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Original Research Article

Fermented soybean meal improved laying performance and egg quality of laying hens by modulating cecal microbiota, nutrient digestibility, intestinal health, antioxidant and immunological functions

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ABSTRACT

Antinutritional factors in feedstuffs may limit their utilization in livestock production, but fermentation process can be used to improve feed quality; however, studies on fermented soybeans for laying hens remain limited. We investigated the effect of fermented soybean meal (FSBM) at various inclusion levels as a partial replacement for soybean meal (SBM) on egg production, egg quality, amino acid digestibility, gut morphology and microbiota, antioxidant capacity and immune response of young laying hens. A total of 360 Hy-line Brown laying hens aged 18 weeks were selected and divided into 5 groups of 6 replicates each and 12 birds per replicate. The control group received a basal diet while the trial group received the basal diet with FSBM included at 2.5%, 5.0%, 7.5% and 10.0%, respectively, for 12 weeks. Our findings revealed that the nutritional value of FSBM was higher compared to that of SBM in terms of reduced content of trypsin inhibitors and increased contents of crude protein, amino acids and minerals. FSBM enhanced egg production ($P < 0.05$), feed-to-egg ratio ($P < 0.05$), and albumen quality (albumen height and Haugh unit) ($P < 0.05$). Furthermore, FSBM improved apparent fecal amino acid digestibility ($P < 0.05$), gut morphology (increased villus height, villus width, villus height-to-crypt depth ratio and decreased crypt depth) ($P < 0.05$), antioxidant capacity (reduced malondialdehyde and increased catalase, total superoxide dismutase, glutathione peroxidase and total antioxidant capacity) ($P < 0.05$) and immune function (increased concentrations of IgG, IgA, and IgM; increased levels of transforming growth factor beta and Toll-like receptor 2; and reduced levels of interleukin 1β and tumor necrosis factor alpha) ($P < 0.05$). Further analysis showed that FSBM altered the composition of the gut microbiota favoring beneficial microbes. These findings suggest that probiotic fermentation improved the nutritional value of SBM. The inclusion of FSBM in the diets of laying hens at 2.5% or 5.0% improved amino acid digestibility, gut health, immune function, egg production and egg quality.

1. Introduction

Poultry farmers worldwide, including China, use corn–soybean meal diets. Due to its high nutritional value, digestibility and absorption, soybean meal (SBM) is the main vegetable protein source in poultry diets (Chachaj et al., 2019a). Despite their high nutritional value, raw soybeans contain several antinutritional factors, including lectin, phytate, trypsin inhibitors, and glycinin and β-conglycinin, the main soybean antigens (Seo and Cho, 2016; Su

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et al., 2018). These antinutritional factors can limit the bioavailability of nutrients, cause an allergic immune response in animals (Cheng et al., 2019), and stimulate inflammatory reactions that impair gut morphology and function (Drażbo et al., 2018; Soumeh et al., 2019). Therefore, the use of raw soybeans in the diets of poultry, including young laying hens, is limited due to the absence of pelleting or heat treatment to deactivate antinutritional elements in the marsh diets supplied to laying hens. Hence, improving the nutritional value of SBM becomes crucial to animal production.

Food processing techniques such as boiling, roasting, soaking, microwaving, autoclaving and fermentation could be used to mask the undesirable effects of the antinutritional factors in feedstuff (Khattab et al., 2009). Microbial fermented feedstuffs are associated with reduced content of antinutritional factors (Medeiros et al., 2018; Yang et al., 2018). When fed to animals, microbial fermented feedstuffs often result in higher synthesis of lactic acid, which lowers gut pH to exclude pathogens (Su et al., 2018; Sugiharto and Ranjitkar, 2019). Fermented feeds have many benefits, including high mineral, vitamin, and amino acid contents (Zhang et al., 2013), an abundance of small peptides with lower molecular weights (Feng et al., 2007; Tsai et al., 2021), a reduced trypsin inhibitors content (Jazi et al., 2018a; Chachaj et al., 2019a; Liu et al., 2021), the probiotic-like effect (Sembratowicz et al., 2020), and the anti-inflammatory and antioxidant effect (Champagne et al., 2010).

The impact of fermented soybean meal (FSBM) and other fermented feed on production performance, egg quality and physiological functions have been reported for poultry. Fermented feedstuffs including corn–soybean meal (Yang et al., 2022), feed additives (Tian et al., 2022), sesame seed (Jazi and Bishesari, 2022) and SBM (Hong et al., 2010; Xu et al., 2012) increased egg production in laying hens. Fermented phyto-additives improved eggshell quality (Park et al., 2016), while fermented feed improved albumen quality (Guo et al., 2021; Kopacz et al., 2021; Lv et al., 2022; Tian et al., 2022). On the other hand, FSBM had no effect on egg quality (Fujiwara et al., 2008). Furthermore, fermented feed augmented the antioxidant capacity and immune function in laying hens (Karaffová et al., 2022; Zhu et al., 2020). FSBM enhanced immune response in chicks (Chachaj et al., 2019a), and antioxidant capacity in turkeys (Chachaj et al., 2019b). The impact of fermented feed (Guo et al., 2021; Lv et al., 2022), fermented soy bean meal (Xu et al., 2012), and fermented corn–soybean mixed feed (Liu et al., 2021) on gut morphology of laying hens with a consequent positive effect on nutrient utilization have been documented. Additionally, fermented feed altered gut microbiota which culminated in the exclusion of pathogens and improved health status of birds (Omar et al., 2021; Peng et al., 2022; Tian et al., 2022). Also, there is evidence that gut microbiota can alleviate inflammatory response and reduce oxidative stress via the microbiota-gut immunity axis (Brandsma et al., 2015), enhance growth performance and serum immunity via altered composition of cecal microbiota (Li et al., 2020a). Hence, the significant impact of gut microbes on the performance and overall poultry health cannot be overemphasized (Niba et al., 2009). All of these indicate that egg production and egg quality may be modulated by the physiological status and nutrient utilization capacity of the laying hens.

Despite the benefits of fermented feed to poultry nutrition, they contain compounds that may decrease feed palatability, adversely influence metabolic reactions (Canibe and Jensen, 2012) and degrade nutrients like free lysine (Canibe and Jensen, 2003). Also, fermentation of SBM may result in the production of biogenic amines (Putrescine, histamine and tyramine) which negatively affects poultry health and performance (Mah et al., 2019). Therefore, selecting an appropriate dietary inclusion level of FSBM as a partial replacement for SBM in poultry diet becomes expedient. Inclusion of FSBM at 2.5% to 7.5% (Guo et al., 2020) and 6% (Sembratowicz et al., 2020) in broiler diets, and 2.5% (Xu et al., 2012) 3% and 6% (Chachaj et al., 2019a) in laying hen diets had no adverse effects on the birds. However, 9% to 10% of FSBM increased oxidative and inflammatory effects in turkey (Chachaj et al., 2019b). The present study was conducted to investigate the chemical composition of FSBM and the efficacy of partial replacement of SBM with FSBM at inclusion rates of 2.5%, 5.0%, 7.5% and 10.0% on performance, egg quality, amino acid digestibility, serum immune and antioxidant response, gut morphology and microbiota in young laying hens.

2. Materials and methods

2.1. Animal ethics statement

All protocols adopted in the current feeding trial were approved by the Animal Ethic Committee of Feed Research Institute, Chinese Academy of Agricultural Science, Beijing China. The animal ethics approval number CAAS. No. 20210620.

2.2. Experimental design and animal management

A total of 360 Hy-Line Brown laying hens at 18 week old were randomly allocated to 5 dietary groups with 6 replicates each (1 replicate consists of 12 birds, i.e., 4 cages with 3 hens each) following a completely randomized design. The birds were housed in a cage (3-tier battery cages: 40 cm width × 40 cm length × 35 cm height), kept in an environmentally controlled room (light: 16 h, humidity: 50% to 80%), and had access to daily fresh feed and water ad libitum. The experimental diets consist of corn–soybean meal as the basal diet (control), and 4 treatment diets: basal diet with FSBM at inclusion levels of 2.5%, 5.0%, 7.5% and 10.0%, respectively. The FSBM were provided by Wilmar (Shanghai) Biotechnology Research & Development Center Co., Ltd., Shanghai, China. The feeding trial lasted for 13 weeks (1 week for adaptation period and 12 weeks-experimental period). The diets were in marsh form, and the formulations of the diets were isonitrogenous and isocaloric. The analytical chemical and nutrient composition are shown in Table S1. The basal diets presented in Table 1 were formulated to meet the nutritional requirements according to the recommendations of Chinese Ministry of Agriculture (2004) and National Research Council (NRC, 1994).

