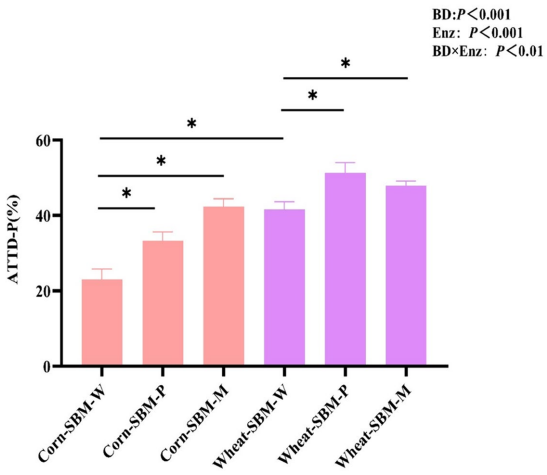

FIGURE 1
Effects of diet type and enzyme supplementation on the apparent total tract digestibility of phosphorus (ATTD of P) in growing pigs. Values are the means ($n = 6$ replicates per treatment). ATTD-P, apparent total tract digestibility of *P*. Statistical significance was assessed at *p < 0.05.

3.2 Digestibility of phosphorus

As shown in Figure 1, a significant interaction ($p = 0.01$) was observed between diet type and enzyme supplementation on ATTD of P. Compared to pigs fed the corn-soybean meal diet without enzymes, the ATTD of P was improved ($p < 0.05$) by 10.3 and 19.5% points with Phy and MCPC, respectively. In comparison to pigs fed the wheat-soybean meal diet without enzymes, the addition of phytase and MCPC improved the ATTD of P by 9.8 and 6.4% points, respectively.

3.3 Intestinal gene expression

The effects of diet type and enzyme supplementation on the relative expression of genes related to calcium and phosphorus absorption in the duodenum, jejunum and ileum of growing pigs are presented in Table 3. Significant interactions ($p < 0.05$) were observed between diet type and enzyme supplementation on the expression of *TRPV6* and *VDR* in the ileum and *FGF23* in the duodenum. Compared with the corn-soybean meal-based diet, the relative expression of *SLC34A2* and *VDR* genes in the ileum and *SLC34A3* in jejunum of growing pigs fed the wheat-soybean meal-based diet was lower (Table 3, $p < 0.05$). The addition of MCPC in the diet significantly reduced ($p < 0.05$) the relative expression of *TRPV5* and *CALB1* genes in the ileum and increased the expression of *CALB1* in the duodenum compared to control diet. The addition of phytase in the diet increased ($p < 0.05$) the relative expression of *SLC34A1* gene in the duodenum in comparison to control diet and MCPC-supplemented diet.

3.4 Plasma indexes

There was a significant interaction ($p < 0.05$) between diet type and enzyme supplementation on plasma P content and ALP activity (Table 4). Pigs fed wheat-soybean meal-based diet without enzyme supplementation or supplemented with MCPC exhibited lower ($p < 0.05$) plasma P content compared with pigs fed corn without enzyme supplementation. Additionally, plasma ALP activity was lower ($p < 0.05$) in pigs fed corn-soybean meal diet without enzyme supplementation or wheat-soybean meal-diet supplemented with phytase compared with pigs fed corn- or wheat-supplemented with MCPC. Compared with pigs fed corn-soybean meal-based diet, pigs fed wheat-soybean meal-based diet presented lower ($p < 0.05$) plasma Ca content and higher ($p < 0.05$) PTH and CT. Compared with pigs fed diets without enzyme addition, plasma Ca content decreased ($p = 0.01$) by MCPC supplementation, while it was not influenced by phytase supplementation.

3.5 Femur mineralization

There were significant interactions ($p < 0.05$) between diet type and enzyme supplementation on femur weight, femur P and Ca contents (Table 5). Compared with pigs fed the corn-soybean meal-based diet, the bone strength, the contents of Ca and P in the femurs were significantly reduced ($p < 0.05$) in growing pigs fed the wheat-soybean meal-based diet. The addition of MCPC to the diet significantly increased ($p < 0.05$) the length of the femur. Supplementation of both enzymes to the diet significantly increased

TABLE 3 Effects of diet type and enzyme supplementation on gene expressions of intestinal calcium and phosphorus absorption in growing pigs.

