An evaluation of graded levels of beta-sitosterol supplementation on growth performance, antioxidant status, and intestinal permeability-related parameters and morphology in broiler chickens at an early age

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ABSTRACT This study was designed to examine the effects of different levels of beta-sitosterol (**BS**) supplementation on growth performance, serum biochemical indices, redox status, and intestinal permeability-related parameters and morphology of young broilers. Two hundred and forty male Arbor Acres broiler chicks were allocated into 5 groups of 6 replicates with 8 birds each, and fed a basal diet supplemented with 0, 25, 50, 75, and 100 mg/kg BS for 21-d, respectively. The BS quadratically decreased feed conversion ratio during 1 to 14 d and 1 to 21 d, with its effect being more prominent at 25 or 50 mg/kg ($P < 0.05$). The BS linearly and quadratically reduced 14-d plasma diamine oxidase activity and D-lactate level, and this effect was more pronounced when its supplemental level was 25 or 50 mg/kg ($P < 0.05$). The BS linearly increased duodenal villus height (**VH**) and quadratically increased jejunal VH and ratio of VH and crypt depth (**CD**) at 14 d, and these effects in 25 mg/kg group were more remarkable ($P < 0.05$). Similarly, BS linearly or quadratically increased VH and ratio of VH and CD, but decreased CD in the jejunum and ileum at 21 d, with these effects being more pronounced at 50 mg/kg ($P < 0.05$). The BS supplementation especially at 50 or 75 mg/kg linearly or quadratically reduced 14-d serum and 21-d hepatic malondialdehyde concentration, and increased serum glutathione peroxidase and catalase activities at 14 and 21 d ($P < 0.05$). Moreover, the BS administration linearly and/or quadratically increased glutathione peroxidase, catalase, and superoxide dismutase activities and glutathione level, and reduced malondialdehyde accumulation in the intestinal mucosa at 14 and/or 21 d, and these consequences were more significant in 50 to 100 mg/kg BS-supplemented groups ($P < 0.05$). The results demonstrated that BS administration could improve growth performance, intestinal barrier function, and antioxidant status of broilers at an early age, with these effects being more pronounced at a level of 50 mg/kg.

Key words: beta-sitosterol, growth performance, antioxidant status, intestinal barrier, broilers

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INTRODUCTION

Phytosterols (plant sterols) are widely available in a great variety of plant-derived foods, especially in oil seeds such as peanut, soybean, rapeseed and sunflower, and they resemble cholesterol in vertebrates in terms of their chemical structures and physiological function (Shahzad et al., 2017; Yang et al., 2019; Bai et al., 2021). The main sources of phytosterols are edible oils since their chemical synthesis is cost-prohibitive, and therefore the properties and compositions of phytosterol mixture always fluctuate depending on their sources as well as the refining and frying process of edible oils (Bai et al., 2021). Up to now, more than two hundred and fifty different types of phytosterols have been already discovered and identified in a variety of vegetation and aquatic flora, with beta-sitosterol (**BS**), campesterol, and stigmasterol being the most common types of phytosterols. Aside from cholesterol-lowering effects, cumulative clinical and experimental evidence has confirmed multiple biological activities of phytosterols, including anti-inflammatory, antioxidant, immunomodulatory, antimicrobial, anticancer, and gut- and liver-protective properties (Othman and Moghadasian, 2011; Shahzad et al., 2017; Plat et al., 2019; Feng et al., 2020a). Phytosterols have been incorporated in animal diets as feed additives for both poultry and livestock animals, and available literature has recently shown that dietary supplementation with phytosterols improved growth

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performance, lipid metabolism, digestive function, antioxidant status, immune response, intestinal morphology, and meat quality in broiler chickens and/or piglets (Hu et al., 2017a,b; Zhao et al., 2019; Ding et al., 2021; Yuan et al., 2021). Although these beneficial effects have been reported, several studies have found that phytosterols administration actually exerted no or limited positive effects in animals (Liu et al., 2010; Li et al., 2015a; Feng et al., 2020b). These discrepancies among the practical application of phytosterols could be mainly traced to their complicated ingredients and variations in composition, which definitely limit their usage in animal feed to a certain extent. It is, therefore, urgently needed to exploit and develop a single phytosterol source and evaluate its application effects in livestock and poultry, aiming to better utilize the biological effects of phytosterols and improve growth performance and health status of animals.

Usually, BS is the most abundant phytosterol and its daily consumption by humans accounts for more than 65% of the total ingested phytosterols (Jiménez-Escrig et al., 2006; Martins et al., 2013). Available studies have demonstrated that BS administration could lower blood cholesterol (Feng et al., 2018; Chen et al., 2020a), attenuate insulin resistance and reduce circulating glucose level (Ponnulakshmi et al., 2019; Babu et al., 2020), prevent bacterial and viral infections (Li et al., 2015b; Zhou et al., 2020), inhibit inflammatory responses (Kurano et al., 2018; Zhou et al., 2020), and counteract oxidative stress (Yin et al., 2018; Devaraj et al., 2020) in rodents. In an oral gavage study, the distribution pattern of radioactivity in the tissues of rats given ^{14}C-labelled BS is similar, but the highest level and the longest retention of radioactivity is observed in the intestinal epithelia (Sanders et al., 2000), which, in turn, indicates that the intestine is a major organ responsible for the adsorption and metabolism of BS. Actually, BS has been reported to effectively ameliorate dextran sulfate sodium- and high-fat-diet-induced colitis by suppressing inflammation through the inactivation of nuclear factor-kappa B pathway, inhibiting the survival of pathogenic bacteria, and maintaining intestinal barrier function and integrity (Kim et al., 2014; Feng et al., 2017; Ding et al., 2019). Additionally, the BS also exhibits in vivo antioxidant properties, and studies have demonstrated that BS inhibited lipid peroxidation and improved redox status in the intestine, liver, or brain of rodent animals subjected to dimethylhydrazine−(Baskar et al., 2012), alcohol−(Chen et al., 2020b), carbon tetrachloride −(Devaraj et al., 2020), or vanadium-induced oxidative stress (Adebiyi et al., 2020).

In swine, BS treatment could improve immune response of pigs when vaccinated against porcine reproductive and respiratory syndrome virus (Fraile et al., 2012). Likewise, the administration of BS has also been demonstrated to attenuate high grain diet-induced inflammatory stress and beneficially regulate rumen fermentation and microbiota in sheep (Xia et al., 2020). To date, information about the application of BS in poultry is scarce. A limited study has shown that dietary supplementation with emulsified sitosterol to a standard chicken diet reduced egg cholesterol level in laying hens (Clarenburg et al., 1971). The early grower period of broiler chicks, a period characterized by low feed intake, poor growth, and a relative high mortality, is vital for the growth and development of body and even determine subsequent growth performance (Noy and Sklan, 1997; Yegani and Korver, 2008; Shang et al., 2018). However, the young broiler chickens is functionally immature during the early growth period and is, therefore, highly vulnerable to various harmful stimuli (Yegani and Korver, 2008; Jeni et al., 2021). Necessary nutritional interventions have been proposed and practiced to modulate early growth and development of broiler chicks and to optimize subsequent growth performance. In this study, we, therefore, hypothesized that dietary BS would bring beneficial consequences to young broilers chickens, and the current study was firstly conducted to evaluate the effects of different levels of BS on growth performance, blood biochemical parameters, redox status, and intestinal barrier integrity of broiler chickens at an early age, which would provide scientific basis for the future rational utilization of BS in broiler feed.