2.3. Chemical composition of fermented soybean meal

The samples were analyzed for crude protein (CP), crude fiber (CF), ether extract (EE) and dry matter (DM) in accordance with analytical methods of the Association of Official Agricultural Chemists AOAC (1990). Nitrogen content was determined by combustion using method (990.03; AOAC, 1990) with a nitrogen analyzer (Model CNS-2000; LECO Corp., St. Joseph, MO), while the CP is calculated as nitrogen × 6.25. The EE determined according to method (920.39; AOAC, 1990) after hexane extraction in an Ankom extraction system (Macedon, NY). The DM content was determined by oven drying a 5.0 g sample at 105 °C overnight (method 925.09; AOAC, 1990). Metabolizable energy (ME) is calculated according to NRC (1994). The mineral contents (Ca, K, P, Mg, Fe, Zn, Mn and Cu) were analyzed after ashing at 600 °C for 12 h using a muffle furnace. The inductively coupled plasma mass spectrometry (ICP-AES; Vista, Varian, Palo Alto, CA) was used to determine the minerals (method; 985.01; AOAC, 2005). The activity of trypsin inhibitors in the samples was determined using the outlined method proposed by Smith et al. (1980) and the values are expressed as milligram trypsin inhibited per gram of the feed sample. The amino acid content analysis for both fermented and unfermented SBM samples

Table 1
Ingredients and nutrient levels of experimental diets (air-dry basis).[1]

Item	Fermented soybean meal				
	0	2.5%	5.0%	7.5%	10.0%
Ingredients, %					
Corn	63.12	63.64	64.16	64.67	65.20
Soybean meal	25.86	22.87	19.87	16.88	13.85
Fermented soybean meal		2.50	5.00	7.50	10.00
Limestone	9.20	9.19	9.19	9.19	9.19
Soybean oil	0.09	0.07	0.04	0.02	
L-Lysine·HCl			0.01	0.01	0.02
DL-Methionine	0.20	0.20	0.20	0.20	0.20
L-Tryptophan	0.02	0.02	0.02	0.02	0.03
L-Threonine	0.04	0.04	0.04	0.04	0.04
Calcium hydrophosphate	0.90	0.90	0.90	0.90	0.90
Salt	0.30	0.30	0.30	0.30	0.30
Premix[2]	0.25	0.25	0.25	0.25	0.25
Phytase	0.02	0.02	0.02	0.02	0.02
Total	100.00	100.00	100.00	100.00	100.00
Nutrient levels,[3] %					
Crude protein	16.52	16.47	16.53	16.48	16.60
Calcium	3.42	3.45	3.39	3.52	3.55
Total phosphorus	0.48	0.47	0.46	0.48	0.47
Metabolizable energy, MJ/kg	11.27	11.27	11.27	11.27	11.27
SID methionine	0.43	0.43	0.43	0.43	0.43
SID lysine	0.76	0.76	0.76	0.76	0.76
SID tryptophan	0.17	0.17	0.17	0.17	0.18
SID threonine	0.55	0.55	0.55	0.55	0.55
SID methionine + cysteine	0.66	0.66	0.66	0.66	0.65

SID = standard ileal digestibility.
[1] The experimental diets were formulated according to the measured nutrient levels of feedstuffs.
[2] Premix provided the following per kilogram of diets: vitamin A, 12,500 IU; vitamin D_3, 4125 IU; vitamin E, 15 IU; vitamin K, 2 mg; vitamin B_1, 1 mg; vitamin B_2, 8.5 mg; vitamin B_6, 8 mg; vitamin B_{12}, 5 mg; calcium pantothenate, 50 mg; niacin, 32.5 mg; biotin, 2 mg; folic acid, 5 mg; choline, 500 mg; Mn, 65 mg; I, 1 mg; Fe, 60 mg; Cu, 8 mg; Zn, 66 mg; phytase, 350 IU.
[3] Crude protein, calcium, and total phosphorus are analyzed values. Metabolizable energy and SID AA are calculated values.

was determined using the method of AOAC (1997, method No. 994.12), with the aid of an automated amino acid analyzer, after the samples were hydrolyzed with 6 mol/L HCl at 110 °C for 24 h.

2.4. Performance and egg quality parameters

The production performance indices were obtained at four-week intervals all throughout the period of the feeding trial. Eggs were collected on a daily basis; egg number and egg weight were recorded on a replicate basis. Hen day egg production, average egg weight, average daily feed intake, egg mass and feed-to-egg ratio were deduced and computed based on 4-week intervals (week 1 to 4, week 5 to 8, and week 9 to 12).

Egg quality was determined at the interval of 4 weeks. The collection of eggs (18 eggs per treatment: 3 eggs per replicate), with weight of similar range was done on the last day of every 4 weeks. The eggs were collected when freshly laid, and egg quality was determined within 12 h of collection. All sample eggs for egg quality evaluation were kept at room temperature prior to egg quality assessment. The eggs were each weighed with a sensitive electronic scale and eggshell strength was measured with an Egg Force Reader (ORKA Technology Ltd., Ramat HaSharon, Israel). The eggs were then broken, albumen height, yolk color score and Haugh unit value were measured with an Automatic Egg Analyzer (ORKA Food Technology Ltd., Ramat HaSharon, Israel). The albumen and yolk were then separated using an egg separator, and each individual component were weighted. The eggshell was weighted after air-drying for 48 h. The percentage proportion of each component was obtained by subtracting the weight from the whole egg weight and multiply by 100 (Sarlak et al., 2021). The thick and thin albumen were separated with a sieve (40-mesh) and the weight of each fraction was measured (Zhou et al., 2021).

2.5. Blood and serum biochemical indices

At the end of 12 and 31 weeks of age, 30 birds (1 bird per replicate, 6 birds per treatment) were randomly selected. The birds were fasted for 10 h prior to slaughtering and then approximately 5 mL of blood were collected from the wing veins of the selected animals. The collected blood samples in the micro-anticoagulant tubes were kept in a fixed slant position for 30 min and then centrifuged (at 3000 × *g* for 15 min), the separated serum was collected in Eppendorf tubes and stored at −20 °C until analyzed. The whole blood samples were transferred to the laboratory in an ice pack within 1 h of collection for hematology analysis. The hematology analysis was performed with an automated hematology analyzer (BC-2800 Vet, Mindray, Shenzhen, China). Prior to the analysis of the serum indices, the serum was thawed and sustained at 4 °C to avoid activation of enzymes. The concentrations of glutathione transferase (GST), glutathione peroxidase (GSH-Px), catalase (CAT), total superoxidase dismutase (T-SOD), malondialdehyde (MDA) and total antioxidant capacity (T-AOC) in the serum were analyzed with corresponding ELISA kits and spectrophotometrically measured (Shimadzu, model UV-1800, Tokyo, Japan). Serum concentrations of immunoglobulins (IgM, IgA, and IgG), complement proteins (C3 and C4), inflammatory cytokines (interlukin 1β [IL-1β], interleukin 10 [IL-10] and interlukin 6 [IL-6]), and (tumor necrosis factor alpha [TNF-α], transforming growth factor beta [TGF-β], and Toll-like receptor 2 [TLR2]) were determined with the appropriate ELISA kits respectively. All kits were purchased from ML Bio and Jiancheng Bioengineering Institute (Nanjing, China). All protocols according to the manufacturer's instructions were strictly adhered to and used during analysis.

2.6. Morphology analyses of the jejunal villi

Likewise, the jejunal samples were collected from the slaughtered animals. For evaluation of the jejunal villus morphometrics, about 1-cm segment of the midpoint of the jejunum was removed from each bird and fixed in a 10% buffered formaldehyde for about 48 h. The segments were embedded in paraffin and about 6-μm section of each sample were placed on a glass slide, stained with hematoxylin and eosin, then examined under a light microscope (Olympus BX43 microscope; Olympus Corp., Tokyo, Japan). For the villus morphometrics, a total of 10 intact crypt–villus units were selected for each sample and the mean values were obtained for each bird. The villus height measurement was taken from the tip of the villus to the top of the villus–crypt junction. The villus width was taken at the middle point of the villus. The crypt depth measurement was taken from the base upwards to the transition region between the crypt and the villus. All villus morphometrics were measured using an image software (Caseviewer Image). Also, the villus surface area was deduced from the equation ($\pi \times$ villus width $\times$ villus height), while the ratio of villus height to crypt depth was obtained by villus height/crypt depth (Thiam et al., 2021).