Item[1]	Gut site	Diet type (DT)		Enzyme (Enz)			SEM[2]	*P*-value		
		Corn	Wheat	Without	Phy	MCPC		BD	Enz	BD × Enz
SLC34A1	Duodenum	2.86	3.97	2.97^{ab}	5.66^{a}	1.62^{b}	1.11	0.23	0.01	0.72
	Jejunum	1.85	1.55	1.58	2.28	1.23	4.58	0.51	0.18	0.80
	Ileum	1.17	2.04	1.59	2.38	0.85	16.53	0.14	0.13	0.11
SLC34A2	Duodenum	2.03	1.87	2.10	2.23	1.51	0.84	0.86	0.65	0.50
	Jejunum	1.25	1.38	1.19	1.03	1.72	2.13	0.87	0.41	0.07
	Ileum	0.75^{A}	0.38^{B}	0.72	0.57	0.39	0.46	0.002	0.08	0.38
SLC34A3	Duodenum	1.02	2.89	1.92	1.31	2.63	1.18	0.06	0.57	0.68
	Jejunum	0.98^{A}	0.68^{B}	0.87^{ab}	0.55^{b}	1.07^{a}	0.30	0.05	0.04	0.56
	Ileum	0.96	0.77	0.89	0.94	0.77	0.68	0.43	0.80	0.40
TRPV5	Duodenum	1.65	1.60	1.76	2.10	1.02	0.66	0.91	0.30	0.38
	Jejunum	1.55	1.57	1.25	2.10	1.32	1.23	0.95	0.41	0.86
	Ileum	0.97	0.98	1.58^{a}	0.86^{ab}	0.47^{b}	0.41	0.99	0.02	0.62
TRPV6	Duodenum	1.29	1.65	1.57	1.77	1.07	0.37	0.26	0.19	0.33
	Jejunum	2.15	1.83	1.76	2.30	1.91	1.43	0.55	0.77	0.22
	Ileum	0.90	3.76	5.21^{a}	1.12^{b}	0.67^{b}	0.79	<0.001	<0.001	<0.001
CALB1	Duodenum	1.09	1.00	1.06	0.81	1.26	0.21	0.57	0.13	0.27
	Jejunum	1.29	1.53	1.59	1.73	0.92	0.65	0.54	0.32	0.25
	Ileum	1.02	0.91	1.41^{a}	1.03^{a}	0.45^{b}	0.32	0.55	<0.001	0.06
PMCA1b	Duodenum	0.96	1.08	0.99^{ab}	0.78^{b}	1.28^{a}	0.15	0.34	0.01	0.14
	Jejunum	0.96	1.13	1.14	0.96	1.03	0.25	0.33	0.68	0.75
	Ileum	1.19	1.11	1.12	1.37	0.95	0.23	0.65	0.16	0.42
VDR	Duodenum	1.07	0.97	1.05	0.96	1.04	0.13	0.39	0.84	0.26
	Jejunum	1.09	0.86	1.10	1.00	0.82	0.33	0.21	0.42	0.90
	Ileum	1.26	0.85	1.19	1.21	0.77	0.20	0.02	0.06	0.05
FGF23	Duodenum	2.12	1.91	1.11^{b}	2.91^{a}	2.03^{ab}	0.64	0.61	0.03	0.01
	Jejunum	1.58	1.49	1.40	2.08	1.12	2.21	0.84	0.26	0.90
	Ileum	1.61	1.45	2.07	1.62	0.89	14.21	0.93	0.58	0.19

Means ($n = 6$ replicates per treatment) with different superscripts (a, b) in the same row differ ($P < 0.05$). [1]SLC34A1, Na^+-Pi cotransporter 1, SLC34A2, Na^+-Pi cotransporter 2, SLC34A3, Na + -Pi cotransporter 3, TRPV5, transient receptor potential vanilloid 5, TRPV6, transient receptor potential vanilloid 6, CALB1, calbindin, PMCA1b, plasma membrane Ca^{2+} adenosintriphosphatase, VDR, vitamin D receptor, FGF23, fibroblast growth factor 23. [2]SEM, standard error of the mean. Phy, phytase; MCPC, a multi-carbohydrase and phytase complex.

TABLE 4 Effects of diet type and enzyme supplementation on plasma indexes in growing pigs.

