Effects of phytosterol supplementation on growth performance, serum lipid, proinflammatory cytokines, intestinal morphology, and meat quality of white feather broilers

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ABSTRACT The aim of this study was to evaluate the effects of dietary phytosterol (**PS**) addition at different levels on growth performance, serum lipid, proinflammatory cytokines, intestinal morphology, and meat quality in broilers. A total of 600, 1-day-old male broilers were allocated into five groups with six replicates and were fed a basal diet supplemented with 0 (control group), 10, 20, 40, or 80 mg/kg PS for 42 days. Compared with the control group, the administration of PS at doses of 40 and 80 mg/kg significantly increased the average daily feed intake and average daily gain of broilers during the experimental period. Similarly, PS at a dosage of 20 and 40 mg/kg increased the concentrations of interleukin-1β, interferon-γ, interleukin-2, and interleukin-6 but decreased triglyceride, total cholesterol, and low-density lipoprotein cholesterol content of serum ($P < 0.05$). Dietary PS at less than or equal to 40 mg/kg level increased ($P < 0.05$) villus height, and villus height to crypt depth ratio in the duodenum and ileum. Supplementing PS increased the pH value at 45 min post-mortem and decreased drip loss and shear force of breast muscle ($P < 0.05$). Dietary PS administration at 20 and 40 mg/kg decreased malondialdehyde accumulation but increased total antioxidant capacity and superoxide dismutase activity of breast muscle compared with the control group ($P < 0.05$). PS increased the concentrations of total amino acids and flavor amino acids as well as eicosapentaenoic acid, docosahexaenoic acid, and total polyunsaturated fatty acids but decreased saturated fatty acids in breast muscle ($P < 0.05$). It was concluded that dietary PS supplementation, especially at 40 mg/kg, could improve growth performance, serum lipid, proinflammatory cytokines, intestinal morphology, and meat quality in broilers, providing insights into its application as a potential feed additive in broiler production.

Key words: phytosterol, proinflammatory cytokine, intestinal morphology, meat quality, broiler

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INTRODUCTION

Phytosterols (**PS**) are a class of steroid compounds with plant active ingredients. PS usually exist in plant foods such as nuts, grains, beans, and plant seeds in free form and esterified form, and are an important component of plant cells. The most common PS in nature are β-sitosterol, stigmasterol, campesterol, and sitostanol, of which β-sitosterol is the main component, accounting for 60%−90% of PS (Moreau et al., 2002). From the 1950s to the present, many clinical studies and animal experiments have shown that PS and their products have good prevention and treatment effects on hypercholesterolemia and cardiovascular diseases, and exhibit extremely high safety (Pollak, 1953; Normén et al., 2000; Jones, 2007). PS can inhibit the absorption of cholesterol in the intestine or affect the cholesterol metabolism of the liver and gallbladder, can reduce cholesterol in the blood and regulate growth, and have anti-oxidative, anti-inflammatory, and antipyretic effects (Brüll et al., 2009; Choudhary and Tran, 2011). Shi et al. (2014) observed that long-term use of high-dose PS (up to 800 mg/kg) did not induce any toxicological effects.

In recent years, PS have been considered an effective feed additive because of their ability to enhance animal performance and improve the quality of livestock and poultry products. Many previous studies have shown that PS can increase the antioxidant capacity, metabolism, intestinal morphology and gut microflora of animals. Zhao et al. (2019b) reported that supplementation with PS improved meat quality, growth performance, and antioxidant status of Partridge Shank chickens. The

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optimum level of PS for Partridge chicken was recommended to be 40 mg/kg. Feng et al. (2020) also observed that PS could enhance gut microflora but did not affect the growth performance of yellow-feather broilers. Moreover, a main component of PS (β-sitosterol) has been demonstrated to not only inhibit the production of tumor necrosis factor α, interleukin-1β, and interleukin-6 in RAW 264.7 mouse macrophages under immune stress but can also efficiently ameliorate the depletion of glutathione and reduce antioxidant enzymes, including superoxide dismutase and glutathione peroxidase, in the colonic and hepatic tissues of rats induced by 1,2-dimethylhydrazine (Baskar et al., 2012; Choi et al., 2012). In addition, the integrity of intestinal mucosa morphology is the basis of maintaining normal intestinal physiological activity in animals, improvement on gut morphology of weaned pigs fed PS has been shown by Hu et al. (2017).

Nevertheless, it remains to be determined whether dietary PS supplementation can improve meat quality and flavor, and the amino acid and fatty acid composition of the broiler meat. Therefore, in this study, we hypothesized that dietary supplementation with PS would improve the quality and flavor of the broiler by increasing the antioxidant capacity, amino acid, and fatty acid profile of muscles. We aimed to evaluate the effects of PS supplementation on broiler growth performance, serum lipid metabolism, proinflammatory cytokines, intestinal morphology, and meat quality in broilers.

MATERIALS AND METHODS

The present study was approved by the Animal Care and Welfare Committee and the Scientific Ethical Committee of the Zhejiang University (No. ZJU2013105002).