2.7. Apparent fecal amino acid digestibility

At the end of the feeding trial (week 12), a total of 90 birds (18 birds for each treatment, 3 birds per replicate) were selected and

kept in another cage fitted with a fecal sample collection tray for 3 d. At an interval of 12 h, the fecal samples were collected in tight-closed plastic bags and kept at −20 °C. During the collection process, all external components such as feathers and any other substances were carefully removed to avoid contamination. At the end of d 3, all fecal samples were thawed, weighed and put in the oven dryer for 72 h at 65 °C. Then weighed again, and pulverized into a powder that can pass through a mesh of 0.05 mm. The feed intake and feces weight (DM basis) for each replicate were recorded and used for the determination of apparent fecal amino acid digestibility. According to the method described by Varzaru et al. (2013), the fecal and feed samples were further analyzed for amino acid contents using HPLC.

Apparent fecal amino acid digestibility coefficient = [1 − (Amino acid content in feces × Feces weight)/(Amino acid content in feed × Feed intake)] × 100.

2.8. Cecal microbiome analysis

At the end of the feeding trial, one laying hen for each repetition was randomly selected, fasted for 12 h and bled from the carotid artery. The laying hens were dissected on a sterile operating table, and the cecum was cut at the same site with sterilized scissors and a scalpel. The cecal digesta was removed and placed in a sterile tube for temporary storage in liquid nitrogen. The sample was then transferred to −80 °C freezer and frozen for 16S rRNA gene sequencing. After slaughtering, cecal giblets were collected from laying hens and stored at −80 °C.

The DNA of the cecal digesta microbial genome was extracted using QIAamp PowerFecal Pro DNA Kit and sequenced at Shanghai Meiji Biomedical Technology Co., Ltd. The extracted DNA samples were used as templates to amplify the 16S rRNA V4 region using primers 515F/806R. The library was constructed using TurSeq DNA PCR-Free Sample Preparation Kit, and qualified for sequencing using NovaSeq 6000 after quantitative analysis by Qubit and qPCR. Sequences were clustered by QIIME 1.9.0 for operable taxonomic units (OTUs), followed by species annotation and abundance analysis in order to analyze the diversity of microorganisms. Linear discrimination of microorganisms (from phylum to genus) among the test groups was performed using a cloud-based platform to analyze the effect size (LEfSe) and perform significance tests.

2.9. Statistical analysis

Data of laying hens on performance, egg quality, and blood biochemical indices were analyzed by one-way ANOVA and Duncan multiple range test using SPSS 26.0 (SPSS Inc., Chicago, IL, USA). Results are listed as means with standard error of mean (SEM). Statistical significance for treatments was considered at *P*-value of less than 0.05. Furthermore, species for different classification levels (Phylum and genus) and *t*-test was performed among the groups and species with significant differences were found. PLSD-A was used to identify the separation of the samples.

3. Results

3.1. Nutritional value of the fermented soybean meal

The nutrient contents (trypsin inhibitors, CP, EE, NDF, ash, mineral, and amino acids) of fermented and ordinary SBM used in the study were respectively presented in Table S1. The FSBM had a lower content of trypsin inhibitors but a higher content of ash and starch. The contents of minerals (Ca, total P, K, Mg, Mn, and Zn) were higher for FSBM. Also, the contents of CP and amino acids, e.g., methionine, cysteine, methionine + cysteine, threonine, tryptophan, arginine, histidine, isoleucine, leucine, and valine for FSBM were higher. Whereas, the SBM had higher contents of EE, CF, neutral detergent fiber, Cu, and Fe.

3.2. Laying performance

The effect of FSBM on egg production and laying performance is presented in Table 2. At week 1 to 4, FSBM increased average egg weight, average egg mass ($P < 0.05$), whereas, there was no influence on average daily feed intake, laying rate and feed-to-egg ratio ($P > 0.05$). The 5.0% inclusion group presented a higher average egg weight and average egg mass numerically, compared to other groups. At week 5 to 8, FSBM increased laying rate and improved feed-to-egg ratio ($P < 0.05$). The 10.0% inclusion group presented a higher laying rate numerically. There was no significant effect of FSBM on average egg weight, average egg mass, average daily feed intake and mortality ($P > 0.05$). At week 9 to 12, FSBM increased egg weight, egg mass, laying rate, enhanced feed-to-egg ratio and reduced the percentage of broken eggs ($P < 0.05$). The 2.5% and 10.0% inclusion groups presented a higher egg weight and egg mass compared to the other treatment groups. There was no effect of FSBM on feed intake and mortality ($P > 0.05$). Assessment of the overall effect which pooled weeks 1 to 12 together, presented a significant effect of FSBM on egg mass, laying rate, and feed-to-egg ratio ($P < 0.05$). The 10.0% inclusion group presented a numerical increase in egg mass, laying rate and improved feed-to-egg ratio compared to other groups. Zero mortality was notable in the treatment groups at the end of week 12.

3.3. Egg quality assessment

The results of egg quality determination are presented in Table 3. At the end of week 4, there was no significant effect of FSBM on all egg quality indices measured ($P > 0.05$). At the end of week 8, FSBM influenced proportions of albumen and yolk, thick and thin albumen fraction, thick to thin albumen ratio, albumen height, Haugh unit and shell strength ($P < 0.05$). Yolk proportion was lower in 5.0%, 7.5% and 10.0% groups while 2.5% was higher and comparable to control group. For albumen proportion, all FSBM groups had higher values following this descending order: 7.5%, 10.0%, 2.5% and 5.0%. The percentage of thick albumen was of higher values for all FSBM groups in this descending order: 7.5%, 10.0%, 5.0% and 2.5%. Whereas, the percentage of thin albumen fraction was lower for all FSBM groups following this descending trend: 7.5%, 10.0%, 2.5% and 5.0%. The ratio of thick to thin albumen fraction was higher for all FSBM groups and in this order: 7.5%, 10.0%, 5.0% and 2.5%. Increased shell strength was notable for all FSBM groups with numerical increase in this trend: 7.5%, 10.0%, 5.0% and 2.5%. Also, albumen height and Haugh unit were higher for all FSBM groups and the increasing trend was: 7.5%, 10.0%, 5.0% and 2.5%. However, there was no significant effect of FSBM on yolk color and shell proportion ($P > 0.05$). At the end of week 12, the significant influence of FSBM on thick and thin albumen fractions, the ratio of thick to thin albumen, yolk color, albumen height and Haugh unit were obvious ($P < 0.05$). There were no variations among FSBM treatments for thick and thin albumen fractions, the ratio of thick to thin albumen and albumen height. For the Haugh unit, the values are numerically higher but in the following order: 10.0%, 7.5%, 5.0% and 2.5%. Nevertheless, there were no significant effect of FSBM on proportions of egg components (shell, yolk and albumen proportions) and shell strength ($P > 0.05$).

Table 2
Effects of dietary fermented soybean meal on performance of laying hens.

Item	Fermented soybean meal					SEM	*P*-value
	0	2.5%	5.0%	7.5%	10.0%		
Week 1 to 4							
Average egg weight, g	40.07^{b}	42.15^{a}	43.18^{a}	41.50^{ab}	41.96^{ab}	0.88	0.018
Average egg mass, g	25.77^{c}	28.38^{a}	28.80^{a}	27.56^{ab}	28.64^{a}	1.35	0.011
Average daily feed intake, g	78.79	75.44	79.92	78.42	80.08	1.87	0.005
Laying rate, %	66.57	63.05	65.63	66.80	68.35	3.53	0.225
Feed-to-egg ratio, g/g	2.79	2.94	2.78	2.87	2.81	0.15	0.513
Abnormal to broken eggs	0.02^{b}	0.00^{c}	0.02^{b}	0.05^{ab}	0.08^{a}	0.03	0.002
Mortality	0.01	0.00	0.00	0.01	0.00	0.00	0.567
Week 5 to 8							
Average egg weight, g	54.30	54.79	54.48	54.51	54.79	0.29	0.094
Average egg mass, g	45.98	48.46	47.40	47.62	49.63	1.90	0.087
Average daily feed intake, g	105.05	99.64	105.00	100.85	103.97	3.81	0.223
Laying rate, %	84.78^{b}	88.47^{a}	88.21^{a}	88.60^{a}	89.39^{a}	2.15	0.021
Feed-to-egg ratio, g/g	2.30^{a}	2.06^{b}	2.10^{ab}	2.13^{ab}	2.09^{b}	0.12	0.044
Abnormal to broken eggs	0.06	0.07	0.02	0.10	0.10	0.04	0.081
Mortality	0.02	0.01	0.02	0.01	0.01	0.02	0.494
Week 9 to 12							
Average egg weight, g	54.50^{b}	56.36^{a}	55.11^{ab}	55.43^{ab}	56.90^{a}	0.48	0.004
Average egg mass, g	49.14^{b}	52.73^{a}	50.16^{ab}	52.59^{a}	52.88^{a}	1.67	0.003
Average daily feed intake, g	125.91	123.99	127.13	125.14	124.67	4.35	0.838
Laying rate, %	88.31^{b}	93.55^{a}	92.35^{a}	94.09^{a}	92.93^{a}	2.21	0.004
Feed-to-egg ratio, g/g	2.57^{a}	2.35^{b}	2.54^{ab}	2.38^{b}	2.36^{b}	0.10	0.002
Abnormal to broken eggs	0.05^{c}	0.10^{b}	0.02^{c}	0.20^{a}	0.12^{b}	0.06	0.002
Mortality	0.01	0.00	0.00	0.00	0.00	0.00	0.068
Week 1 to 12							
Average egg weight, g	51.29	51.76	51.62	51.30	51.68	0.38	0.216
Average egg mass, g	41.17^{b}	42.32^{ab}	42.12^{ab}	42.59^{ab}	43.72^{a}	1.14	0.034
Average daily feed intake, g	103.25	99.69	104.02	101.47	102.91	2.21	0.063
Laying rate, %	79.89^{b}	81.69^{a}	82.06^{a}	83.16^{a}	83.56^{a}	1.81	0.042
Feed-to-egg ratio, g/g	2.51^{a}	2.36^{b}	2.48^{ab}	2.39^{b}	2.35^{b}	0.08	0.017
Abnormal to broken eggs	0.04^{bc}	0.06^{b}	0.02^{c}	0.11^{a}	0.10^{ab}	0.03	0.002
Mortality	0.02	0.00	0.00	0.01	0.00	0.01	0.446