MATERIALS AND METHODS

Animal, Diets, and Experimental Design

All experiments involving animals were strictly performed in accordance with guideline set by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. The serial number of Laboratory Animal Use License of Nanjing Agricultural University was SYXK-2017-0007, which was licensed by the Jiangsu Provincial Department of Science and Technology, P.R. China.

One-day-old male Arbor Acres Plus broiler chicks with a similar body weight (41.94 ± 0.19 g) were randomized against the experimental diets in a completely randomized design for a 21-d feeding experiment. The chicks were allocated into one of 5 groups of 6 replicates (pens) with 8 birds each, and fed a basal diet supplemented with 0 (Control group), 25, 50, 75, and 100 mg/kg BS of diet, respectively. The BS extracted from tall oil was kindly gifted from Yichun Dahaigui Life Science Co., Ltd (Yichun, Jiangxi Province, P.R. China), with a purity of approximately 99%. The supplemental levels of BS used in this study were selected according to the available findings relevant to the evaluation of phytosterol mixture in broiler chickens' diets (Zhao et al., 2019; Ding et al., 2021; Yuan et al., 2021). To ensure its uniform mixing, the BS power with good flowability was firstly mixed thoroughly with corn gluten meal in a small double screw cone mixer until homogeneous prior to feed preparation. The basal diet used in the current study was a corn-soybean meal diet formulated according to the requirements of broiler chickens (0−3 wk) (National Research Council, 1994), and its composition and nutrient level are presented in the Table 1. All birds were raised in stainless steel cages

Table 1. Composition and nutrient level of basal diet.

Ingredients, %	Content
Corn	57.01
Soybean meal	31.50
Corn gluten meal	3.40
Soybean oil	3.10
Limestone	1.20
Dicalcium phosphate	2.00
L-Lysine	0.34
DL-Methionine	0.15
Sodium chloride	0.30
Premix[1]	1.00
Total	100
Calculated nutrient levels	
Apparent metabolizable energy, MJ/kg	12.55
Crude protein, %	21.33
Calcium, %	1.00
Total phosphorus, %	0.68
Available phosphorus, %	0.46
Lysine, %	1.21
Methionine, %	0.50
Methionine + cystine, %	0.86
Analyzed nutrient levels[2]	
Gross energy, MJ/kg	15.48
Crude protein, %	20.79
Calcium, %	1.06
Total phosphorus, %	0.72
Lysine, %	1.24
Methionine, %	0.48

[1]Premix provided per kilogram of diet: vitamin A (transretinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-rac-α-tocopherol), 30 IU; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 600 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulphate), 8.0 mg; Mn (from manganese sulphate), 110 mg; Zn (from zinc oxide), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

[2]Results are the average values of triplicate measurements.

with plastic floors (150 cm [length] × 70 cm [width] × 50 cm [height]) put in a temperature-controlled house. The birds were allowed free access to water through nipple drinkers, and the complete mash diets were given ad libitum to broiler chickens during the whole period of experiment except when the feed deprivation was performed. Aside from the initial 3 d, the broiler chickens were subjected to a light program of 23 h light and 1 h dark. The indoor temperature of chicken house was set between 33 and 34°C during the first week post-hatch, which was then reduced by 2°C to 3°C at weekly interval. The room relative humidity was maintained at around 70% during the first 3 d after entering, and was then kept at 60% to 65% thereafter.

Sample Collection

One bird was randomly selected from each replicate (cage) corresponding to dietary treatments for sampling collection and analysis at 14 and 21 days of age, respectively. Blood samples were collected from wing veins using 10-mL sterilized tubes coated with or without heparin anticoagulant after weighing. After centrifugation at 3,000 × g for 15 min at 4°C, the plasma and serum samples were separated, aliquoted, and then stored at −80°C for being used to measure relevant parameters. Birds were then euthanized by cervical dislocation. After necropsy liver was dissected free from connective tissues, washed twice in ice-cold phosphate buffer solution (pH = 7.4), and lightly surface-dried with filter-paper. The left lateral lobe of liver samples was collected from individual birds, immediately snap frozen, and stored in liquid nitrogen until analysis. After collection of liver tissues, the duodenum, jejunum, and ileum were excised and placed a chilled stainless-steel tray. A 2-cm-long segment of the mid-intestine (duodenum, jejunum, and ileum) was dissected, flushed gently with physiological saline, and fixed in fresh neutral-buffered formalin (10%) for the evaluation of intestinal morphology. The intestinal tracts were then longitudinally opened along its mesenteric border and cleaned of residual digesta by rinsing it with buffer. The intestinal mucosa was scraped with a sterile microscope slide, and the mucosal scrapings were placed into cryopreservation tubes, immediately snap-frozen in liquid nitrogen, and then stored at liquid nitrogen tank for further measurement.

Determination of Growth Performance

At 14 and 21 d of age, broiler chickens were weighed on replicate (cage) basis in the early morning after a 12-h period of feed withdrawal, and feed consumption of individual replicates were recorded to calculate average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**) during each experimental period (1−14 d and 15−21 d) and the entire experimental period (1−21 d) after adjusting the weight of mortalities and culled birds.

Measurement of Serum Biochemical Parameters

The levels of circulating total protein (cat. No. A045-4-1), albumin (cat. No. A028-2-1), glucose (cat. No. F006-1-1), triglyceride (cat. No. A110-2-1), and total cholesterol (cat. No. A111-1-1), and the activities of aspartate aminotransferase (cat. No. C010-1) and alanine aminotransferase (cat. No. C009-2) were colorimetrically quantified with corresponding commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, P.R. China), strictly according to the manual provided, using a microplate reader (MODEL 680, Bio-Rad Laboratories Inc., Hercules, CA) at different absorbance levels.

Quantification of Diamine Oxidase (DAO) Activity and D-lactate Level

The plasma DAO activity and D-lactate level were measured to reflect the degree of intestinal permeability. The measurement of plasma DAO activity was performed by employing a commercial kit specific for chickens (cat. No. A088-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, P. R. China), strictly following the instructions of

manufacture. The plasma D-lactate concentration was assayed using a general-purpose kit provided by AAT Bioquest (cat. No. AAT-13811, Sunnyvale, CA).