Experimental Design, Animals, and Diets

A total of six hundred, 1-day-old male, white feather broilers of similar body weight and healthy age were assigned to a completely randomized design with five treatments for a 42 d feeding trial after individual weighing. Each treatment consisted of six replicates (cages) with 20 birds each. During the starter phase, the 20 broilers in each replicate were reared in a single cage (120 cm × 60 cm × 50 cm), whereas during the grower phases, each replicate was housed in 2 cages of this size with 10 birds per cage. Broilers had free access to mashed feed and water throughout the whole period. In a controlled environment with a relative humidity of 45% to 55% and a temperature of 25°C to 34°C, the broilers were maintained on a 18 h light and 6 h dark cycle. In the first week of the experiment, the ambient temperature was maintained at 34°C, then gradually decreased to 25°C after 21 d, and maintained thereafter. Broilers were fed a corn-soybean meal basal diet supplemented with 0 (control group), 10, 20, 40, and 80 mg/kg PS (Zhejiang Delekang Food Co., Ltd., Zhejiang, China), respectively. The dosage of PS was selected according to previous studies (Bo et al., 2015). The composition and nutrient content of the corn-soybean meal basal diet (starter diet and grower diet) are presented in Table 1. All birds in each replicate (cage) were weighed individually after a 12 h feed deprivation at 21 d and 42 d of age, and the consumption of feed by birds was recorded on a cage (replicate) basis to calculate average daily feed intake (**ADFI**), average daily gain (**ADG**), and feed conversion rate (**FCR**) during the starter (0−21 d), grower (22−42 d), and overall (0−42 d) periods.

Sample Collection

At 21 d and 42 d, two bird of moderate weight was randomly picked from each replicate. Blood samples (5 mL) were collected from the brachial vein into a 10-mL anticoagulant-free Vacutainer tube (Greiner Bio-One GmbH, Kremsmunster, Austria). After standing 37°C for 2 hours, the serum was separated by centrifugation at 3000 rpm for 15 min at 4°C and stored in a refrigerator at -80°C for further analysis. The broilers were euthanized by cervical dislocation and necropsied immediately. The left breast muscle samples were quickly collected into self-sealing bags with corresponding labels and frozen at -20°C for further analysis of meat quality, antioxidant capacity, amino acid, and fatty acid composition. Meanwhile, the pH values, meat color, shear force, and drip loss of meat were measured on the right breast muscle samples that were stored at 4°C. After removing the intestinal contents with pre-cooled saline, an intestinal segment 1 cm from the middle of the duodenum, jejunum, and ileum was collected and fixed in 4% paraformaldehyde solution. Histological slides were prepared from 4 cross-sections (5 μm thick) of each intestinal sample, which were processed in low-melt paraffin and stained with hematoxylin-eosin. The histologic sections were examined with an Axioplan 2 optical microscope (Carl Zeiss Jena GmbH, Oberkochen, Germany) equipped with a refrigerated QImaging Retiga-4000R digital camera (QImaging, Surrey, Canada) coupled with a charge-coupled device detector. The image was analyzed with MShot Image Analysis System software. Villus height was determined as the distance between the tip of the villi and the villus−crypt junction. Crypt depth was measured as the distance of the invagination between 2 adjacent villi (Uni et al., 1999). Villus width was measured at the middle part of the villi. Distance between villi was determined as the distance between the adjacent villi at the base of the villi. The villus height and crypt depth of 10 well-oriented villi were measured per section.

Serum Lipid Metabolites, Antioxidant Parameters, and Enzyme-Linked Immunoassay

The serum lipid metabolites of high-density lipoprotein cholesterol (**HDLC**), low-density lipoprotein cholesterol (**LDLC**), total cholesterol (**TC**), triglyceride

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

Items	Starter (1 to 21 D)	Grower (22 to 42 D)
Ingredients (%)		
Corn	55.00	60.00
Soybean meal	34.00	29.00
Fish meal	4.00	3.00
Soybean oil	3.00	4.00
Lysine	0.20	0.25
Methionine	0.25	0.20
Threonine	0.05	1.30
Dicalcium phosphate	1.50	1.00
Limestone	1.00	0.25
Premix [1]	1.00	1.00
Total	100.00	100.00
Nutrient composition		
ME [2] (Mcal/kg)	3.00	3.10
Crude protein (%)	22.00	20.00
Calcium (%)	1.00	0.90
Available phosphorus (%)	0.67	0.65
Methionine (%)	0.60	0.50
Lysine (%)	1.30	1.25
Methionine + Cystine (%)	0.93	0.80
Threonine (%)	0.86	0.76

[1]Premix provided the following per kilogram of diet: vitamin A, 6000 IU; vitamin D3, 3,000 IU; vitamin E, 30 IU; vitamin B1, 3 mg; vitamin B12, 1 mg; vitamin B2, 6 mg; vitamin K, 2 mg; vitamin B6, 2.5 mg; choline chloride, 1 mg; biotin, 0.2 mg; folic acid, 1 mg; niacin, 30 mg; pantothenic acid, 15 mg; lysine, 2 mg; methionine, 0.5 mg; threonine, 0.8 mg; Nacl, 2.5 mg; Fe, 60 mg; Zn, 70 mg; Mn, 80 mg; Cu, 6mg; I, 1.1 mg; Se, 0.3 mg.
[2]ME based on calculated values; others were analyzed values.