^{a,b,c}Means within a row with different superscripts differ significantly ($P < 0.05$).

3.4. Haematological and serum biochemical profiles

The haematological indices of laying hens fed dietary FSBM are presented in Table 4. The blood indices of white blood cell count and neutrophil-to-lymphocytes ratio were significantly influenced by FSBM and there were variations among the treatments ($P < 0.05$). Whereas, there was no significant effect of FSBM on other blood indices including red blood cells count, hemoglobin count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, neutrophils and lymphocytes ($P > 0.05$).

The influence of dietary FSBM on the serum antioxidant, immune and inflammatory parameters of laying hens are presented in Table 5. The effect of FSBM on the antioxidant system (T-SOD, GSH-Px, CAT and T-AOC) and oxidant cue (MDA) was highly significant ($P < 0.05$). On the other hand, the 10.0% inclusion group recorded the lowest concentration of MDA in the serum. Concentrations of serum IgM, IgG and IgA, and complement proteins (C3 and C4) were significantly influenced by FSBM ($P < 0.05$). The 10.0% inclusion group recorded significantly higher values compared to other treatment groups. In addition, the pro-inflammatory cytokines of IL-1β, and TNF-α were influenced by dietary FSBM ($P < 0.05$), the treatment group recorded the lowest values for IL-1β and TNF-α. Also, the anti-inflammatory cytokines of TGF-β and TLR2 were influenced by dietary FSBM ($P < 0.05$). The 10.0% group recorded the lowest and highest values, for both pro-inflammatory and anti-inflammatory cytokines, respectively. The effect of FSBM on anti-oxidant and immune function were increased in the following manner: 10.0%, 7.5%, 5.0% and 2.5%.

3.5. Apparent amino acid digestibility

The apparent whole gut amino acid digestibility of laying hens fed FSBM diets is presented in Table 6. The effects of FSBM on apparent digestibility of most amino acids (asparagine, threonine, serine, alanine, cysteine, valine, methionine, tyrosine, histidine, lysine and tryptophan) was highly significant ($P < 0.05$). Significant improvements in the digestibility of all amino acids both essential and non-essential, were highest in the 2.5% group followed by the 5.0% and 7.5% groups. There was no significant effect of FSBM on amino acids including glutamine, isoleucine, leucine, phenylalanine and arginine ($P > 0.05$).

3.6. Jejunal villus morphological structure

The effects of FSBM on morphological characteristics of the jejunal villi of laying hens are presented in Table 7. There were significant effects of FSBM on the villus indices (villus height, villus width, villus surface area), crypt depth, villus height-to-crypt depth ratio, and lamina propria thickness. FSBM inclusion led to significant increases in villus height, villus width, villus surface area and villus height-to-crypt depth ratio ($P < 0.05$), but caused a reduction in crypt depth compared to the control ($P < 0.05$). There were no significant variations among the treatment groups for villus height and crypt depth ($P > 0.05$). The higher values for villus height-to-crypt depth ratio, villus width and villus surface area were notable for 10.0% group followed by 7.5%, 5.0% and 2.5% groups. Nevertheless, there was no effect of FSBM on epithelial thickness ($P < 0.05$).

Table 3
Effects of dietary fermented soybean meal on egg quality.

Item	Fermented soybean meal					SEM	*P*-value
	0	2.5%	5.0%	7.5%	10.0%		
Week 4							
Shell, %	9.60	9.50	9.40	9.56	9.47	0.54	0.117
Yolk, %	27.80	27.57	27.70	27.71	27.78	0.97	0.451
Albumen, %	62.60	62.93	62.90	62.73	62.75	1.05	0.666
Thick albumen, %	57.27	57.80	54.81	57.62	57.98	2.20	0.071
Thin albumen, %	42.73	42.20	45.19	42.38	42.02	2.18	0.117
Thick to thin albumen ratio	1.34	1.36	1.21	1.35	1.37	0.11	0.152
Shell strength, N/m^2	37.67	35.27	38.06	38.27	38.38	2.90	0.621
Yolk color	5.72	5.72	5.72	5.72	5.72	0.50	1.000
Albumen height, mm	7.48	7.88	7.96	7.80	7.82	0.46	0.539
Haugh unit	87.01	89.49	89.82	87.81	88.10	2.55	0.588
Week 8							
Shell, %	9.28	9.39	9.46	9.28	9.56	0.31	0.549
Yolk, %	28.19^a	27.44^{ab}	27.40^b	26.02^c	26.07^c	0.82	0.004
Albumen, %	61.71^c	63.17^b	63.14^b	64.70^a	64.37^a	0.87	0.003
Thick albumen, %	56.99^c	58.82^b	63.33^b	66.55^a	64.48^a	3.12	<0.001
Thin albumen, %	43.01^a	41.18^{ab}	36.67^b	33.45^c	35.52^c	3.06	<0.001
Thick to thin albumen ratio	1.32^c	1.43^{ab}	1.73^b	1.99^a	1.82^a	0.22	0.001
Shell strength, N/m^2	35.26^c	38.17^b	43.83^a	40.92^{ab}	41.35^a	3.17	0.002
Yolk color	5.22	5.17	5.72	5.72	5.89	0.55	0.185
Albumen height, mm	7.11^c	7.22^c	7.97^b	8.92^a	8.68^a	0.65	0.001
Haugh unit	84.39^c	85.09^c	89.06^b	94.14^a	93.22^a	3.57	0.003
Week 12							
Shell, %	9.54	9.71	9.45	9.53	9.57	0.25	0.655
Yolk, %	28.21	26.25	26.27	26.10	26.23	0.71	0.296
Albumen, %	62.65	64.04	64.28	64.37	64.20	0.69	0.403
Thick albumen, %	58.77^b	62.52^a	62.69^a	63.24^a	62.42^a	3.32	0.042
Thin albumen, %	41.23^a	37.48^b	37.31^b	36.76^b	37.58^b	2.20	0.048
Thick to thin albumen ratio	1.43^b	1.67^a	1.68^a	1.72^a	1.66^a	0.23	0.033
Shell strength, N/m^2	42.19	45.31	43.51	42.29	45.93	3.78	0.261
Yolk color	5.17^b	5.00^b	6.33^a	5.61^{ab}	5.44^b	0.51	0.011
Albumen height, mm	7.97^b	8.70^a	8.76^a	8.60^a	8.80^a	0.46	0.043
Haugh unit	89.47^b	90.25^{ab}	91.01^{ab}	92.80^a	93.68^a	2.51	0.031

^{a,b,c}Means within a row with different superscripts differ significantly ($P < 0.05$).

Table 4
Effects of dietary fermented soybean meal on blood routine examination of laying hens.