Histological Measurement

The histological measurement was performed as reported previously (Zha et al., 2022). In detail, the formalin-fixed small intestinal segments were dehydrated in ethanol series of increasing concentrations up to absolute, cleared in xylene, and embedded in paraffin wax prior to sectioning. A 5-μm serial section cut from the paraffin block was then deparaffinized with xylene, rehydrated in the serial dilutions of ethanol, and stained with hematoxylin & eosin. The stained slides were visualized using a Nikon ECLIPSE 80i light microscope equipped with a computer-assisted morphometric system (Nikon Corporation, Tokyo, Japan). The villus height (**VH**) and crypt depth (**CD**) of 8 well-preserved villi and crypts in individual slide were measured and means calculated for each.

Evaluation of Antioxidant Status

The homogenate of liver and intestinal mucosal scrapings were prepared by homogenizing liquid nitrogen frozen tissues with an ice-cold homogenate medium (physiological saline solution) at a ratio of 1:9 (liver, wt/vol) or 1:4 (intestinal mucosal, wt/vol) until no tissue particles were visible (around 35 s), using a motor-driven homogenizer (PRO-PK-02200D, Pro Scientific, Inc., Monroe, CT). After centrifugation at 4,450 × g for 15 min, the supernatant of homogenate was then separated, aliquoted, and stored at −80°C for the subsequent determination of antioxidant parameters, including superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), catalase (**CAT**), reduced form of glutathione (**GSH**), and malondialdehyde (**MDA**).

The activities of SOD (cat. No. A001-1-1), GSH-Px (cat. No.A005-1-2), and CAT (cat. No. A007-1-1), and the concentrations of GSH (cat. No. A006-1) and MDA (cat. No. A003-1) in the serum, liver, and small intestinal mucosa samples were colorimetrically measured with corresponding commercial kits at the recommended absorbance levels according to the standardized protocols provided by manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, P.R. China). In detail, a classic hydroxylamine method (Kono, 1978) was adopted for the measurement of SOD activity. The 5, 5′-dithiobis (2-nitrobenzoic acid) method (Owens and Belcher, 1965) was used for the assay of both GSH-Px activity and GSH level. As for the quantification of CAT activity, the ammonium molybdate method (Góth, 1991) was performed. The thiobarbituric acid method (Placer et al., 1966) was employed to measure MDA level in the serum and tissues. All results in the liver and mucosa samples were adjusted against the corresponding total protein concentration prior to inner comparisons, and the total protein concentration was determined with the Bradford assay method (Kruger, 1994), using crystalline bovine serum albumin as a reference standard.

Statistical Analysis

Data were analyzed by one-way analysis of variance using SPSS statistical software (Ver.22.0 for windows, SPSS Inc., Chicago, IL). The statistical model for data analysis was: $Y_{ij} = \mu + T_i + \varepsilon_{ij}$, where Y_{ij} = dependent observation; μ = overall mean; T_i = effect of treatment; ε_{ij} = the random error. A pen (replicate) was the experimental unit for the growth performance data, while an individual bird from each pen was the experimental unit for other measured parameters. Orthogonal polynomial contrasts were also employed to test the linear and quadratic effects of the increasing levels of BS. Differences among treatments were tested using Duncan's multiple range test. The differences were considered as statistically significant when $P < 0.05$, and results were presented as means with their pooled standard errors.

RESULTS

Growth Performance

Dietary supplementation with BS quadratically decreased FCR of broilers during 1 to 14 d and 1 to 21 d of age, respectively (Table 2, $P < 0.05$). Compared with the control group, the FCR of broilers fed a basal diet supplemented with 25 to 75 mg/kg BS was lower than their counterparts fed a control diet only during 1 to 14 d ($P < 0.05$), and there was no significant difference among these BS-supplemented groups ($P > 0.05$). The highest FCR of broilers during 1 to 14 d was found in the 100 mg/kg BS-supplemented group when compared with other BS-supplemented treatments ($P < 0.05$), with its value being similar to that of the control group ($P > 0.05$). Supplementing 25 or 50 mg/kg BS decreased FCR of broilers during 1 to 21 d of age in comparison with the control group ($P < 0.05$), and the value of this parameter in these two BS-supplemented groups was lower than that of 100 mg/kg BS group ($P < 0.05$), with its level being intermediate in 75 mg/kg BS treatment ($P > 0.05$). However, dietary treatment did not affect ADG or ADFI during each experimental period and the entire experimental period ($P > 0.05$).

Serum Biochemical Parameters

As shown in the Table 3, the administration of BS quadratically reduced 14-d total cholesterol level and 21-d alanine aminotransferase activity, and linearly increased 21-d total protein concentration in the serum of broiler chickens ($P < 0.05$), but these parameters did not differ among groups ($P > 0.05$). Dietary treatment did not alter circulating albumin concentration, glucose level, triglyceride density or aspartate aminotransferase activity at both 14 and 21 d of age ($P > 0.05$).

Table 2. Effects of graded levels of dietary beta-sitosterol supplementation on the growth performance of broiler chickens.

Items[1]	Beta-sitosterol level (mg/kg)					SEM[2]	*P*-values		
	0	25	50	75	100		ANOVA[3]	Linear	Quadratic
1 to 14 d									
ADFI (g/d/bird)	35.52	35.29	36.31	36.06	35.88	0.45	0.963	0.661	0.773
ADG (g/d/bird)	27.58	29.34	29.95	29.61	27.94	0.40	0.248	0.726	0.025
FCR (g:g)	1.29[a]	1.20[b]	1.21[b]	1.22[b]	1.29[a]	0.01	0.007	0.919	<0.001
15 to 21 d									
ADFI (g/d/bird)	52.11	51.70	50.25	49.03	50.26	0.72	0.691	0.234	0.576
ADG (g/d/bird)	33.79	34.57	32.92	31.74	32.41	0.59	0.599	0.198	0.958
FCR (g:g)	1.54	1.51	1.53	1.55	1.55	0.01	0.922	0.663	0.546
1 to 21 d									
ADFI (g/d/bird)	40.74	40.47	40.62	40.13	40.39	0.35	0.989	0.698	0.896
ADG (g/d/bird)	29.65	31.09	30.94	30.32	29.43	0.31	0.344	0.581	0.056
FCR (g:g)	1.37[a]	1.31[b]	1.31[b]	1.33[ab]	1.37[a]	0.01	0.014	0.753	0.001

[a-b]Means within a row with different superscripts are different at $P < 0.05$.
[1]ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.
[2]SEM, standard error of the mean (n = 6).
[3]ANOVA, analysis of variance.

Intestinal Permeability-Related Parameters

The administration of BS linearly and quadratically reduced plasma DAO activity and D-lactate level at 14 d of age (Table 4, $P < 0.05$), and the values of these 2 parameters in the BS-supplemented groups, regardless of its dosage, were all lower than those of control group ($P < 0.05$). The 14-d plasma DAO activity and D-lactate level of birds in the 25 mg/kg BS group was lower and higher than those of birds receiving 50 mg/kg BS, respectively ($P < 0.05$), with their values in the other two BS-supplemented groups being intermediate ($P > 0.05$). Although no significant difference was detected, dietary BS supplementation quadratically reduced DAO activity and linearly decreased D-lactate level in the plasma at 21 d of age ($P < 0.05$).