Item[1]	Diet type (DT)		Enzyme (Enz)			SEM[2]	*P*-value		
	Corn-SBM	Wheat-SBM	Without	Phy	MCPC		BD	Enz	BD × Enz
Ca, μmol/mL	0.72^{A}	0.56^{B}	0.75^{a}	0.63^{ab}	0.53^{b}	0.07	0.01	0.02	0.51
P, mmol/L	3.12	2.68	2.82	3.04	2.84	0.12	<0.001	0.15	0.05
ALP, U/L	19.00	19.09	15.38	16.33	25.42	2.39	0.79	<0.001	0.04
PTH, ng/L	22.45^{B}	24.42^{A}	22.59	23.73	23.99	0.79	0.006	0.20	0.70
$1,25(OH)_2D$, ng/L	29.83	31.07	31.28	30.11	29.97	1.14	0.24	0.55	0.09
CT, ng/L	20.24^{B}	22.66^{A}	21.54	21.26	21.54	0.79	0.001	0.92	0.94

Means ($n = 6$ replicates per treatment) with different superscripts (A, B or a, b) in the same row differ ($P < 0.05$). [1]ALP, alkaline phosphatase; PTH, parathyroid hormone; CT, calcitonin. [2]SEM, standard error of variance. Phy, phytase; MCPC, a multi-carbohydrase and phytase complex.

TABLE 5 Effects of diet type and enzyme supplementation on femur parameters in growing pigs.

Item[1]	Diet type (DT)		Enzyme (Enz)			SEM[2]	*P*-value		
	Corn	Wheat	Without	Phy	MCPC		BD	Enz	BD × Enz
Weight, g	207.32	204.71	200.38	205.51	212.14	5.88	0.60	0.15	0.03
Length, cm	16.80	16.66	16.43^{b}	16.68^{ab}	17.07^{a}	0.20	0.40	0.01	0.10
Bone strength,	3620.49^{A}	3019.28^{B}	2762.50^{b}	3742.07^{a}	3455.08^{a}	328.46	0.04	0.02	0.32
CSA, mm^2	232.71	221.65	214.06	234.50	233.00	14.82	0.38	0.33	0.29
MRWT, mm	1.20	1.13	1.16	1.20	1.15	0.08	0.28	0.83	0.16
CI, %	77.35	67.53	66.95	74.77	75.60	6.56	0.09	0.38	0.36
P, % of ash	9.25	8.92	9.20	9.59	8.47	0.14	0.01	<0.001	0.001
Ca, % of ash	18.94	18.16	18.23	19.16	18.26	0.27	0.001	0.003	<0.001

Means ($n = 6$ replicates per treatment) with different superscripts (A, B or a, b) in the same row differ ($P < 0.05$). [1]CSA, cross-sectional area, MRWT, mean relative wall thickness, CI, cortical index. [2]SEM, standard error of variance. Phy, phytase; MCPC, multi-carbohydrase and phytase complex.

($p < 0.05$) bone strength in the femur. Supplementation of phytase in the diet significantly increased ($p < 0.05$) calcium and phosphorus content in the femur (Table 5). Femur weight was higher in the wheat-soybean meal diet supplemented with MCPC than those in the other treatments.

3.6 Fecal microbiota

Figure 2 shows the fecal microorganisms (top 10 phyla levels) in growing pigs fed different types of diets with or without enzyme supplementation, we can find Firmicutes and Bacteroidota predominant. After adding enzyme in corn-soybean meal group, the abundance of Bacteroidota in feces increased and the abundance of Firmicutes decreased, but this phenomenon was not observed after adding enzyme in wheat-soybean meal group. Table 6 shows the fecal microbial alpha diversity of growing pigs fed different types of diets (with or without enzyme preparations). Compared with the corn-soybean meal-based diet, the Shannon and Simpson indices of fecal microorganisms of growing pigs fed the wheat-soybean meal-based diet was increased ($p < 0.05$), and the diversity of fecal microorganisms was significantly increased ($p < 0.05$). Also adding both enzymes to the diet significantly increased the fecal microbial Shannon and Simpson indices. Fecal microbial Shannon and Simpson indices were higher in the MCPC-fed group than in the other treatments.