Item	Fermented soybean meal					SEM	*P*-value
	0	2.5%	5.0%	7.5%	10.0%		
WBC, $\times 10^9$ mL^{-1}	12.12^c	17.22^b	18.46^{ab}	18.51^{ab}	19.97^a	1.84	<0.001
RBC, $\times 10^{12}$ mL^{-1}	2.27	2.31	2.23	2.23	2.31	0.12	0.792
HGB, g/dL	71.00	69.80	73.67	73.17	73.33	3.76	0.551
HCT, %	35.05	34.38	34.68	35.15	35.47	2.12	0.957
MCV, fL	154.93	160.42	155.63	157.38	153.72	7.01	0.804
MCH, pg	31.43	30.28	33.37	32.75	31.78	1.54	0.109
MCHC, g/L	202.67	199.40	213.67	208.00	206.67	6.92	0.188
PLT, $\times 10^9$mL^{-1}	10.60	11.40	11.67	11.60	12.00	2.14	0.086
HT, %	52.43	50.50	48.12	50.00	44.46	9.17	0.773
LYM, %	37.20	36.07	35.10	40.55	45.54	7.24	0.232
H/L ratio	1.41^c	1.40^c	1.37^b	1.23^b	0.97^a	0.20	0.048
MONO, %	1.30	3.52	2.18	2.43	5.23	2.24	0.237
EO, %	0.43	0.98	0.72	2.05	1.47	1.33	0.501
BASO, %	7.34	5.52	7.47	12.47	6.72	4.84	0.283

SEM = standard error; RBC = red blood cells count; HBG = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLT = platelets; HT = heterophil; LYM = lymphocytes; H/L ratio = heterophil-to-lymphocyte ratio; MONO = monocytes; EO = eosinophils; BASO = basophils.
^{a,b,c} Means within a row with different superscripts differ significantly ($P < 0.05$). WBC-white blood cells count.

3.7. Microbial analysis

The microbiota analysis for both phylum and genus levels analyzed using Partial-Least Squares Discriminant analysis (PLS-DA) were presented in Fig. 1. The results showed that at phylum level, 5.0% and 7.5% FSBM inclusion groups were not distinct from the control group, whereas the 2.5% group was distinctly separated from other groups, which may imply variations in the abundance of microorganisms present. At the genus level, the separation effect between CON and other FSBM groups was obvious. Hence, cecal microflora significantly differed among CON and fermented feed replacement groups ($P < 0.05$). The analysis of various species of microbes found in the cecum is presented in Fig. 2. There was significant variation among the species examined at both phylum and genus levels, relative to each treatment group ($P < 0.05$). At phylum and genus levels, there were variations in abundance of

Table 5
Effects of dietary fermented soybean meal on serum antioxidant capacity of laying hens.

Item	Fermented soybean meal					SEM	*P*-value
	0	2.5%	5.0%	7.5%	10.0%		
MDA, nmol/mL	11.20^{a}	9.07^{b}	8.10^{b}	7.70^{b}	5.51^{c}	1.75	<0.001
CAT, U/mL	8.51^{c}	10.50^{b}	13.02^{a}	11.26^{b}	13.48^{a}	1.17	<0.001
T-SOD, U/mL	114.02^{c}	120.12^{b}	128.60^{ab}	135.97^{a}	137.27^{a}	6.05	<0.001
GSH-Px, ng/mL	41.78^{d}	44.20^{cd}	47.20^{c}	59.28^{b}	76.87^{a}	7.10	<0.001
T-AOC, U/mL	10.80^{c}	11.78^{b}	11.56^{b}	13.10^{a}	13.40^{a}	0.79	0.002
IgG, μg/mL	60.18^{c}	60.65^{c}	68.64^{b}	69.90^{ab}	71.68^{a}	5.77	0.017
IgM, ng/mL	1698^{c}	2,515^{b}	2,530^{b}	3,618^{a}	3,620^{a}	402	<0.001
IgA, ng/mL	4620^{c}	5,254^{b}	5,485^{b}	5,870^{a}	5,980^{a}	386	0.011
C3	75.77^{b}	79.78^{b}	89.07^{ab}	92.83^{a}	97.49^{a}	7.63	0.003
C4	19.56^{b}	20.63^{b}	20.41^{b}	22.20^{ab}	25.36^{a}	3.04	0.052
IL-1β	1.00^{a}	0.91^{ab}	0.81^{b}	0.65^{c}	0.66^{c}	0.10	<0.001
IL-6	1.00	1.09	0.94	1.20	1.06	0.14	0.057
TNF-α	1.00^{a}	0.77^{b}	0.66^{bc}	0.50^{c}	0.57^{c}	0.08	<0.001
IL-10	1.00	0.97	1.17	0.99	0.82	0.21	0.216
TGF-β	1.00^{c}	1.33^{b}	1.36^{b}	1.55^{a}	1.58^{a}	0.14	<0.001
TLR2	1.00^{c}	2.55^{b}	2.76^{ab}	2.70^{b}	2.94^{a}	0.18	<0.001

SEM = standard error; MDA = malondialdehyde; CAT = catalase; T-SOD = total superoxide dismutase; GSH-Px = glutathione peroxidase; T-AOC = total antioxidant capacity; IgG = immunoglobulin G; IgM = immunoglobulin M; IgA = immunoglobulin A; C3 and C4 = complement proteins; IL-1β = interlukin 1β; IL-10 = interleukin 10; IL-6 = interlukin 6; TNF-α = tumor necrosis factor alpha; TGF-β = transforming growth factor beta; TLR2 = Toll-like receptor 2.
[a,b,c,d]Means within a row with different superscripts differ significantly ($P < 0.05$).

Table 6
Effects of dietary fermented soybean meal on apparent digestibility of whole gut amino acids of laying hens (%).

Item	Fermented soybean meal				SEM	*P*-value
	0	2.5%	5.0%	7.5%		
Asparagine	67.68^{c}	74.38^{a}	72.79^{ab}	69.60^{bc}	3.33	0.049
Threonine	59.61^{b}	67.44^{a}	67.19^{a}	60.28^{b}	4.39	0.035
Serine	68.89^{b}	74.50^{a}	73.37^{a}	67.90^{b}	3.25	0.036
Glutamic acid	77.27	81.55	80.73	78.30	2.31	0.071
Proline	73.20^{b}	77.07^{a}	76.05^{a}	72.16^{b}	2.62	0.044
Alanine	62.74^{b}	68.90^{a}	67.86^{a}	61.96^{b}	4.20	0.034
Cysteine	62.49^{b}	67.85^{a}	66.18^{a}	45.80^{c}	5.63	<0.001
Valine	61.59^{b}	68.48^{a}	67.25^{a}	60.85^{b}	4.31	0.040
Methionine	63.80^{c}	78.60^{a}	74.50^{a}	70.68^{b}	4.25	0.001
Isoleucine	66.16	72.28	70.64	65.95	3.91	0.088
Leucine	74.54	78.05	77.76	73.49	2.81	0.110
Tyrosine	70.90^{b}	79.89^{a}	78.85^{a}	72.30^{b}	3.41	0.042
Phenylalanine	71.15	75.89	76.63	72.71	2.96	0.078
Histidine	76.24^{b}	83.70^{a}	80.10^{a}	77.63^{b}	2.40	0.028
Lysine	68.51^{b}	76.50^{a}	74.30^{a}	69.49^{b}	3.10	0.020
Arginine	80.59	84.37	83.28	80.81	2.08	0.080
Tryptophan	85.23^{b}	91.52^{a}	87.24^{b}	87.53^{b}	2.07	0.002

[a,b,c]Means within a row with different superscripts differ significantly ($P < 0.05$).

Wittenberg polluted soil-2 bacteria (WPS-2) ($P < 0.05$), which was significantly higher in 2.5% group and very low for 7.5% group. At phylum level, there was a significant effect of FSBM on *Unclassified_f_Tannaerellaceae* ($P = 0.039$), and the lowest and highest values were recorded for the control and the 5.0% group, respectively. Significant variations were found for *f_Eubacterium_coprostanoligenes_group* ($P = 0.019$), and 2.5% recorded the highest value while the 5.0% group had the lowest value. The genus

Table 7
Effects of dietary fermented soybean meal on jejunal mucosal morphology of laying hens.[1]

Item	Fermented soybean meal					SEM	*P*-value
	0	2.5%	5.0%	7.5%	10.0%		
VH, μm	930.75^{b}	1200.66^{a}	1260.25^{a}	1306.27^{a}	1356.86^{a}	80.89	<0.001
CD, μm	137.11^{a}	110.29^{b}	105.70^{b}	108.23^{b}	105.81^{b}	9.38	<0.001
VH:CD	6.79^{c}	10.89^{b}	11.92^{a}	12.06^{a}	12.82^{a}	1.27	<0.001
VW, μm	129.09^{c}	164.44^{b}	171.07^{a}	173.05^{b}	205.29^{a}	11.65	<0.001
VSA, mm^2	380,864^{c}	623,070^{b}	686,019^{ab}	707,367^{a}	874,931^{a}	69,943	<0.001
LPT, mm	39.63^{b}	50.59^{ab}	49.32^{ab}	51.97^{ab}	60.32^{a}	8.46	0.021
EPT, mm	36.00	48.63	43.82	38.27	44.15	6.82	0.063

SEM = standard error of mean; VH = villus height; CD = crypt depth; VH:CD = villus height-to-crypt depth ratio; VW = villus width; VSA = villus surface area; LPT = lamina propria thickness; EPT = epithelial thickness.
[a,b,c]Means within a row with different superscripts differ significantly ($P < 0.05$).
[1] Data represent mean of 6 replicates of 3 hen each.