Small Intestinal Morphology

At 14 d of age (Table 5), dietary BS administration linearly increased duodenal VH ($P < 0.05$). The value of this parameter in the 25, 75, and 100 mg/kg BS group was all higher than that of control group ($P < 0.05$), and there was no significant difference among the four BS-supplemented groups ($P > 0.05$). The BS supplementation quadratically increased jejunal VH and the ratio between VH and CD at 14 d of age ($P < 0.05$). Compared with the control group, the 14-d jejunal VH was increased by 25 and 50 mg/kg BS ($P < 0.05$) to a similar extent ($P > 0.05$). The BS administration, regardless of its dosage, increased 14-d jejunal ratio of VH and CD ($P < 0.05$), and no significant difference was observed among BS-supplemented groups ($P > 0.05$). The incorporation of BS linearly increased 21-d jejunal VH and the ratio of VH and CD, but linearly decreased 21-d jejunal CD ($P < 0.05$). Compared with the control group, the 21-day jejunal CD was reduced by 25, 50, and 100 mg/kg BS ($P < 0.05$) to a similar value ($P > 0.05$). The 21-d jejunal ratio between VH and CD was increased by BS administration when its level ranged from 50 to 100 mg/kg ($P < 0.05$), but its value in the 75 mg/kg BS-treated group was still lower than that of birds fed a basal diet supplemented with 50 or 100 mg/kg BS ($P < 0.05$). The BS administration

Table 3. Effects of graded levels of dietary beta-sitosterol supplementation on the serum biochemical parameters of broiler chickens.

Items	Beta-sitosterol level (mg/kg)					SEM[1]	*P*-values		
	0	25	50	75	100		ANOVA[2]	Linear	Quadratic
14 d									
Total protein (g/L)	24.67	23.07	23.52	22.78	22.82	0.44	0.661	0.221	0.585
Albumin (g/L)	18.63	18.58	19.85	18.90	19.32	0.21	0.289	0.255	0.464
Glucose (mmol/L)	8.48	9.10	9.38	9.20	9.76	0.24	0.564	0.131	0.772
Triglyceride (mmol/L)	1.47	1.52	1.27	1.37	1.35	0.03	0.155	0.101	0.479
Total cholesterol (mmol/L)	4.13	3.66	3.70	3.51	4.02	0.09	0.170	0.554	0.026
Alanine aminotransferase (U/L)	5.03	4.66	5.03	5.84	5.18	0.17	0.306	0.230	0.922
Aspartate aminotransferase (U/L)	33.05	35.37	27.25	31.02	31.24	0.96	0.087	0.213	0.307
21 d									
Total protein (g/L)	24.35	24.23	24.66	26.14	25.90	0.30	0.127	0.020	0.733
Albumin (g/L)	19.00	19.34	18.95	19.77	19.92	0.24	0.643	0.205	0.689
Glucose (mmol/L)	10.82	10.51	10.93	11.39	11.09	0.16	0.565	0.242	0.964
Triglyceride (mmol/L)	0.91	1.00	0.75	0.89	0.97	0.04	0.296	0.946	0.246
Total cholesterol (mmol/L)	3.78	3.88	3.76	3.82	3.68	0.06	0.906	0.576	0.586
Alanine aminotransferase (U/L)	6.39	5.60	5.18	5.56	6.15	0.15	0.064	0.599	0.004
Aspartate aminotransferase (U/L)	31.84	27.31	31.87	27.81	29.52	1.13	0.598	0.616	0.693

[1]SEM, standard error of the mean (n = 6).
[2]ANOVA, analysis of variance.

Table 4. Effects of graded levels of dietary beta-sitosterol supplementation on the diamine oxidase activity and D-lactate concentration in the plasma of broiler chickens.

Items	Beta-sitosterol level (mg/kg)					SEM[1]	*P*-values		
	0	25	50	75	100		ANOVA[2]	Linear	Quadratic
14 d									
Diamine oxidase (U/L)	18.10[a]	10.19[c]	13.21[b]	11.41[bc]	10.48[bc]	0.66	<0.001	<0.001	0.015
D-lactate (mmol/L)	0.74[a]	0.63[b]	0.48[c]	0.57[bc]	0.58[bc]	0.02	0.001	0.003	0.001
21 d									
Diamine oxidase (U/L)	12.75	7.57	6.58	7.51	9.90	0.79	0.079	0.271	0.009
D-lactate (mmol/L)	0.52	0.49	0.49	0.41	0.43	0.01	0.071	0.011	0.844

[a-c]Means within a row with different superscripts are different at $P < 0.05$.
[1]SEM, standard error of the mean (n = 6).
[2]ANOVA, analysis of variance.

quadratically increased 21-d ileal VH and linearly increased 21-d ileal ratio of VH and CD, and quadratically reduced 21-d ileal CD ($P < 0.05$). Birds in the 50 mg/kg BS group exhibited the highest 21-d ileal VH and CD when compared with other groups ($P < 0.05$). The 21-d ileal ratio of VH and CD was increased by 25, 75, and 100 mg/kg BS ($P < 0.05$), and no significant difference was found among the four BS-supplemented groups ($P > 0.05$).

Serum and Liver Antioxidant Status

Dietary BS incorporation (Table 6) quadratically reduced 14-d MDA concentration, linearly increased 14-d GSH-Px activity, and quadratically elevated 14-d CAT and 21-d GSH-Px and CAT activities in the serum ($P < 0.05$). Compared with the control group, the 14-d serum MDA concentration was reduced by 50 mg/kg BS ($P < 0.05$). The 14-d serum GSH-Px activity was increased to a similar value when supplementing 75 or 100 mg/kg BS in comparison with the control group ($P < 0.05$), with its value in the 50 mg/kg supplemented-BS group being intermediate ($P > 0.05$). The BS supplementation at a level of 50 mg/kg increased the 14-d serum CAT activity when compared with the control group ($P < 0.05$). The 21-d serum GSH-Px activity in the 75 mg/kg BS group was highest when compared with the control and other BS-supplemented groups ($P < 0.05$). Likewise, compared with the control group, supplementing 50 or 75 mg/kg BS increased 21-d serum CAT activity ($P < 0.05$) to a similar value ($P > 0.05$). However, dietary treatment did not affect serum GSH concentration or SOD activity at both 14 and 21 d of age ($P > 0.05$).

Table 5. Effects of graded levels of dietary beta-sitosterol supplementation on the small intestinal morphology of broiler chickens.