As shown in Figure 3 the fecal microbes are located on the coordinates of each treatment to form a clear regional separation, which indicates that there are differences in the composition of fecal microbes among the treatments. Among them, changing the diet type made each coordinate point farther away, which indicated that the diet type treatment had a greater impact on the composition of fecal microbial structure. Further prediction of fecal microbial functions can reveal that metabolism, through KEGG processing analysis, found that these functions are Two-component system, Starch and sucrose, Biosynthesis of secondary metabolites and Bacterial chemotaxis. As shown in Figure 4, the proportion of fecal microorganisms performing the above functions was more in the corn soybean meal without enzyme group.

4 Discussion

It has been widely reported that exogenous phytase and xylanase added alone or in combination in growing pigs' diets could improve the growth performance (17, 18). In this experiment, phytase supplementation tended to increase ADG and final BW, while the addition of MCPC, enriched in xylanase, β-glucanase, and phytase significantly increased final BW and ADG compared with pigs fed the control diet. This result is similar with previous studies that observed that supplementation of multi-carbohydrase and phytase in combination improved the growth performance in pigs probably by targeting the two main antinutritional factors namely the NSP and phytate (17, 18). Previous studies reported that phytase and NSP-degrading enzymes could hydrolyze phytate and NSP which lead to increase the availability of nutrients for pigs and consequently the growth performance (17, 18).

In the present study, the addition of phytase or MCPC increased the ATTD of P, resulting in an increase in the availability of digestible P in the diets which can improve the growth performance of growing pigs. This result is in agree with the previous study (19). Moreover, calcium and phosphorus are actively absorbed in the porcine small intestine mainly through transporter-mediated transcellular pathways. In the present study, the expression levels and signaling of calcium-phosphorus carrier proteins in the gut may represent the actual retention of calcium and phosphorus in the gut. Coupled with the mediation of vitamin D signaling, it can be used for further transport and absorption of calcium and phosphorus in the gut. The SLC34 family of sodium-driven phosphate cotransporters is comprised of three members: NaPi-IIa (SLC34A1), NaPi-IIb (SLC34A2), and NaPi-IIc (SLC34A3) (20). From the gene expression levels in this experiment, the main active carrier proteins mediating calcium and phosphorus transport were highly expressed in the duodenum and jejunum. Compared with the corn-soybean meal-based diet, feeding the wheat-soybean meal-based diet could significantly reduce the expression level of *SLC34A2* gene in the ileum, but feeding the wheat-soybean meal-based diet significantly increased ATTD of P in growing pigs. While reducing the dietary digestible phosphorus level, previous study found that the gene expression of Na+-PiIIb transporter was significantly decreased, but the protein expression level of this protein was significantly increased (21). The Na+-PiIIb transporter is a