(**TG**), and concentrations of malondialdehyde (**MDA**) as well as the activities of superoxide dismutase (**SOD**), glutathione peroxidase, total antioxidant capacity (**T-AOC**), and catalase (**CAT**) in breast muscle, were measured using the corresponding commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following the manufacturer's instructions. Serum levels of interleukin-1β (IL-1β), interleukin-2 (**IL-2**), interleukin-6 (**IL-6**), tumor necrosis factor α (**TNF-α**), and interferon-γ (**IFN-γ**) were determined using enzyme-linked immunoassay (**ELISA**) analysis (double antibody sandwich method) as previously described (Zhang et al., 2020). Briefly, 50 μl of standard solutions or serum samples were added to 96-well plates (coated with purified chicken IL-1β, IL-2, IL-6, TNF-α and IFN-γ antibody). Then, the horseradish peroxidase −labeled second antibody was added to the wells, and the plates were incubated for 60 min at 37°C. The wells were washed 5 times, and chromogen solutions were added and preserved in dark for 15 min at 37°C. Finally, the absorbance was measured at 450 nm using a multifunctional microplate reader (Tecan Infinite M200 PRO; Switzerland) after addition of the stop solution.

Meat Quality

According to the manufacturer's recommendations, the brightness (**L***), redness (**a***), and yellowness (**b***) of three fixed positions post-mortem were measured after 45 min and 24 h using a CR-400 color difference meter (Beijing Kemei Runda Instrument Co., Ltd. Beijing, China), and averaged. The pH value was recorded at 45 min and 24 h postmortem, using a HI199163 portable pH meter (Hanna Instrument Co., Ltd., Beijing, China). The pH meter was first calibrated at chilling temperature using pH 4.00 and pH 7.00 buffers. Then, the pH was measured at upper, middle, and lower points in the left breast muscle, at a depth of 10 mm into the muscle. The 3 measurements within each carcass were averaged for statistical analyses. The shear force was determined using the Warner−Bratzler shear method as previously described (Jiang et al., 2011). The drip loss of the pectoralis major muscle was determined as previously described (Yang et al., 2016). Two grams of muscle samples were collected (W1), then hung in a plastic bag, sealed, stored at 4°C for 24 h, and then weighed again (W2). Drip loss was calculated as:

$$\text{Drip Loss}\,(\%) = (\text{W1} - \text{W2})/\text{W1} \times 100\%.$$

Fatty Acid and Amino Acid Analysis

The fatty acid composition of breast muscle was measured using the method of Folch et al. (1957). Approximately 2 g of muscle sample was extracted for total lipid extraction, and fatty acid methyl esters (FAME) were prepared from total lipid extract using methanolic HCl as the derivatizing agent. FAME analysis was performed using an Agilent 7890-5973 gas chromatograph (Agilent Technologies, Inc., Palo Alto, CA) equipped with an autosampler, flame ionization detector, and fused silica capillary column with a film thickness (Sp−2560; Supelco, Bellefonte, PA) of 30 m × 0.25 mm × 0.2 μm. The temperature of the chromatographic column, injector, and detector were set to 220°C, 230°C, and 250°C, respectively. Helium was used at a flow rate of 1 ml/min, and the result was expressed as the percentage distribution of FAME. By comparing the retention time with

Table 2. Effects of phytosterol supplementation on growth performance of white feather broilers.

Items [1]	PS [2] (mg/kg) 0	10	20	40	80	SEM [3]	*P*-Value
Initial BW (g)	45.52	45.45	45.84	44.84	45.50	0.185	0.719
Final BW (g)	2511.74[b]	2697.28[a,b]	2727.92[a]	2731.96[a]	2670.50[a,b]	17.830	0.035
Starter (1 to 21 D)							
ADFI (g)	62.40[b]	64.09[a,b]	66.10[a,b]	69.38[a]	70.55[a]	0.825	0.002
ADG (g)	42.39[b]	43.60[a,b]	44.12[a,b]	44.66[a]	45.63[a]	0.447	0.025
FCR (g/g)	1.48	1.47	1.50	1.55	1.55	0.019	0.590
Grower (22 to 42 D)							
ADFI (g)	130.26[b]	137.90[a]	140.17[a]	140.35[a]	140.17[a]	1.237	0.018
ADG (g)	72.32[b]	79.92[a]	80.13[a]	79.40[a]	76.97[a]	1.031	0.043
FCR (g/g)	1.80	1.73	1.75	1.76	1.82	0.017	0.196
Overall (1 to 42 D)							
ADFI (g)	101.47[c]	106.42[b]	108.44[a,b]	110.24[a]	110.22[a]	0.853	0.013
ADG (g)	58.72[b]	63.14[a]	63.86[a]	63.98[a]	62.50[a]	0.539	0.001
FCR (g/g)	1.73	1.69	1.70	1.72	1.76	0.011	0.240

[1]ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion rate.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-c]Means with different letters within a row differ significantly ($P < 0.05$).

the standard (Sigma, code no: 189−19; St Louis, MO, USA), each fatty acid was identified as a methyl ester. The derivatized sample and amino acid standard (Sigma-Aldrich, St. Louis, MO) were injected into an HPLC (Agilent HP 1200 series instrument) equipped with a Nova-PakTM C18 column (4 μm, 3.9 × 4.6 mm) for separation and quantification of free amino acids (nmol/g meat), using the method of Salah et al. (2019).

Statistical Analysis

The data were analyzed using one-way analysis of variance (**ANOVA**) and Duncan's multiple range test for multiple comparisons using SPSS version 20.0 (SPSS Inc., Chicago, IL). Results are presented as means with standard error of the mean (**SEM**). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Growth Performance

Compared with the control group, the supplementation of PS at a dosage of 40 and 80 mg/kg significantly increased the ADFI and ADG of broilers during the starter period (Table 2). Moreover, during the grower and the overall periods, birds that received the PS increased ADFI and ADG ($P < 0.05$) when compared with the control group. There were no differences ($P > 0.05$) in FCR among any of the groups during the experimental period.