Items	Beta-sitosterol level (mg/kg)					SEM[1]	*P*-values		
	0	25	50	75	100		ANOVA[2]	Linear	Quadratic
14 d									
Duodenum									
Villus height (μm)	1238.49[b]	1437.91[a]	1318.52[ab]	1400.96[a]	1393.66[a]	21.75	0.018	0.048	0.186
Crypt depth (μm)	143.06	155.65	138.16	145.37	134.00	4.11	0.553	0.346	0.513
Villus height: crypt depth (μm)	9.26	10.13	9.88	10.29	11.05	0.31	0.507	0.106	0.867
Jejunum									
Villus height (μm)	589.56[c]	777.61[ab]	819.31[a]	668.47[bc]	687.12[bc]	22.88	0.005	0.517	0.002
Crypt depth (μm)	141.70	150.39	141.35	134.88	133.95	4.14	0.755	0.317	0.647
Villus height: crypt depth (μm)	4.36[b]	5.41[a]	6.09[a]	5.33[a]	5.30[a]	0.17	0.015	0.082	0.005
Ileum									
Villus height (μm)	578.42	548.35	615.42	559.69	550.60	14.49	0.596	0.676	0.520
Crypt depth (μm)	105.14	104.66	119.91	115.75	110.78	3.37	0.568	0.365	0.332
Villus height: crypt depth (μm)	5.68	5.50	5.40	5.02	5.23	0.15	0.724	0.227	0.704
21 d									
Duodenum									
Villus height (μm)	1644.71	1597.79	1606.42	1588.00	1637.08	22.13	0.922	0.881	0.408
Crypt depth (μm)	131.99	145.12	156.72	131.64	145.27	3.53	0.120	0.581	0.209
Villus height: crypt depth (μm)	12.92	11.96	10.65	12.47	11.91	0.35	0.342	0.543	0.192
Jejunum									
Villus height (μm)	1049.57	1025.07	1173.72	1247.37	1265.89	36.16	0.100	0.011	0.969
Crypt depth (μm)	178.20[a]	146.92[bc]	129.71[c]	160.79[ab]	134.47[c]	4.72	0.002	0.008	0.067
Villus height: crypt depth (μm)	6.31[c]	7.21[bc]	9.65[a]	8.21[b]	9.87[a]	0.32	<0.001	<0.001	0.182
Ileum									
Villus height (μm)	651.45[b]	656.28[b]	931.07[a]	758.14[b]	712.15[b]	25.47	<0.001	0.096	0.001
Crypt depth (μm)	139.77[b]	118.73[b]	174.25[a]	136.04[b]	124.06[b]	5.08	0.001	0.610	0.028
Villus height: crypt depth (μm)	4.88[b]	5.75[a]	5.54[ab]	5.98[a]	6.12[a]	0.14	0.032	0.005	0.436

[a-c]Means within a row with different superscripts are different at $P < 0.05$.
[1]SEM, standard error of the mean (n = 6).
[2]ANOVA, analysis of variance.

Table 6. Effects of graded levels of dietary beta-sitosterol supplementation on the serum antioxidant status of broiler chickens.

Items[1]	Beta-sitosterol level (mg/kg)					SEM[2]	*P*-values		
	0	25	50	75	100		ANOVA[3]	Linear	Quadratic
14 d									
MDA (nmol/mL)	2.26[a]	2.04[ab]	1.75[b]	2.10[ab]	2.42[a]	0.07	0.027	0.393	0.003
GSH (mg/L)	15.37	13.88	15.32	15.21	15.33	0.45	0.825	0.707	0.676
SOD (U/mL)	219.32	225.68	232.46	206.83	242.11	5.91	0.417	0.528	0.611
GSH-Px (U/mL)	1227.37[bc]	1177.13[c]	1348.85[ab]	1463.55[a]	1490.29[a]	31.03	<0.001	<0.001	0.609
CAT (U/mL)	2.76[b]	4.20[ab]	5.35[a]	3.93[ab]	4.07[ab]	0.27	0.036	0.173	0.015
21 d									
MDA (nmol/mL)	6.35	4.90	5.58	5.95	6.33	0.30	0.550	0.650	0.202
GSH (mg/L)	16.36	17.33	16.81	16.08	19.52	0.57	0.339	0.213	0.325
SOD (U/mL)	235.29	232.09	212.59	243.53	237.31	4.04	0.144	0.571	0.176
GSH-Px (U/mL)	801.80[b]	756.72[b]	929.47[b]	1094.80[a]	781.98[b]	32.89	0.001	0.103	0.015
CAT (U/mL)	2.71[b]	4.60[ab]	5.56[a]	5.14[a]	2.85[b]	0.37	0.027	0.725	0.001

[a-c]Means within a row with different superscripts are different at $P < 0.05$.
[1]CAT, catalase; GSH, reduced form of glutathione; GSH-Px, Glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
[2]SEM, standard error of the mean (n = 6).
[3]ANOVA, analysis of variance.

Dietary BS supplementation linearly increased 14-d hepatic CAT activity and decreased 21-d hepatic MDA accumulation level (Table 7, P <0.05). The 21-d hepatic MDA concentration in the 25, 75, and 100 mg/kg BS group was lower than that of control and 50 mg/kg BS-supplemented group ($P < 0.05$), and no significant difference was detected in these three BS-treated groups ($P > 0.05$). However, no significant difference was found in hepatic GSH concentration, SOD activity, or GSH-Px activity at 14 or 21 d ($P > 0.05$).

Small Intestinal Redox Status

Dietary BS administration (Table 8) linearly and quadratically reduced duodenal MDA level and increased duodenal GSH concentration at 14 d of age ($P < 0.05$). The BS supplementation, irrespective of its supplemental level, reduced 14-d duodenal MDA level ($P < 0.05$). The 14-d duodenal GSH concentration in the 50 and 75 mg/kg BS-treated groups was both higher than that of control and other two BS-supplemented groups ($P < 0.05$). Dietary BS linearly increased 14-d and 21-d SOD activity, 14-d GSH-Px activity, and 21-d CAT activity in the duodenum ($P < 0.05$). Compared with the control group, the duodenal SOD activity in the BS-supplemented group at both 14 and 21 d was higher than that of control group, when its supplemental level ranged from 50 to 100 mg/kg ($P < 0.05$). Compared with the control group, the administration of 75 or 100 mg/kg BS increased 14-d duodenal GSH-Px activity ($P < 0.05$). In comparison with the control group, the 21-d duodenal CAT activity was increased by BS supplementation at a level of 25, 75, or 100 mg/kg ($P < 0.05$), with its value in 50 mg/kg BS-supplemented group being intermediate.

The BS supplementation linearly and quadratically decreased MDA concentration and increased SOD activity in the jejunum at 14 d of age (Table 9, $P < 0.05$). The 14-d jejunal MDA level was reduced by BS administration, irrespective of its dosage ($P < 0.05$). A higher 14-d jejunal SOD activity was observed in the 100 mg/kg BS group, when compared with control and other BS-supplemented groups ($P < 0.05$). Dietary BS incorporation linearly increased 14-d GSH-Px activity and 21-d SOD and CAT activities, and quadratically

Table 7. Effects of graded levels of dietary beta-sitosterol supplementation on the hepatic antioxidant status of broiler chickens.