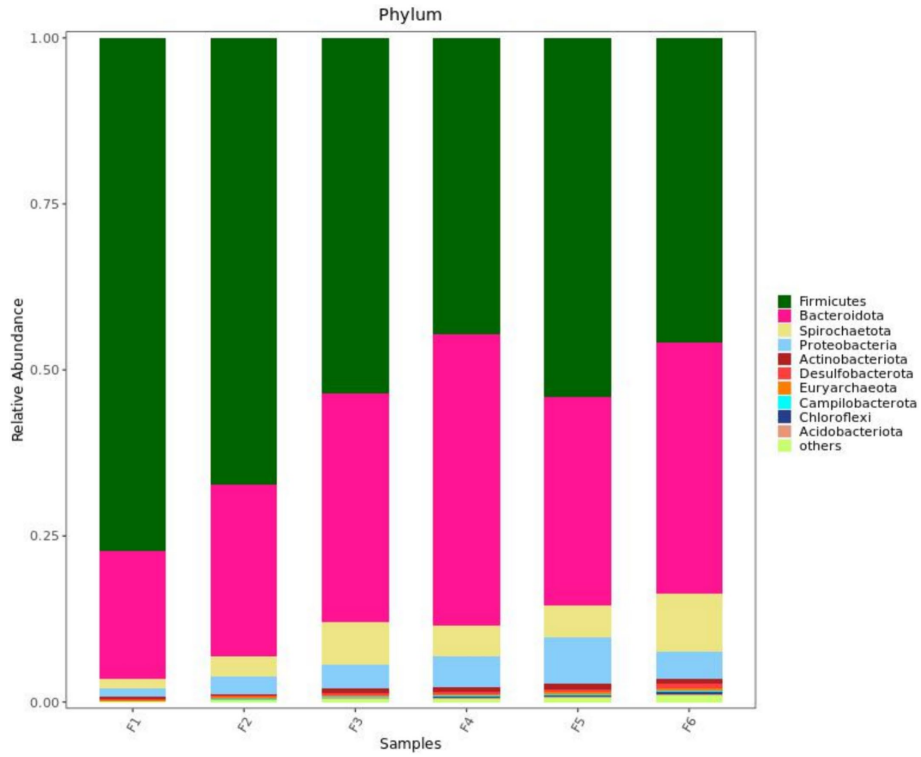

FIGURE 2
Effects of diet type and enzyme supplementation on the relative abundance of fecal microbial phyla (top 10). F1 = fecal microorganisms of corn-soybean meal group, F2 = fecal microorganisms of corn-soybean meal + phytase group, F3 = fecal microorganisms in corn-soybean meal + MCPC group, F4 = fecal microorganisms of wheat-soybean meal group, F5 = fecal microorganisms of wheat-soybean meal + phytase group, F6 = Fecal microorganisms of wheat- soybean meal + MCPC group.

TABLE 6 **Effects of diet type and enzyme supplementation on fecal microbial alpha diversity in growing pigs.**

Item	Diet type (DT)		Enzyme (Enz)			SEM[1]	*P*-value		
	Corn	Wheat	Without	Phy	MCPC		BD	Enz	BD × Enz
Shannon	7.85	8.72	7.87	8.25	8.74	0.121	<0.001	<0.001	<0.001
Simpson	0.97	0.99	0.97	0.99	0.99	0.004	<0.001	<0.001	<0.001

member of the SLC family (22). This suggests that more *SLC34A3* mRNA translated to functional protein, and improve the digestibility of phosphorus (22). The addition of MCPC to the diet significantly increased the expression level of the *SLC34A3* gene in the jejunum, which was consistent with the changes in our ATTD of P. During calcium-saturated transcellular processes, calcium is taken up by enterocytes through calcium channels such as *TRPV6* located in the brush border membrane. In this experiment, the relative expression of *TRPV6* gene in the ileum of growing pigs was significantly increased by feeding the wheat-soybean meal-based diet, indicating that calcium absorption was enhanced in this treatment group. The relative expression of the ileal *TRPV6* gene was significantly reduced by the addition of both enzymes in the diet.

Compared with the corn-soybean meal-based diet, feeding the wheat-soybean meal-based diet significantly reduced plasma calcium and phosphorus levels in growing pigs. While increasing the intestinal absorption of calcium, there will be also a feedback regulation system to maintain the level of calcium in the plasma (22). The CT content in the plasma of the wheat-soybean meal group was significantly increased, which could also maintain the calcium concentration in the plasma at a certain level. Secondly, the digestion and absorption of phosphorus is also affected by many factors. After changing the type of diet, PTH in the plasma of the wheat-soybean meal group was significantly increased, and PTH could reduce the number of NaPi-IIA and NaPi-IIC proteins on the brush border membrane (BBM) (23, 24), thereby limiting the absorption of part of P, It was finally reflected in the decrease of Ca and P contents in the plasma of the wheat-soybean meal group.

In this study, diet type and enzymes (with and without supplementation) had no significant effect on femur length, cross-sectional area (CSA), cortical index (CI) and mean relative wall thickness (MRWT) general characteristic parameters of growing pigs. Since bone strength is provided by an inorganic component consisting of hydroxyapatite and insoluble salts containing