Lipid Metabolic Status

Dietary PS supplementation decreased ($P < 0.05$) TG content in the serum (Table 3). Meanwhile, 20 and 40 mg/kg of dietary PS reduced ($P < 0.05$) LDLC and TC content of serum. Compared with the control group, dietary supplementation with PS decreased HDLC, but the differences were not statistically significant ($P > 0.05$).

Serum Cytokine Concentrations

Dietary supplement PS did not influence ($P > 0.05$) the TNF-α, but increased ($P < 0.05$) the IFN-γ level compared to the control group (Table 4). Meanwhile, the concentrations of IL-1β, IL-2, and IL-6 in broilers receiving PS at doses of 20 and 40 mg/kg were higher than those of the control group ($P < 0.05$).

Table 3. Effects of phytosterol supplementation on serum lipid level of white feather broilers.

Items [1] (mmol/L)	PS [2] (mg/kg) 0	10	20	40	80	SEM [3]	*P*-Value
HDLC	5.44	5.16	4.65	5.24	5.36	0.143	0.473
LDLC	1.87[a]	1.70[a]	1.37[b]	1.34[b]	1.45[a,b]	0.080	0.033
TC	6.71[a]	6.18[a,b]	5.46[b]	5.50[b]	6.29[a,b]	0.164	0.047
TG	1.02[a]	0.52[b]	0.52[b]	0.51[b]	0.56[b]	0.479	0.021

[1]HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-b]Means with different letters within a row differ significantly ($P < 0.05$).

Table 4. Effects of phytosterol supplementation on serum cytokines of white feather broilers.

	PS [2] (mg/kg)						
Items[1](ng/L)	0	10	20	40	80	SEM [3]	*P*-Value
IL-1β	76.73[b]	99.56[a,b]	104.21[a]	113.58[a]	109.19[a]	4.776	0.032
IL-2	22.05[b]	31.33[a,b]	38.40[a]	42.30[a]	34.94[a,b]	2.487	0.020
IL-6	63.85[b]	72.52[a,b]	88.74[a]	84.41[a]	77.90[a,b]	2.763	0.017
TNF-a	76.81	72.12	84.65	80.34	69.14	7.249	0.973
IFN-γ	169.90[b]	215.47[a]	217.63[a]	230.96[a]	212.13[a]	9.170	0.005

[1]IL-1β, interleukin-1β; IL-2, interleukin-2; IL-6, interleukin-6; TNF-a, tumor necrosis factor α; INF-γ, interferon-γ.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-b]Means with different letters within a row differ significantly ($P < 0.05$).

Small Intestinal Histomorphology

The VH:CD values in both the duodenum and ileum of chickens were enhanced by PS supplementation ($P < 0.05$) (Table 5 and Supplementary Figure 1). Compared with the control group, dietary PS at less than or equal to 40 mg/kg level improved ($P < 0.05$) VH in both the duodenum and ileum. However, broilers fed different levels of PS exhibited no difference in intestinal CD value compared with those fed the basal diet ($P > 0.05$).

Quality of Breast Muscle

PS addition did not alter muscular b * ($P > 0.05$), but decreased ($P < 0.05$) drip loss and shear force of breast muscle and increased the pH value at 45 min (Table 6). Compared with the control group, broilers receiving PS administration at doses of 20 and 40 mg/kg had lower muscular a* and L* values at 45 min and 24 h, respectively ($P < 0.05$). There were no statistically significant differences in pH and a* value at 24 h and L* value at 45 min among all groups ($P > 0.05$).

Antioxidant Capacity of Breast Muscle

In breast muscle, PS supplementation increased the T-AOC activity in broilers ($P < 0.05$; Table 7). Compared with the control group, dietary supplementation with PS at 20 and 40 mg/kg increased SOD activity and decreased MDA concentrations ($P < 0.05$). However, the glutathione peroxidase and CAT activity of chickens were not changed by PS addition ($P > 0.05$).

Amino Acid Profile of Breast Muscle

There were differences ($P < 0.05$) in some amino acid concentrations after dietary supplementation of PS compared with the control (Table 8). The total amino acid and flavor amino acid (FAA) concentrations increased ($P < 0.05$) with dietary supplementation of PS (40 mg/kg) compared with the control group, whereas no differences ($P > 0.05$) were found in the contents of the essential amino acids among all groups. Moreover, the concentrations of isoleucine, arginine, glycine, and cystine in breast muscle of broilers receiving PS were higher than those of the control group ($P < 0.05$). Compared with the control, dietary supplementation with PS at 20 and 40 mg/kg increased arginine and serine concentrations, respectively ($P < 0.05$). Interestingly, dietary supplementation of PS increased the concentrations of valine, phenylalanine, methionine, glutamic acid, and alanine ($P > 0.05$) compared with the control group, but the differences were not statistically significant ($P > 0.05$).

Table 5. Effects of phytosterol supplementation on intestinal morphology of white feather broilers.