Items[1]	Beta-sitosterol level (mg/kg)					SEM[2]	*P*-values		
	0	25	50	75	100		ANOVA[3]	Linear	Quadratic
14 d									
MDA (nmol/mg protein)	0.48	0.43	0.41	0.60	0.33	0.03	0.073	0.571	0.344
GSH (mg/g protein)	4.11	3.46	3.66	4.41	4.61	0.16	0.085	0.064	0.072
SOD (U/mg protein)	112.80	107.11	109.15	111.62	110.71	1.62	0.850	0.979	0.490
GSH-Px (U/mg protein)	41.83	44.91	35.60	41.58	45.88	1.56	0.260	0.659	0.174
CAT (U/mg protein)	4.69	6.73	5.89	6.04	6.76	0.26	0.062	0.048	0.411
21 d									
MDA (nmol/mg protein)	0.57[a]	0.27[b]	0.61[a]	0.31[b]	0.24[b]	0.04	<0.001	<0.001	0.349
GSH (mg/g protein)	2.92	2.90	3.03	2.51	3.24	0.09	0.126	0.680	0.244
SOD (U/mg protein)	75.02	87.80	93.45	82.24	88.50	2.40	0.136	0.193	0.127
GSH-Px (U/mg protein)	46.30	53.34	49.29	46.57	48.65	1.47	0.593	0.847	0.501
CAT (U/mg protein)	9.75	10.54	11.47	9.83	9.66	0.34	0.430	0.718	0.132

[a-b]Means within a row with different superscripts are different at $P < 0.05$.
[1]CAT, catalase; GSH, reduced form of glutathione; GSH-Px, Glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
[2]SEM, standard error of the mean (n = 6).
[3]ANOVA, analysis of variance.

Table 8. Effects of graded levels of dietary beta-sitosterol supplementation on the duodenal mucosal antioxidant status of broiler chickens.

Items[1]	Beta-sitosterol level (mg/kg)					SEM[2]	*P*-values		
	0	25	50	75	100		ANOVA[3]	Linear	Quadratic
14 d									
MDA (nmol/mg protein)	0.37[a]	0.21[b]	0.25[b]	0.22[b]	0.23[b]	0.02	0.005	0.008	0.025
GSH (mg/g protein)	11.22[c]	10.78[c]	18.49[b]	23.31[a]	12.32[c]	1.07	<0.001	0.002	<0.001
SOD (U/mg protein)	367.49[b]	394.29[ab]	431.94[a]	433.02[a]	428.06[a]	7.36	0.006	0.001	0.056
GSH-Px (U/mg protein)	23.01[c]	25.70[bc]	25.95[bc]	51.72[a]	34.23[b]	2.44	<0.001	<0.001	0.274
CAT (U/mg protein)	0.88	0.69	0.86	1.02	0.87	0.04	0.122	0.236	0.802
21 d									
MDA (nmol/mg protein)	0.16	0.15	0.12	0.12	0.16	0.01	0.357	0.637	0.095
GSH (mg/g protein)	12.48	10.76	11.33	11.42	11.56	0.42	0.809	0.707	0.388
SOD (U/mg protein)	328.09[b]	339.92[b]	414.48[a]	388.91[a]	411.36[a]	8.74	<0.001	<0.001	0.132
GSH-Px (U/mg protein)	7.36	6.66	7.36	8.00	6.85	0.23	0.394	0.843	0.621
CAT (U/mg protein)	0.86[b]	1.19[a]	1.06[ab]	1.28[a]	1.22[a]	0.05	0.038	0.013	0.261

[a-c]Means within a row with different superscripts are different at $P < 0.05$.
[1]CAT, catalase; GSH, reduced form of glutathione; GSH-Px, Glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
[2]SEM, standard error of the mean (n = 6).
[3]ANOVA, analysis of variance.

increased 21-d GSH-Px activity in the jejunum ($P < 0.05$). The 14-d and 21-d jejunal GSH-Px activity was increased by 100 and 50 mg/kg BS, respectively ($P < 0.05$). The 21-d jejunal SOD activity was increased by 25, 50, or 100 mg/kg BS, when compared with the control group ($P < 0.05$). Additionally, birds fed a basal diet supplemented with 25, 75, or 100 mg/kg BS exhibited a higher 21-d jejunal CAT activity in comparison with the control group ($P < 0.05$).

Dietary BS administration (Table 10) quadratically increased 14-d SOD and 21-d GSH-Px activities, linearly increased 21-d SOD activity, and linearly and quadratically increased 14-d GSH-Px activity and reduced 21-d MDA level in the ileum ($P < 0.05$). Compared with the control group, the 14-d ileal GSH concentration was increased by 25 or 50 mg/kg BS ($P < 0.05$) to a similar value ($P > 0.05$). The similar effect was also observed for 14-d ileal GSH-Px activity, when its supplemental level was 50 or 75 mg/kg ($P < 0.05$). The 21-d ileal MDA concentration was reduced by BS administration, regardless of its supplemental level ($P < 0.05$). In contrast, the 21-d ileal GSH-Px activity was increased by 25 or 50 mg/kg BS ($P < 0.05$) to a similar value ($P > 0.05$).

DISCUSSION

Up to date, multiple studies have investigated the consequences of dietary administration with different levels of phytosterol on growth performance of different animal species including poultry (Zhao et al., 2019; Ding et al., 2021; Yuan et al., 2021), swine (Hu et al., 2017a, b), and aquatic animals (Couto et al., 2014; He et al., 2022), but the used phytosterol in these studies is almost a kind of phytosterol mixture varying in compositions, rather than a single phytosterol source. As for broilers, Ding et al. (2021) have reported that the administration of phytosterol at the doses of 40 and 80 mg/kg significantly increased feed intake and weight gain in white-feathered broilers. In agreement with this finding, Zhao et al. (2019) have shown that dietary supplementation with phytosterol quadratically increased ADG of Partridge Shank chickens during the grower and overall

Table 9. Effects of graded levels of dietary beta-sitosterol supplementation on the jejunal mucosal antioxidant status of broiler chickens.

Items[1]	Beta-sitosterol level (mg/kg)					SEM[2]	*P*-values		
	0	25	50	75	100		ANOVA[3]	Linear	Quadratic
14 d									
MDA (nmol/mg protein)	0.39[a]	0.22[b]	0.15[b]	0.17[b]	0.20[b]	0.03	0.021	0.013	0.016
GSH (mg/g protein)	12.52	11.26	11.61	11.31	13.46	0.37	0.273	0.457	0.052
SOD (U/mg protein)	431.02[bc]	425.82[c]	420.90[c]	466.78[b]	536.31[a]	9.73	<0.001	<0.001	<0.001
GSH-Px (U/mg protein)	10.55[b]	11.44[b]	11.84[b]	13.93[ab]	16.99[a]	0.66	0.008	0.001	0.201
CAT (U/mg protein)	0.45	0.42	0.49	0.59	0.56	0.03	0.490	0.120	0.867
21 d									
MDA (nmol/mg protein)	0.38	0.29	0.37	0.34	0.31	0.02	0.484	0.533	0.982
GSH (mg/g protein)	12.48	10.76	11.33	11.42	11.56	0.42	0.809	0.707	0.388
SOD (U/mg protein)	397.28[c]	448.63[ab]	451.55[ab]	432.25[bc]	484.26[a]	8.33	0.010	0.004	0.722
GSH-Px (U/mg protein)	7.15[b]	10.31[ab]	13.08[a]	9.71[b]	9.74[b]	0.55	0.009	0.170	0.003
CAT (U/mg protein)	0.86[b]	1.19[a]	1.06[ab]	1.28[a]	1.22[a]	0.05	0.038	0.013	0.261

[a-c]Means within a row with different superscripts are different at $P < 0.05$.
[1]CAT, catalase; GSH, reduced form of glutathione; GSH-Px, Glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
[2]SEM, standard error of the mean (n = 6).
[3]ANOVA, analysis of variance.