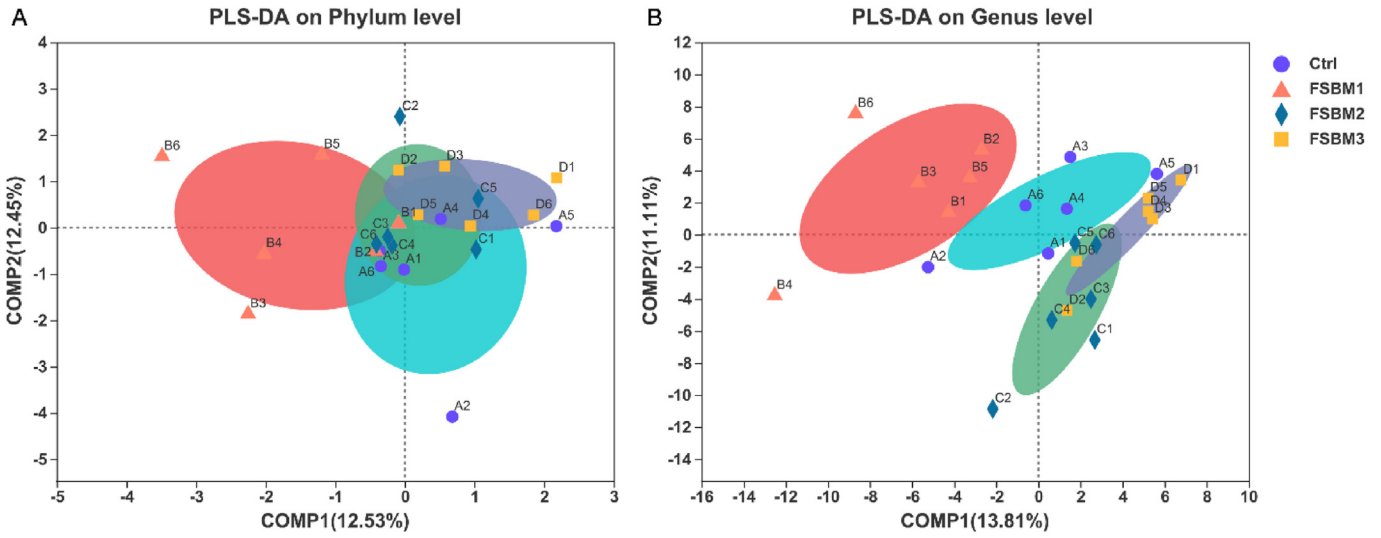

Fig. 1. Effects of dietary fermented soybean meal (FSBM) on cecal microbial diversity of laying hens. The abscissa and ordinate represent the 2 chosen principal components (COMP 1 and COMP2); the percentage indicates the contribution of the principal component to the variations in the sample composition; the different colors represent samples from various treatment groups; the closer the sample groups, the more similar the composition of the 2 groups and vice versa. (A) PLS-DA on phylum level; (B) PLS-DA on genus level. Ctrl = control; FSBM1 = 2.5% FSBM; FSBM2 = 5.0% FSBM; FSBM3 = 7.5% FSBM.

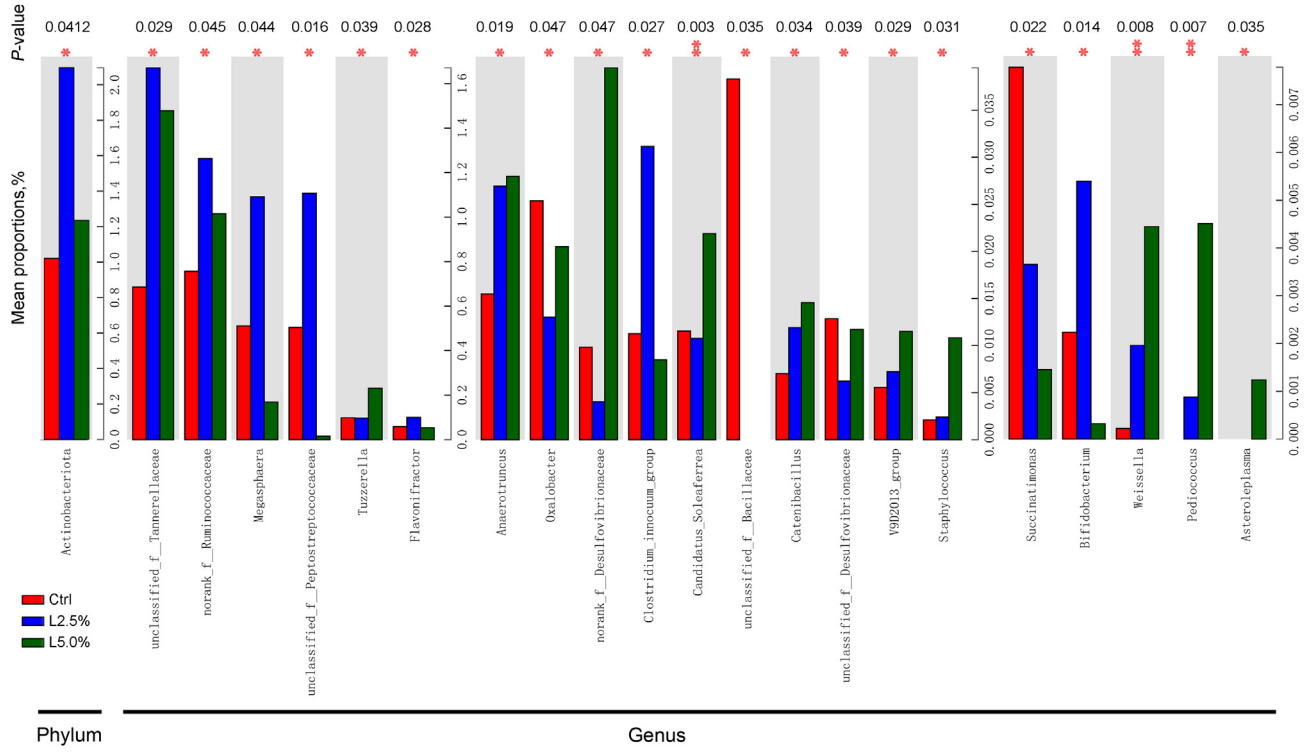

Fig. 2. Effects of dietary fermented soybean meal (FSBM) on cecal microorganisms of laying hens at phylum and genus levels. Ctrl = control; FSBM1 = 2.5% FSBM; FSBM2 = 5.0% FSBM; FSBM3 = 7.5% FSBM; WPS-2 = Wittenberg polluted soil-2 bacteria; CAG-56 = *Firmicutes bacterium CAG-56*. *: significant ($P < 0.05$); **: highly significant ($P < 0.01$).

f_nonrank_o_Saccharimondales was influenced by FSBM ($P = 0.031$), and the control and 7.5% groups had the highest values while 2.5% and 5.0% recorded almost zero values. The abundance of *Firmicutes bacterium CAG-56* differed significantly among the groups ($P = 0.019$), and 2.5% group recorded the highest value while the 7.5% group was lower than the control and the 5.0% groups. *Anaerostipes* differed among the groups ($P = 0.009$), and the values for the control and 2.5% groups were comparable but the 7.5% group recorded the lowest value. The abundance of Firmicutes was found to be significantly affected by treatments ($P = 0.023$), 2.5% and 5% groups were comparable while that of 7.5% group had the lowest value. *Staphylococcus* was found to differ among the groups ($P = 0.027$), and the control group was almost zero value. The abundance of *Weissella* was found to be significantly different among the groups ($P = 0.034$), the 7.5% group recorded the highest value while control was lower compared to other groups.

Pediococcus differed among the groups ($P = 0.002$), notably this genus was absent in the control group, while the 7.5% group recorded the highest value. *Lachinospira* varied significantly among groups ($P = 0.013$), was absent in the control and the 7.5% group, the 2.5% group was higher than the 5.0% group which was very low.

4. Discussion

Diets containing FSBM have a high nutritional value, resulting in improved animal health and performance. Thus, the utilization of FSBM in diet for poultry, including broilers (Chachaj et al., 2019a; Guo et al., 2020), quails (Jazi et al., 2018a), and laying hens (Hong et al., 2010; Xu et al., 2012), have gained focus in poultry research. Nevertheless, available data on laying hens are scarce. The present study presents ample evidence that fermentation process can decrease the antinutritional factors such as trypsin inhibitors present in SBM and improve its nutritional value with positive effects on the physiological response and health of the animal.