	PS [2] (mg/kg)						
Items[1]	0	10	20	40	80	SEM [3]	*P*-Value
Duodenum							
VH	1439.72[b]	1667.45[a]	1702.73[a]	1764.73[a]	1476.49[b]	30.140	0.001
CD	187.68	178.86	169.66	164.24	171.74	4.074	0.411
VH:CD	7.85[c]	9.46[b]	10.07[a,b]	10.78[a]	8.82[a,b]	0.225	<0.001
Jejunum							
VH	1507.63	1465.64	1499.23	1511.92	1504.03	15.383	0.897
CD	170.38	161.67	151.58	159.07	163.07	2.983	0.397
VH:CD	9.02	9.10	9.98	9.44	9.32	0.151	0.287
Ileum							
VH	987.03[c]	1255.48[a]	1298.56[a]	1162.15[a,b]	1112.57[b,c]	25.150	<0.001
CD	134.33	139.81	125.98	119.83	122.32	2.770	0.131
VH:CD	7.46[c]	9.09[b]	10.41[a]	9.83[a,b]	9.17[b]	0.231	<0.001

[1]VH, villous height; CD, crypt depth; VH:CD, villous height: crypt depth.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-c]Means with different letters within a row differ significantly ($P < 0.05$).

Table 6. Effects of phytosterol supplementation on meat quality of white feather broilers.

Items [1]	PS [2] (mg/kg) 0	10	20	40	80	SEM [3]	*P*-Value
pH_{45min}	6.67[b]	6.91[a]	6.95[a]	6.96[a]	6.92[a]	0.039	0.041
L^*_{45min}	53.29	51.84	52.84	52.00	52.07	0.259	0.421
a^*_{45min}	5.86[b]	6.24[a]	6.37[a]	6.48[a]	6.08[a,b]	0.596	0.022
b^*_{45min}	10.09	9.18	9.60	9.05	9.15	0.201	0.496
pH_{24h}	5.79	5.89	5.81	5.82	5.82	0.016	0.360
L^*_{24h}	57.76[a]	56.02[a,b]	54.83[b]	55.26[b]	57.22[a]	0.374	0.037
a^*_{24h}	4.17	4.24	4.45	4.55	4.38	0.110	0.557
b^*_{24h}	12.51	10.80	10.63	10.74	11.38	0.457	0.175
Drip loss (%)	4.67[a]	2.33[b]	1.88[b]	2.20[b]	3.15[b]	0.274	0.004
Shear force (kgf)	4.07[a]	2.97[b]	2.80[b]	2.95[b]	3.45[a,b]	0.309	0.014

[1]L^*, Luminance; a^*, redness; b^*, yellowness.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-b]Means with different letters within a row differ significantly ($P < 0.05$).

Table 7. Effects of phytosterol supplementation on antioxidant capacity in breast muscle of white feather broilers.

Items [1]	PS [2] (mg/kg) 0	10	20	40	80	SEM [3]	*P*-Value
SOD (U/mgprot)	57.99[c]	61.67[b,c]	64.66[b]	72.40[a]	61.22[b,c]	1.834	0.014
MDA (nmol/mgprot)	1.54[b]	1.55[b]	0.81[a]	0.90[a]	1.17[a]	0.079	<0.001
GSH-PX (U/mgprot)	6.63	6.07	6.44	6.49	6.51	0.541	0.999
CAT (U/mgprot)	0.74	0.73	0.84	0.75	0.62	0.046	0.698
T-AOC (μmol/gprot)	3.17[c]	4.88[b]	5.02[b]	6.50[a]	4.97[b]	0.408	0.012

[1]SOD, superoxide dismutase; MDA, malondialdehyde; GSH-Px, glutathione peroxidase; CAT, catalase; T-AOC, total antioxidant capacity.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-c]Means with different letters within a row differ significantly ($P < 0.05$).

Table 8. Effects of phytosterol supplementation on amino acids in breast muscle of white feather broilers (g/100g).

Items [1]	PS [2] (mg/kg) 0	10	20	40	80	SEM [3]	*P*-Value
Valine	0.656	0.671	0.676	0.659	0.680	0.004	0.306
Isoleucine	0.722[c]	0.747[b]	0.741[b]	0.739[b]	0.763[a]	0.004	0.002
Leucine	1.489[b]	1.538[a,b]	1.521[a,b]	1.568[a]	1.501[a,b]	0.011	0.026
Phenylalanine	0.860	0.886	0.893	0.878	0.882	0.005	0.169
Methionine	0.573	0.587	0.583	0.592	0.583	0.002	0.118
Threonine	0.893	0.873	0.899	0.894	0.881	0.004	0.127
Lysine	1.616	1.617	1.592	1.600	1.599	0.010	0.935
Arginine	1.217[b]	1.308[a]	1.441[a]	1.219[a]	1.345[a]	0.024	0.002
Histidine	0.877	0.851	0.853	0.868	0.875	0.004	0.068
Aspartic	1.972	1.888	1.906	1.959	1.911	0.117	0.473
Serine	0.836[b]	0.854[b]	0.887[a]	0.856[a,b]	0.859[a,b]	0.005	0.011
Glutamic	2.755	2.793	2.830	2.903	2.831	0.019	0.125
Proline	1.099	1.082	1.102	1.19	1.090	0.009	0.783
Glycine	0.832[b]	0.885[a]	0.881[a]	0.877[a]	0.888[a]	0.006	0.003
Alanine	1.085	1.148	1.160	1.200	1.153	0.015	0.225
Cystine	0.382[b]	0.410[a]	0.410[a]	0.400[a]	0.406[a]	0.003	0.014
Tyrosine	0.680	0.670	0.684	0.692	0.679	0.003	0.346
EAA	8.902	9.078	9.008	9.017	9.110	0.044	0.674
FAA	8.183[b]	8.270[a,b]	8.354[a]	8.509[a]	8.344[a]	0.044	0.036
TAA	18.542[b]	18.807[b]	18.867[a,b]	19.273[a]	18.927[a,b]	0.080	0.044

[1]EAA, essential amino acids; FAA, flavor amino acids; TAA, total amino acids.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-c]Means with different letters within a row differ significantly ($P < 0.05$).