Table 10. Effects of graded levels of dietary beta-sitosterol supplementation on the ileal mucosal antioxidant status of broiler chickens.

Items[1]	Beta-sitosterol level (mg/kg)					SEM[2]	*P*-values		
	0	25	50	75	100		ANOVA[3]	Linear	Quadratic
14 d									
MDA (nmol/mg protein)	0.54	0.56	0.53	0.39	0.47	0.04	0.632	0.272	0.940
GSH (mg/g protein)	5.68[b]	8.76[a]	9.20[a]	5.56[b]	7.66[ab]	0.44	0.009	0.768	0.058
SOD (U/mg protein)	215.64	247.85	260.00	247.29	246.30	5.34	0.094	0.094	0.037
GSH-Px (U/mg protein)	16.01[b]	16.83[b]	24.79[a]	22.06[a]	20.19[ab]	1.01	0.020	0.036	0.036
CAT (U/mg protein)	3.51	3.15	3.58	2.66	3.64	0.16	0.268	0.828	0.312
21 d									
MDA (nmol/mg protein)	0.27[a]	0.16[b]	0.16[b]	0.11[b]	0.19[b]	0.01	0.003	0.016	0.002
GSH (mg/g protein)	11.99	11.98	8.78	11.25	10.04	0.47	0.139	0.155	0.391
SOD (U/mg protein)	250.62	336.07	325.06	318.48	349.59	12.39	0.092	0.035	0.287
GSH-Px (U/mg protein)	11.70[c]	17.33[a]	16.38[ab]	13.11[bc]	12.06[c]	0.64	0.004	0.348	0.001
CAT (U/mg protein)	2.48	2.78	2.36	2.25	2.68	0.12	0.608	0.865	0.561

[a-c]Means within a row with different superscripts are different at $P < 0.05$.
[1]CAT, catalase; GSH, reduced form of glutathione; GSH-Px, Glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
[2]SEM, standard error of the mean (n = 6).
[3]ANOVA, analysis of variance.

periods, but linearly decreased FCR during the starter period, with its beneficial effects being more pronounced, when its supplemental level was 50 mg/kg. However, Feng et al. (2020b) reported that dietary phytosterol incorporation at a level of 25 mg/kg did not affect ADG, ADFI, or FCR of yellow-feathered broilers. These inconsistent results can be attributed to the broiler species, supplemental level and composition of phytosterols, diets, feeding environment, and management. We found that BS administration quadratically reduced FCR during 1 to 14 d or 1 to 21 d, and supplementing 25 to 75 mg/kg BS and 25 to 50 mg/kg BS to broiler diet significantly reduced FCR during 1 to 14 d and 1 to 21 d, respectively, when compared with their counterparts fed a control diet only. This result was consistent with an earlier finding of Cheng et al. (2019). The improved feed utilization efficiency resulting from BS administration observed in this study could be explained by the increased nutrient digestibility, elevated pancreatic and small intestinal digestive enzyme activities, improved antioxidant capacity and immune function, and the better intestinal morphology, as reported previously (Hu et al., 2017a,b; Cheng et al., 2019, 2020; Zhao et al., 2019; Ding et al., 2021; Yuan et al., 2021).

The blood cholesterol-lowering property of phytosterols have been extensively reported and continuously investigated since 1953 (Pollak, 1953; Trautwein et al., 2003; Brufau et al., 2008; Feng et al., 2018; Poli et al., 2021). An in vivo study has reported that phytosterol decreased serum triglyceride, total cholesterol, and low-density lipoprotein cholesterol levels in broilers (Ding et al., 2021). In this research, BS quadratically reduced 14-d serum total cholesterol, which is in agreement with the result of a previous study (Cheng et al., 2020).The in vitro cholesterol-lowering effect of BS has been shown to be largely mediated via inhibition of cholesterol biosynthesis, regulation on the key proteins involved in the transportation and metabolism of cholesterol, and prohibition on the conversion of cholesterol to cholesterol esters (Yuan et al., 2020). In rodent animals, the serum cholesterol-lowering activity of BS has been attributed to the reduction of cholesterol absorption and bile acids reabsorption as well as the altered biosynthesis and transportation of cholesterol through the down-regulation of intestinal acyl-CoA: cholesterol acyltransferase 2 and hepatic 3-hydroxy-3-methylglutaryl-CoA reductase and up-regulation of hepatic low-density lipoprotein receptor (Chen et al., 2014, 2020a). In this study, however, the blood cholesterol-lowering was actually not observed at 21 d. The cholesterol metabolism of chickens has been demonstrated be correlated with diet, age, sex, growth rate, physiological status, feeding environment, and management (Wood et al., 1961; Simsek et al., 2009), and serum cholesterol concentration would reduce with the increasing age of chickens, mainly due to the increased cholesterol deposition in the tissues (Wood et al., 1961), which may provide a possible explanation for the unchanged serum cholesterol level at 21 d observed in the current research. However, the exact reason still needs further investigation. The administration of BS also linearly increased total protein level and quadratically reduced alanine aminotransferase activity in the serum of broilers at 21 d of age, indicating that BS could possibly improve the liver function. This finding is partially consistent with the results of Yuan et al. (2021), who have reported that dietary phytosterol at more than or equal to 20 mg/kg level decreased aspartate aminotransferase activity, and increased albumin, immunoglobin A, and immunoglobin G levels in the serum of broilers. The improved liver function, as evident by an increase in total protein level and a decrease in aminotransferase activity, could be explained by the metabolism, cell renewal, immunity, and redox status, which can be beneficially regulated by BS administration, according to available evidence (Feng et al., 2018; Kurano et al., 2018; Yin et al., 2018; Chen et al., 2020b; Devaraj et al., 2020).