4.1. Nutritional value of fermented soybean meal

Chemical composition of feedstuffs provides a clue to their nutritive value. In the study, the nutritional value of FSBM was higher compared to SBM as it contained a reduced level of trypsin inhibitors, increased contents of minerals (K, Mg, Mn and Zn), a higher content of amino acids (Asp, Ser, Ala, Val, Met, Tyr, His, Lys and Trp) and CP, and a reduced level of CF. This supports previous reports that fermentation improves the nutritional value of feedstuffs for better and more efficient nutrient utilization. In agreement with our findings, fermentation process reduced the content of trypsin inhibitors in FSBM compared to raw SBM (Xu et al., 2012; Jazi et al., 2018a; Chachaj et al., 2019a; Sembratowicz et al., 2020; Liu et al., 2021). The positive effect could be attributed to the fermentation process which may induce structural alterations and precipitation, which could inactivate binding sites of trypsin inhibitors, hence, lowering their contents (Chi and Cho, 2016). In addition, a higher content of minerals (Zn and Mn) in FSBM (Sembratowicz et al., 2020), is in support of our findings. An indication that the fermentation process may be efficient in increasing the bioavailability of minerals for the sustenance of animal health and the production of high-quality eggs. Also, the higher contents of amino acids in FSBM compared to SBM (Teng et al., 2012; Jazi et al., 2018a; Chachaj et al., 2019b; Sembratowicz et al., 2020) agrees with our findings. There was an increase in the amino acid levels in hens fed the diets containing FSBM. The fact that microbial fermentation may hydrolyze proteins and create proteases that enhance the levels of peptides and free amino acids in FSBM (Sun et al., 2013; Shi et al., 2017b) may partially explain this finding. The higher CP and lower CF contents in the FSBM compared to normal SBM are in affirmation with previous findings (Chachaj et al., 2019a; Jazi et al., 2018a; Li et al., 2020b; Sembratowicz et al., 2020). These effects may be due to the ability of the microorganisms to degrade fiber to release more utilizable carbohydrates (Mukherjee et al., 2015; Ashayerizadeh et al., 2017; Olukomaiya et al., 2019). Hence, the improvement in the nutritional value of FSBM, i.e., reduced trypsin inhibitors and CF, and increased levels of CP, amino acids and minerals, would, in turn, promote nutrient absorption by the host and enhance laying performance.

4.2. Effect of fermented soybean meal on laying performance

The assessment of laying performance and feed utilization efficiency, which were improved in the study, is the key to enhancing laying performance for increased economic benefits in chicken production. In support of our findings, egg production was improved in laying hens fed fermented feed additives (Kothari et al., 2021), and 3.5% FSBM (Xu et al., 2012). Furthermore, fermented feed improved the feed-to-egg ratio in hens (Xu et al., 2012), feed conversion ratio in quails (Jazi et al., 2018a), and nutrient utilization in pigs (Yuan et al., 2017). Furthermore, fermented corn byproducts (Yang et al., 2022), fermented sesame seed (Jazi and Bishesari, 2022), and fermented corn–soybean mixed feed (Guo et al., 2021; Lv et al., 2022) improved feed efficiency in birds. Moreover, phytogenic feed additive containing *Gynura procumbens* and *Rehmannia glutinosa* enhanced egg weight of Hy-Line brown laying hens (Park et al., 2016), which lends support to our findings. The improvement in egg production and feed efficiency suggests a higher nutritional value of FSBM and a more efficient utilization of nutrients. It is known that trypsin inhibitors cause gut inflammation (Feng et al., 2007), and hence a reduction in their contents increases the level of small-size peptides, digestive enzyme activities, protein synthesis, and amino acid release (Cheng et al., 2019; Feng et al., 2007; Shi et al., 2017a; Liu et al., 2021). Thus, a lower trypsin inhibitors level may have contributed to improvement in laying performance. Fermented feed's probiotic-like effect may have nourished the gut epithelial cells and encouraged the proliferation of beneficial microorganisms to boost nutrient utilization. This claim is supported by earlier reports that fermented feed stimulates the proliferation of lactic acid bacteria and the synthesis of lactic acid, which reduces gut pH (Jazi et al., 2018a; Su et al., 2018; Sembratowicz et al., 2020) and increases beneficial microbe colonization (Omar et al., 2021; Peng et al., 2022; Tian et al., 2022). Conversely, fermented feed (Guo et al., 2021) and FSBM (Fujiwara et al., 2008) did not affect egg production. Also, FSBM did not affect egg weight (Hong et al., 2010; Xu et al., 2012). The variations in the studies could be due to the fermentation process used. It could be inferred that the higher nutritional value, energy utilization and digestibility of the fermented feed, may have resulted in improved laying performance and feed-to-egg ratio. We hypothesized that the improved nutritional value of FSBM could not only boost laying rate but also be associated with egg component formation. As a result, egg quality was investigated.

4.3. Effect of fermented soybean meal on egg quality of laying hens

Egg quality assessment is a primary responsibility for poultry farmers to gain consumers' trust in their preference for table eggs and utilization by the food processing sector. The improvement in eggshell quality was in line with a previous report that fermented phyto-additive improved eggshell strength (Park et al., 2016), and this is crucial to the shelf life of eggs. The improved eggshell quality could be a consequence of increased mineral absorption. In addition, albumen height and Haugh unit are used to measure albumen quality (Shi et al., 2020). Albumen with a higher Haugh unit has a thicker albumen content and a higher viscosity suggests freshness (Zhang et al., 2020). Our results on high-quality albumen conform with other studies which reported that fermented feed improved albumen quality (Guo et al., 2021; Kopacz et al., 2021; Lv et al., 2022; Tian et al., 2022; Yang et al., 2022). The better albumen quality could be attributed to improved CP content and amino acid digestibility. Because high quality albumen is often linked to better protein synthesis (Zhou et al., 2021) and FSBM improves protein metabolism. Oxidative stress impairs oviduct function, including albumen synthesis (He et al., 2017). Thus, the fermented feed's anti-inflammatory and antioxidant properties may have sustained a healthy oviduct for increased albumen secretion. On the other hand, there was no effect of FSBM products on albumen quality (Fujiwara et al., 2008), and fermented feed on eggshell quality (Lv et al., 2022; Tian et al., 2022). The variations in the studies may be due to strains used during fermentation and inclusion levels. The

improved eggshell and albumen quality suggests that the diet augmented the nutritional status of laying hens. Egg formation is a function of various physiological processes which are regulated by an array of blood parameters.

4.4. Effect of fermented soybean meal on antioxidant function, immunoglobulin secretion and immune response

Variations in blood parameters reflect changes in animal body metabolism and absorption. Laying hens are exposed to oxidative stress due to continuous egg production, therefore a homeostatic balance must be maintained. Antioxidant enzymes (SOD, CAT and GSH-Px) are the host's first line of defense against oxidative stress, and the total antioxidant capacity (T-AOC) includes both enzymatic and non-enzymatic components (Zhang et al., 2018). Also, malondialdehyde (MDA) levels rise in response to oxidative stress and lipid peroxidation, hence serving as an oxidative biomarker (Wang et al., 2018). In the study, FSBM significantly upregulated the antioxidant enzymes and reduced the level of MDA. Increased antioxidant function would provide a conducive environment for the overall metabolic processes of the laying hens. In agreement with our results, FSBM (Chachaj et al., 2019b; Sembratowicz et al., 2020), and fermented feed (Zhu et al., 2020) enhanced the antioxidant capacity of poultry species. The strong antioxidant defense system is a key driver involved in oxidative stress pathway. Whereas the high level of MDA and reduced antioxidant enzymes in the control group suggest oxidative stress and could partially explain the inflammatory response notable in the study. The higher antioxidant capacity may be attributed to the presence of efficient reactive oxygen species (ROS) scavengers such as isoflavone in fermented feed (Lin et al., 2006), increased the abundance of antioxidant amino acids such as tryptophan, tyrosine, histidine, lysine, and histidine (Wang and De Mejia, 2005), and the abundance of micronutrients (Zn and Fe) that are essential for antioxidant enzyme activity (Chatterjee et al., 2018). The nutritional value of FSBM may be responsible for the increased antioxidant capacity, which may help to reduce immunological stress and inflammatory chain reactions.