Fatty Acid Profile of Breast Muscle

Dietary supplementation of PS produced differences in the concentration of several fatty acids compared with the control group ($P < 0.05$) (Table 9). The saturated fatty acids (**SFA**) content increased ($P < 0.05$) in the breast muscle of broilers supplemented with PS compared with the control group. Capric acid (C10:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and docosanoic acid (C22:0) were decreased in the broilers fed PS compared with those in the control group. However, the other SFAs showed no differences ($P > 0.05$) among the groups. For monounsaturated fatty acids, the content of margaroleic acid (C17:1) was higher ($P < 0.05$) with PS supplementation. For polyunsaturated fatty acids (**PUFA**), the contents of linoleic acid (C18:2n-6), arachidonic acid (C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3), total PUFA, and total n-3 PUFA content increased ($P < 0.05$) in the broilers fed with PS compared with the control diet. In contrast, the contents of other unsaturated fatty acids showed no differences ($P > 0.05$) in breast muscle among all five groups.

DISCUSSION

PS is a new type of animal growth-promoting agent, which has growth regulation functions and promotes animal health (Emandes and Cabral, 2007). However, their effects on growth performance of animals are not fully understood as conflicting results are often found in the literature. Naji et al. (2014a) reported that consumption of PS improved the growth performance of broilers, which can be demonstrated by an increase in the final weight. In line with our results, different doses of PS improved broiler final body weight, ADFI and ADG to varying degrees. These observed improvements in bird growth performance resulted from PS supplementation can be, at least partially, explained by following two distinct aspects. On the one hand, PS can improve nutrient digestibility and promote protein synthesis in animals (Eugste and Rivara, 1995; Emandes and Cabral, 2007). On the other hand, PS has hormone-like effects and can regulate the functions of the pituitary gland and liver, stimulate the production of insulin-like growth factors, and thus promote animal body development (Jia et al., 2005; Shi et al., 2014). Moreover, the results of the current study showed that feeding the diet supplemented with PS increased the pro-inflammatory cytokines,

Table 9. Effects of phytosterol supplementation on fatty acids in breast muscle of white feather broilers (g/100g).

	PS [2] (mg/kg)						
Items [1]	0	10	20	40	80	SEM [3]	*P*-Value
C10:0	0.055[a]	0.048[a,b]	0.048[a,b]	0.033[b,c]	0.020[c]	0.004	0.001
C12:0	0.025	0.025	0.025	0.027	0.018	0.001	0.183
C14:0	0.275[a]	0.255[a,b]	0.238[b]	0.240[b]	0.220[b]	0.005	0.002
C15:0	0.550	0.580	0.567	0.578	0.507	0.009	0.061
C16:0	21.173[a]	20.563[b]	20.503[b]	20.295[b]	20.288[b]	0.084	<0.001
C17:0	0.028	0.028	0.023	0.023	0.025	0.001	0.351
C18:0	0.268[a]	0.273[a]	0.243[b]	0.223[b]	0.195[c]	0.007	0.047
C20:0	0.350	0.380	0.350	0.335	0.328	0.006	0.075
C22:0	0.045[a]	0.035[a,b]	0.033[b]	0.030[b]	0.023[b]	0.002	0.007
C24:0	0.208	0.215	0.205	0.210	0.203	0.003	0.826
C14:1	0.063	0.050	0.058	0.045	0.050	0.003	0.109
C15:1n-5	0.260	0.248	0.258	0.273	0.275	0.004	0.063
C16:1n-7	0.605	0.540	0.528	0.548	0.545	0.008	0.112
C17:1	0.025[b]	0.035[a]	0.040[a]	0.038[a]	0.038[a]	0.002	0.042
C18:1n-9	22.368	22.530	22.720	22.398	22.363	0.066	0.303
C22:1n-9	0.175	0.185	0.183	0.190	0.198	0.003	0.327
C24:1n-9	0.365	0.373	0.370	0.368	0.380	0.004	0.799
C18:2n-6	33.480[b]	33.575[a,b]	33.533[a,b]	33.930[a]	34.150[a]	0.089	0.045
C20:4n-6	0.765[b]	0.800[a,b]	0.820[a]	0.813[a]	0.810[a,b]	0.007	0.036
C22:2n-6	0.690	0.720	0.738	0.728	0.725	0.006	0.051
C18:3n-3	3.355	3.388	3.485	3.470	3.433	0.024	0.424
C20:3n-3	10.093	10.188	10.315	10.303	10.200	0.031	0.121
C20:5n-3	0.260[b]	0.288[a]	0.278[a]	0.288[a]	0.278[a]	0.004	0.024
C22:5n-3	1.850	1.820	1.683	1.815	1.790	0.012	0.396
C22:6n-3	2.673[b]	2.863[a]	2.877[a]	2.825[a]	2.838[a]	0.016	0.026
SFA	22.975[a]	22.400[b]	22.233[b]	21.985[b]	21.823[b]	0.100	<0.001
MUFA	23.860	23.960	24.155	23.858	23.848	0.062	0.517
PUFA	53.165[c]	53.640[b]	53.613[b]	54.158[a]	54.330[a]	0.111	<0.001
n-3 PUFA	18.230[b]	18.545[a]	18.523[a]	18.700[a]	18.643[a]	0.050	0.010
n-6 PUFA	34.935[b]	35.175[a,b]	35.090[a,b]	35.458[a]	35.688[a]	0.089	0.032
PUFA:SFA	2.314[b]	2.395[a]	2.411[a]	2.463[a]	2.490[a]	0.015	<0.001
n-6:n-3	1.917	1.897	1.895	1.896	1.914	0.006	0.703