The DAO, a unique enzyme, presents in high concentration in the intestinal mucosa of mammals, and its circulating activity has been shown to serve as a sensitive marker of mucosal maturation and integrity, since this enzyme would enter into blood in large

quantities when the intestinal barrier damage occurs (Luk et al., 1980; Honzawa et al., 2011). Usually, D-lactate is predominantly produced by the intestinal microflora in animals, however, this intestine-originated D-lactate would flow into blood through the injured intestinal barrier (Levitt and Levitt, 2020). In the current research, BS linearly and/or quadratically reduced plasma DAO activity and D-lactate level at 14 and 21 d of age, and the values of these two parameters at 14 d were significantly reduced by BS, irrespective of its supplemental level, indicating that BS could beneficially maintain intestinal barrier integrity of broilers at an early age. The improved intestinal integrity and barrier function were further supported by the simultaneously improved intestinal morphology in this research. In detail, BS linearly increased duodenal VH and quadratically increased jejunal VH and ratio of VH and CD at 14 d, and linearly or quadratically increased VH and ratio of VH and CD and decreased CD in the jejunum and ileum at 21 d. Similarly, Ding et al. (2021) have reported that dietary phytosterol supplementation at a level less than or equal to 40 mg/kg increased jejunal and ileal VH and the ratio between VH and CD of broilers. In weaned piglets, Hu et al. (2017a) also observed that phytosterol increased the ratio of VH and CD in both duodenum and jejunum in comparison with their control counterparts. The BS has been shown to effectively improve intestinal barrier integrity and function through the inhibition of inflammatory response by inactivating nuclear factor-kappa B signal transduction pathway, block of the infiltration of inflammatory cells, up-regulation of the expression of antimicrobial peptides, prevention of the colonization of pathogenic bacteria, and improvement in redox status and intestinal microflora composition in rodent animals subjected to different stimuli, including dextran sulfate sodium, high-fat diet (Aldini et al., 2014; Kim et al., 2014; Feng et al., 2017; Ding et al., 2019), and these findings may provide an explanation for the improved intestinal barrier function in this study. Moreover, the intestinal antioxidant capacity of broilers has been enhanced when feeding BS, which would also contribute to the improved intestinal barrier function in this study since the intestinal epithelia is continuously exposed to a wide variety of potentially harmful oxidative insults and redox balance is crucial to the maintenance of intestinal integrity and barrier function, as summarized previously (Mishra and Jha, 2019; Bacou et al., 2021).

The animal body is subjected to constant oxidative attack and, therefore, a complex antioxidant defense system has evolved to generally hold this inevitable attack in balance (Burton and Jauniaux, 2011; Sies et al., 2017). Among them, SOD, a key antioxidant enzyme, is capable of eliminating cellular superoxide anion radicals by converting them to less harmful hydrogen peroxide, which could be further decomposed by CAT or GSH-Px (Sies et al., 2017). The GSH, the most abundant cellular nonprotein thiol antioxidant, serves as a main intracellular redox buffer (Lu, 2013), while MDA is one of the final products of cellular lipid peroxidation (Del Rio et al., 2015). The BS administration linearly or quadratically reduced 14-d serum and 21-d hepatic MDA concentration, and increased serum GSH-Px and CAT activities at 14 and 21 days as well as 14-day hepatic CAT activity. Moreover, BS linearly and/or quadratically increased GSH-Px, CAT and SOD activities and GSH level, and reduced MDA accumulation in the intestinal mucosa at 14 and/or 21 days. The previous findings related to the beneficial consequences of phytosterols on the antioxidant status in livestock and poultry are mostly evaluating phytosterol mixture. In broilers, Zhao et al. (2019) reported that phytosterol addition linearly increased serum GSH-Px activity at 21 and 50 d and hepatic GSH-Px and SOD activities at 21 d, but linearly decreased MDA concentration in the breast muscle at 50 d. Also, Ding et al. (2021) found that phytosterol decreased MDA accumulation, but increased SOD activity in the breast muscle of white-feathered broilers. Moreover, phytosterol has been also shown to inhibit MDA accumulation in both serum and liver of crossbred piglets (Hu et al., 2017a). The improved antioxidant status in this study would be partially associated the direct antioxidant properties of BS. The BS is considered as a modest antioxidant in lipid media, and it could prevent oxidative damage to biological molecules such as cell membrane lipids (Ortiz-escarza et al., 2021). The BS isolated from *Polygonum hydropiper* could effectively scavenge 1,1-diphenyl-2-picrylhydrazyl free radicals, 2, 2′-azinobis 3-ethylbenzthiazoline-6-sulfonic acid radicals, and hydrogen peroxide in vitro (Ayaz et al., 2017). And the BS extracted from *Asclepias curassavica* exhibits a good in vitro free radical scavenging ability against 1,1-diphenyl-2-picrylhydrazyl free radicals and nitric oxide (Baskar et al., 2010). The BS can inhibit the production of cellular reactive oxygen species in keratinocytes subjected to peptidoglycan-induced oxidative stress by up-regulating heme oxygenase-1 protein expression (Liao et al., 2018). Consistently, the administration of BS alleviates lipopolysaccharide/D-galactosamine-induced acute hepatic oxidative stress by up-regulating the expression of hepatic nuclear respiratory factor 2 and heme oxygenase-1 in mice, as evident by the dramatic increases in the activities of SOD and CAT and GSH level as well as the reduced MDA level (Yin et al., 2018). These findings, in turn, suggest that BS could improve cellular antioxidant capacity by activating antioxidant signal pathway, which may also contribute to the improved redox status in this study. In a cell culture study, the BS has been reported to effectively alleviate phorbol ester-induced oxidative stress in RAW 264.7 macrophages by beneficially regulating antioxidant enzyme response, and these effects depend on the estrogen/phosphatidylinositol 3-kinase pathway, probably due to its estrogen-like structure and activity (Vivancos and Moreno, 2005). Additionally, the incorporation BS using 2-hydroxypropyl-β-cyclodextrin as a carrier has also been reported to counteract glucose

oxidase-induced oxidative stress and lipid peroxidation in hippocampal neuronal cells via regulating estrogen receptor-mediated phosphatidyl inositol 3-kinase/glycogen synthase kinase 3 signaling, since the BS is an estrogen agonist for estrogen receptor α and β, and preferentially binds estrogen receptor β (Gutendorf and Westendorf, 2001; Shi et al., 2013), and this finding may also provide an scientific explanation for the elevated antioxidant capability in this research. According to measured parameters, the beneficial effects of BS in regulating antioxidant status are more pronounced in the small intestinal mucosa when compared with liver or serum in the current study. The bioavailability of BS, unlike cholesterol, is only 5% or even less in rodent animals and human, and the gut has been demonstrated to play an important role in the adsorption and metabolism of BS (Borgström, 1968; Salen et al., 1970; Ikeda and Sugano, 1978; Heinemann et al., 1993). In an isotope labeling experiment, the elimination of BS from the body has been shown mainly through feces, and the highest level and the longest retention of radioactivity is observed in the intestinal epithelia in comparison with tissues, urine, and blood (Sanders et al., 2000). And the difference in the pattern of absorption, disposition, and metabolism of BS in animal body would, therefore, partially explain the disparate improvement in the antioxidant capacity in blood, liver, and intestinal mucosa.

In summary, this study suggested that dietary supplementation with BS could increase feed conversion efficiency, improve intestinal barrier function and integrity, and enhance antioxidant capacity of broiler chickens at an early age, and these beneficial effects were more pronounced when its supplemental level was 50 mg/kg of diet.

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DISCLOSURES

The authors declare no conflict of interest.

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