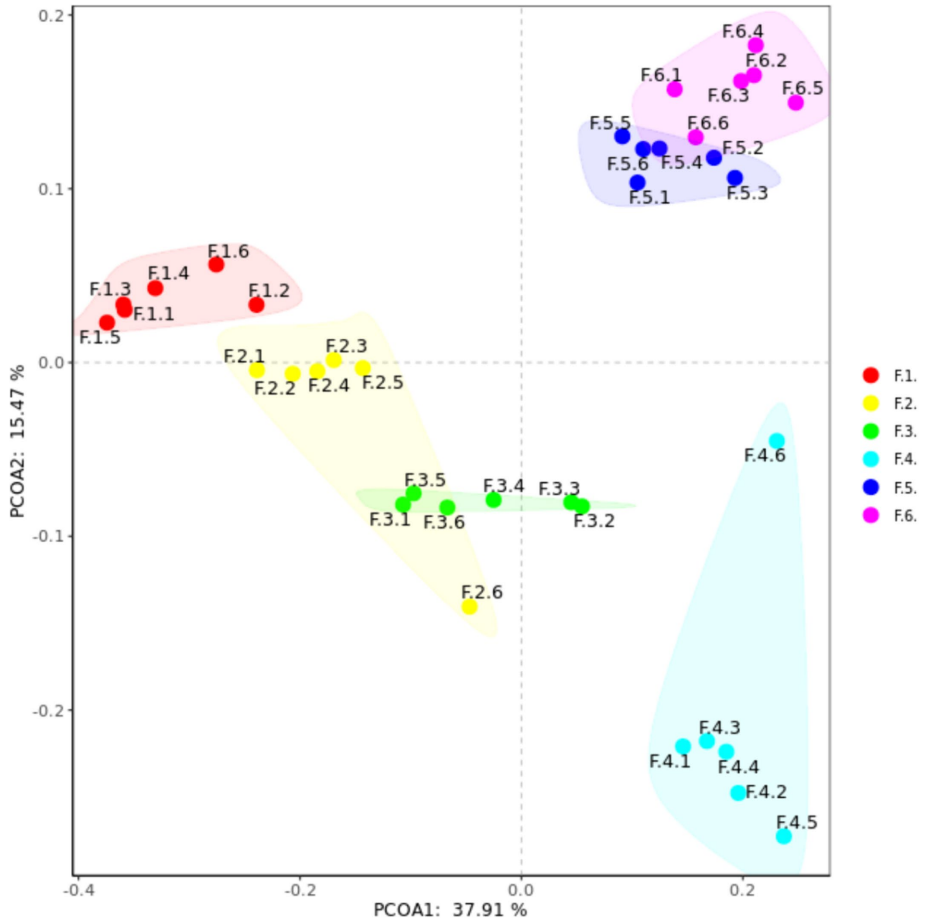

FIGURE 3
Principal coordinate analysis (PCoA) composition of fecal microorganisms in each group. F1=fecal microorganisms of corn-soybean meal group, F2=fecal microorganisms of corn-soybean meal + phytase group, F3=fecal microorganisms in corn-soybean meal + MCPC group, F4=fecal microorganisms of wheat-soybean meal group. F5=fecal microorganisms of wheat-soybean meal + phytase group, F6=fecal microorganisms in wheat-soybean meal + MCPC group.

calcium and phosphorus, the content of phosphorus and calcium in the femur increased after dietary supplementation of phytase, with a corresponding increase in bone strength. In the previous studies (25), it was also found that adding phytase significantly increased the content of calcium and phosphorus in the tibia of piglets after the 95-day experiment in piglets. Similarly, after the 185-day experiment in fattening pigs, adding phytase significantly increased fattening. Calcium and phosphorus content were increased in pig metacarpal bones, indicating that the addition of phytase in the diet significantly increased the deposition of calcium and phosphorus in the femur, improved the bone strength, and was beneficial to the bone development of growing pigs. Also, the interaction between diet types and enzymes on P content and femur weight were observed in this study, showing a better effect to add enzymes in corn-soybean meal diet. The addition of phytase in the diet based on barley - wheat - soybean meal also significantly increased the content of calcium and phosphorus in the bone (25). There was also a significant interaction between diet type and enzyme in Ca and P contents in the bones, showing more Ca and P in the bone of pigs fed corn-soybean meal diet or diets with enzyme supplementations.

In this experiment, it is not difficult to see from the results and analysis of 16S sequencing that changing the type of diet and adding phytase or MCPC can significantly affect the alpha diversity of fecal microorganisms, such as the Shannon and Simpson indices are significantly increased. Secondly, we can also see from the Beta analysis that changing the type of diet and adding phytase or MCPC can produce significant differences in the structural composition of fecal microorganisms, but changing the type of diet has a greater impact on the difference in the structural composition of pig feces in terms of microbiota. This may be because wheat contains a large amount of NSP, and NSP is the main component of dietary fiber. They are not digested and absorbed in the small intestine, but are fermented by the resident microbiota in the gut (26). Most gut bacteria preferentially ferment carbohydrates, which also include NSP, implying that diets containing large amounts of complex structural NSP may nourish a greater number and variety of microbes (27).