[1]SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
[2]PS, phytosterols.
[3]SEM, total standard error of means.
[a-c]Means with different letters within a row differ significantly ($P < 0.05$).

which was in contrast with the findings of Nashed et al. (2005), who reported that PS can regulate immune system, has disease resistance and anti-inflammatory effects, thereby promoting animal growth. The discrepancy may be attributed to animal species and age, feeding management, compositions of the basal diet and PS level.

In the current study, dietary PS supplementation decreased the LDLC, TC, and TG content of broilers. This result concurs with the findings of Sachiko et al. (2000), who summarized experimental research on the effect of PS on cholesterol metabolism in the past 50 years, and concluded that PS could significantly reduce the concentration of TC and LDL-C, and have no effect on the concentration of HDL-C. Moreover, the reduction of TG content by PS is mainly accomplished by interfering with the absorption of TG in the intestinal lumen. PS can inhibit lipase activity and reduce the hydrolysis of TG in food, and can also affect the expression of fatty acid synthesis-related genes and fatty acid intake-related genes, thereby achieving the effect of reducing TG content (Kim et al., 2014; Feng et al., 2018). Many previous clinical studies have shown that an appropriate amount of PS can reduce the absorption of intestinal cholesterol, slow down the esterification rate of cholesterol in intestinal epithelial cells, and increase the reverse transport and excretion of cholesterol (Sachiko et al., 2000; Kim et al., 2014; He et al., 2018). Cholesterol is an insoluble substance that requires the help of intestinal bile acids, which are emulsified into globular micelles before they are absorbed by intestinal cells. The structure of PS is similar to cholesterol, so it can compete with cholesterol in the formation of spherical micelles, thereby effectively reducing cholesterol and LDLC levels in the body (Arienne et al., 2003; He et al., 2018). The decreased LDLC, TC, and TG content in serum by PS inclusion in this study suggested that dietary PS addition is of great significance for maintaining the health of broilers.

Proinflammatory cytokines are essential in initiating immune responses and eliminating pathogens from the host. However, their exaggerated or prolonged secretion may be detrimental to the host (Smith and Humphries, 2009). It has become apparent that interactions between proinflammatory and antiinflammatory mediators regulate the inflammatory response (Piotr et al., 2014). The results of previously published studies on the impact of various feed additives on the immune system of poultry indicate that they stimulate proliferation of Th1/Th2 cells and the release of proinflammatory and anti-inflammatory cytokines (Holian et al., 1997, Pavelić et al., 2001). In the present study, a similar effect is exerted by dietary supplementation of PS, which could significantly affect the levels of serum inflammatory cytokines and increase synthesis of IL-1β, IL-6, IL-2 and IFN-γ. Among the cytokines, IFN-γ has been shown to play a crucial role in clearing the inflammation caused by primary salmonella infection in chickens (Withanage et al., 2005). IL-2 is an important immunomodulatory cytokine, closely related to the occurrence of avian diseases and immunit (Buonocore et al., 2020). IL−6, and IL-1β, a proinflammatory cytokine, has a stimulating effect on the defense mechanisms of the body (Haseeb et al., 2018). The increase in the concentration of these pro-inflammatory cytokines stimulates the body's immune response and causes the secretion of related anti-inflammatory cytokines, resulting in inhibiting proinflammatory cytokine synthesis and adhesion molecule expression while increasing the levels of specific cytokine inhibitors, thereby creating a positive feedback-loop to maintain the balance between pro-inflammatory and anti-inflammatory (Srinivasan et al., 2017). Although a proinflammatory response is a part of natural immune defense, it is important to note that the secretion of long-term and high-concentration cytokines may not only trigger the body's inflammatory response, but may also lead to cellular injury by excessive generation of ROS (Khan et al., 2016).

In the present study, the VH:CD and VH values in both the duodenum and ileum of chickens were enhanced by supplementation with PS, which was consistent with the results of previous studies. Cheng et al. (2020) found that β-sitosterol could increase the surface area of the small intestine and enhance the digestion and absorption of nutrients. The intestine is not only the body's largest digestion and absorption site, but also the body's largest immune organ (Wang et al., 2017). This is the first barrier to prevent the invasion of intestinal pathogens. Intestinal morphology is an important indicator that reflects the health of the digestive tract and the response of the intestine to certain feed substances (Boguslawska-Tryk et al., 2012; Cao et al., 2015). The longer the VH and the shallower the CD in the small intestine, the greater the ability to absorb nutrients. The ratio of villus height to crypt depth can reflect the function of the small intestine. Decreased ratios indicate damage to the mucosa, reduced digestion and absorption and are often accompanied by diarrhea and growth retardation (Ding et al., 2020). Our research showed that dietary PS supplementation improved the immune function of the intestines, contributing to better intestinal health status. Similarly, Hu et al. (2017) has demonstrated that dietary PS supplementation decreased lipid peroxidation and provide protection for improving the intestinal morphology of piglets. The amelioration of intestinal morphology also supports our results with respect to an improved the VH:CD of the duodenum and ileum in broilers fed a PS diet. However, in terms of the effects of PS on broilers, more investigations are needed to understand the relationship between morphology, immune function, and intestinal oxidation state.

Previous studies have reported that muscle pH, meat color, water, and tenderness can comprehensively reflect meat quality (Ding et al., 2020). A low pH is related to the accumulation of lactic acid caused by anaerobic respiration, which may affect water retention capacity (Qi et al., 2010). In this study, the pH of muscles supplemented with PS in the diet was higher than that of the control group, indicating that the supplementation of

PS is beneficial to muscle storage and prevents muscle corruption. Water-holding capacity (measured by drip loss) and shear force are important variables that reflect the juiciness and tenderness of meat products (Yang et al., 2010). According to some research conclusions on animals by Wyrwisz et al. (2012), the pH value of meat is negatively related to its drip loss, L* value, and shear force. The results of this study showed that supplementation with PS decreased the L* value, drip loss, and shear force of the muscles 24 h post-mortem. These findings are similar to those of Wu et al. (2012), who reported that the addition of dietary PS reduced muscle loss 24 h and 48 h post-mortem in ducks. Moreover, Descalzo and Sancho (2008) showed that meat quality is closely related to the antioxidant function of muscles. Lipid peroxidation is one of the important reasons for the deterioration of meat quality. It can reduce the nutritional value of muscles, affect the flavor and texture, and change the appearance of muscles. MDA is one of the representative products of lipid peroxidation metabolism, and its content directly reflects the degree of lipid peroxidation damage. Both CAT and SOD are antioxidant enzymes in the body and can remove oxygen free radicals in the body. T-AOC is a comprehensive indicator of the body's antioxidant system (Kim et al., 2016). The current study showed that dietary supplementation of PS increased the activity of SOD and T-AOC, while reducing muscle MDA content. Similar results were reported by Gu et al. (2008), who demonstrated that adding 40 or 80 mg of PS per kilogram of feed significantly increased SOD activity. Based on the above results, dietary supplementation with PS can improve the meat quality of broilers by increasing the antioxidant capacity of muscles.

The content of amino acids and fatty acids in muscles is a key indicator of meat quality, especially diet quality (Yin et al., 2017). The present authors are unaware of any comprehensive evaluation of the effects of PS on profiles of muscle amino acids and fatty acids in broilers, although other effects have been measured on meat quality, antioxidants, and other related indicators in chickens (Zhao et al., 2019b; Cheng et al., 2020). In this study, dietary PS supplementation increased the concentrations of isoleucine, leucine, cystine glycine, serine, and arginine in the muscles, which may improve muscle quality. The present results are consistent with those of Ma et al. (2010) and Madeira et al. (2014), where arginine and isoleucine improved meat quality. Glycine, aspartic acid, alanine, phenylalanine, glutamic acid, and tyrosine are collectively referred to as the FAA (Sabagh et al., 2016). Their composition and content directly affect the freshness of food. The increased levels of FAA following PS supplementation in the current study improved the flavor of broiler meat, making it more favorable to consumers. Dietary modification of fatty acids may be an effective way to obtain healthy broiler products for human consumption. Dietary supplementation with PS can change the fatty acid profile of broiler muscle and reduce its SFA content. Previous studies have shown that dietary SFA intake is closely related to vascular heart disease (Ramasamy et al., 2006). Therefore, from a health perspective, meat with relatively low SFA content can help reduce the risk of cardiovascular heart disease (Cheng et al., 2017). The n-3 PUFA have preventive and therapeutic effects on cardiovascular diseases, while DHA and EPA are currently the most important n-3 PUFAs (Colussi et al., 2017). Egert et al. (2009) reported that DHA influences normal blood lipids and in people with hyperlipidemia, has a significant effect on lowering blood lipids. The present results show that dietary supplementation with PS can improve meat quality by increasing n-3 PUFA, DHA, EPA, and PUFA:SFA of breast muscle. The increase in PUFA content may be because PS enhances the antioxidant defense system of broiler muscles, and rich, long-chain, PUFA in broiler meat may be beneficial to human health, but the specific mechanism of action requires further research. In addition, PS can increase the ratio of PUFA: SFA by changing the fatty acid composition of broiler muscles. To the best of our knowledge, the present study provides the first evidence that dietary PS supplementation can beneficially improve profiles of amino acids and fatty acids in broilers.

In conclusion, the results of the present study indicate that dietary PS supplementation can improve growth performance, serum lipid level, proinflammatory cytokine levels, and intestinal morphology of white feather broilers. Furthermore, PS indirectly improve the quality and flavor of chicken by improving the antioxidant capacity, amino acid, and fatty acid profiles of muscles, thereby increasing its economic value as a food commodity.

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DISCLOSURES

No conflict of interest exits in the submission of this manuscript, and the manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101096.

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