Taken together, the results of the present study demonstrated that diet type and exogenous enzymes supplementation could differently influence the growth performance, utilization of P, intestinal transporter gene expression, bone mineralization and microbial diversity and composition in growing pigs.

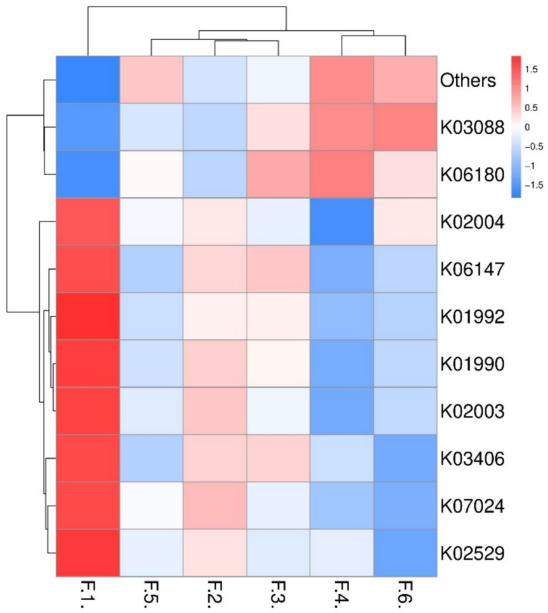

FIGURE 4
Prediction of fecal microbial function (top 10). F1 = fecal microorganisms of corn-soybean meal group, F2 = fecal microorganisms of corn-soybean meal + phytase group, F3 = fecal microorganisms of corn-soybean meal + MCPC group, F4 = fecal microorganisms of wheat- soybean meal group. F5 = fecal microorganisms of wheat-soybean meal + phytase group, F6 = fecal microorganisms in wheat-soybean meal + MCPC group.

Data availability statement

The datasets for this study can be available on request to the corresponding author. The raw sequencing data are available from NCBI repository: https://www.ncbi.nlm.nih.gov/, under accession number PRJNA1123310.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Sichuan Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YY: Data curation, Investigation, Visualization, Writing – original draft. MJ: Conceptualization, Data curation, Software, Validation, Visualization, Writing – review & editing. BY: Data curation, Investigation, Methodology, Writing – review & editing. YL: Data curation, Software, Writing – review & editing. JH: Investigation, Writing – review & editing. PZ: Investigation, Writing – review & editing. XM: Investigation, Writing – review & editing. HY: Writing – review & editing. AW: Investigation, Writing – review & editing. SB: Data curation, Software, Writing – original draft. ED: Project administration, Validation, Writing – original draft. JY: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

MJ and ED were employed by Adisseo France S.A.S.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1413920/full#supplementary-material

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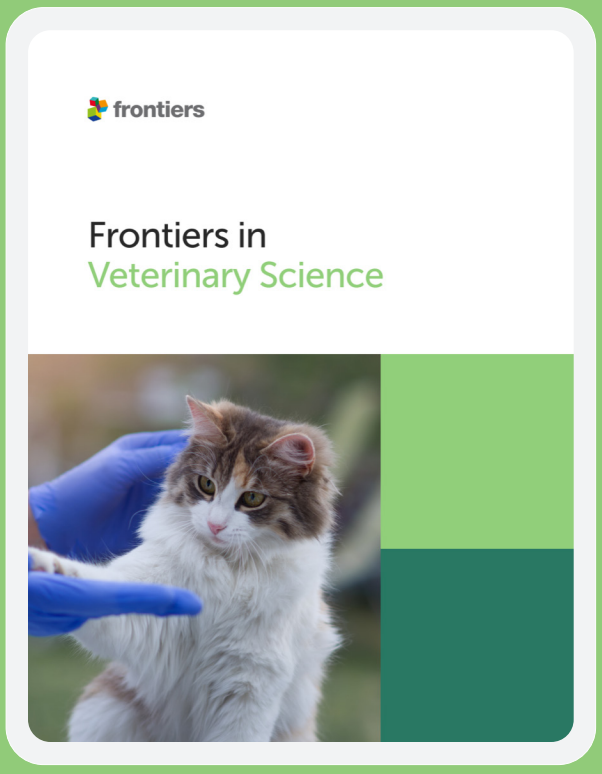

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