Immunoglobulins (IgA, IgG, and IgM) and complement components are critical to the immune system, and thus can be used to assess the humoral immunity status of the animal (Lu et al., 2019). IgA protects the mucosal surface against pathogen invasion (de Sousa-Pereira and Woof, 2019), IgG is mainly involved in the recognition of antigens on the surfaces of invading viruses and can be transferred to the offspring (Shah et al., 2020), and IgM is in the first line of defense against invading pathogens and regulates immune response (Gong and Rurecht, 2020). In the study, FSBM increased the serum concentrations of immunoglobulins, an indication of improved health status and performance. Dietary FSBM increased serum IgM and IgG (Feng et al., 2007), IgA and IgG in laying hens (Xu et al., 2012), which lends support to our findings. In addition, fermented feed increased the serum concentrations of IgA, IgG, and IgM in pullets (Zhu et al., 2020), and IgM and IgG in pigs (Lu et al., 2019). Increased immunoglobulin concentrations may be attributed to abundance of small peptides produced during the fermentation process, which have been found to boost immunoglobulin synthesis (Feng et al., 2007; Xu et al., 2012). As a result, an optimal level of FSBM inclusion could improve immunological function. Conversely, FSBM did not influence serum IgA (Feng et al., 2007) and caused a reduction in serum IgM and IgG (Chachaj et al., 2019a). The variations may be due to the age of the birds. Furthermore, complement proteins including C3 and C4 are found in the blood and are part of the complement system, a system that is a component of the immune system (Morgan et al., 2005); complement proteins have crucial functions in immune function; hence, they are frequently employed to evaluate the immunological status of animals. Fermented cottonseed meal enhanced levels of C3 and C4 proteins (Tang et al., 2012), which supports our findings, suggesting a boost to the immune status. It is known that the abundance of small peptides and variations in the composition of intestinal microbiota may play a key role in immunity enhancement (Sugiharto and Ranjitkar, 2019). Therefore, the positive effect of FSBM on complement proteins may suggest the ability of the fermentation process to improve the immune system due to the presence of small peptides and an abundance of beneficial microbes. Serum immunoglobulins are important inflammation regulators because they play an important role in the feedback system that maintains an equilibrium between the activities of pro-inflammatory (IL-1, IL-6, and TNF-α) and anti-inflammatory (IL-10, TGF-β, and IL-4) cytokines (Smith and Humphries, 2009).

Cytokines are essential components of the cell-mediated immune response. In the current study, FSBM reduced TNF-α and IL-1β levels while increased the levels of TGF-β and TLR2 in the serum. The reduction in the levels of pro-inflammatory cytokines (TNF-α and IL-1β) and increased the level of anti-inflammatory cytokines (TGF-β and TLR2), suggests a better immune response and oxidative eustress for oxidative balance for enhanced health status. On the other hand, the increased concentration of pro-inflammatory cytokines in the serum of birds in the control group suggest an inflammatory response, which could be a consequence of reduced antioxidant capacity. Previous reports are in line with our findings on anti-inflammatory effects which were notable in pigs fed FSBM (Zhang et al., 2018), and broilers fed fermented wheat bran (An et al., 2022; Wang et al., 2022). The ability of fermented feed to induce anti-inflammatory response could be attributable to the presence of small peptides and reduced the content of trypsin inhibitors (Feng et al., 2007). These effects are known to enhance animal production performance via improved immune response and reduced inflammatory effect. Similarly, the impact of amino acids on downregulation of nuclear factor kappa B (NF-kB) pathway could be a contributory factor. Although NF-kB was not evaluated, it is known that activation of NF-κB may lead to the production of pro-inflammatory cytokines. Previous research has shown that soy peptides have higher concentrations of glutamine and asparagine that can downregulate the activity of NF-kB (Ren et al., 2014). It could be inferred that the same effect was notable since the FSBM has high contents of these amino acids. However, fermented wheat bran did not affect pro-inflammatory factors in broiler birds (Wang et al., 2022) and FSBM did not affect serum IL-6 of broiler chicks (Chachaj et al., 2019a). The reduction of proinflammatory cytokines due to decreased trypsin inhibitors and enhanced immunoglobulin synthesis indicates that the anti-inflammatory and better immune response may promote gut health for efficient nutrient digestion and absorption.

4.5. Effect of fermented soybean meal on apparent digestibility of amino acids

Amino acids are crucial to improved efficiency and sustenance of egg production. In the study, the apparent digestibility of most essential and non-essential amino acids was improved. Amino acids are critical to egg formation; thus, the enhanced digestibility reflects the high amount of amino acids available for maintenance and egg production. Data are scarce on the effect of FSBM on apparent amino acid digestibility for comparison. Also, the digestibility of total sulfur amino acids (TSAA), which play a key role in albumen synthesis (Parenteau et al., 2020), were notable. Therefore, the higher egg production and albumen synthesis are attributable to the increased bioavailability of amino acids for the physiological functions of the animal. There is a relationship

between the increased digestibility of amino acids and contents of free amino acids (Sembratowicz et al., 2020). Also, the reduced content of trypsin inhibitors in the FSBM is conducive to protein digestibility and bioavailability of amino acids (de Coca-Sinova et al., 2008). Similarly, the ability of FSBM to stimulate the activity of digestive enzymes for better protein metabolism (Soumeh et al., 2019), may be a contributory factor. Thus, reduced trypsin inhibitors in FSBM as well as its positive effects on digestive enzymes and amino acid oxidation may promote protein metabolism and amino acid digestibility, which, in turn, meant improved amino acid digestibility and higher gut integrity for bioavailability and absorption.

4.6. Effect of fermented soybean meal on gut morphology

Gut health indicators such as villus height, crypt depth, villus height-to-crypt depth ratio are often assessed since they are directly related to nutrient absorption capacity of the intestinal mucosa (Chuang et al., 2021). In the study, FSBM increased villus height, villus height-to-crypt depth ratio and reduced crypt depth, all of which have positive effects for gut function. The improved villus morphology reflects improved absorptive surface area necessary for the expression of brush border enzymes, absorptive capacity, and villus renewal in response to inflammatory cues or epithelial exfoliation, which are all crucial for nutrient utilization. Indeed, FSBM enhanced jejunal villus morphology in chicks (Chachaj et al., 2019a), laying hens (Xu et al., 2012), and quails (Jazi et al., 2018a). Additionally, the positive effects of feedstuffs such as fermented corn–soybean mixed feed (Liu et al., 2021), probiotic fermented feeds (Guo et al., 2021; Lv et al., 2022) and fermented plant product containing probiotics and Chinese medicinal herb (Tian et al., 2022) on gut morphology of laying hens have been reported. Fermented feed in the gut enhance microbial synthesis of short chain fatty acids, which supply energy to the epithelial gut cells for villi renewal (Ashayerizadeh et al., 2017; Jazi et al., 2018a; Liu et al., 2021), thus could account for the enhancement effect on gut morphology. It could be related to enhanced proliferation and differentiation of gut enterocytes (Jazi et al., 2018b) as a result of fermented feed's effect on gut microbiota and metabolites (Peng et al., 2022; Tian et al., 2022). However, there is an evidence that gut morphology may be impaired due to the presence of trypsin inhibitors, which can cause villus atrophy (Xu et al., 2003; Chiang et al., 2009) and there exists a negative correlation between trypsin inhibitors and villus height (Zarkadas and Wiseman, 2005). Similarly, high-level expression of proinflammatory cytokines can trigger an immune response and damage gut epithelial cells and disrupt intestinal integrity (Wang et al., 2012; Nagano et al., 2019). The findings suggest that enhanced gut morphology is linked to decreased antinutritional factors, increased antioxidant capacity, improved immunoglobulin secretions and elevated microbial fermentation, which preserve gut integrity. Thus, a healthy gut may improve nutrient utilization and absorption, enhancing laying performance and egg quality. We speculate that fermented feeds' probiotic-like effect on gastrointestinal intestinal cells and beneficial microbial colonization may have improved gut morphology.

4.7. Effect of fermented soybean meal on composition of cecal microbiota

Gut microbiota provides nutrients from diet and regulates digestive and immune systems; thus, animals Zhu et al., 2022need a healthy gastrointestinal microbiome. Previous research has found that fermented feed can modify cecal microbiota, resulting in a rich diversity of gut microbiota (Lv et al., 2022; ; Tian et al., 2022). In the current study, FSBM modulated the diversity and richness of cecal microbiota; the abundance of *Lachnospiraceae, E. coprostanoligenes, Weissella, Staphylococcus* and *Anaerostipes* were reduced. Members of *Lachnospiraceae* are associated with fermentation of non-starch polysaccharides, producers of short chain fatty acids (acetate and butyric acid), and suppression of inflammatory responses (Vacca et al., 2020). In affirmation to our results, FSBM increased the abundance of *Lachnospiraceae* in broiler birds (Li et al., 2020), thus beneficial to the gut health of the host. *E. coprostanoligenes* was positively correlated with jejunum crypt depth in geese fed a fermented diet (Yan et al., 2019), which supports our findings. An indication of the relationship between *E. coprostanoligenes* and intestinal development. *Weissella* and *Staphylococcus* are critical to the maintenance of gut homeostasis in the host (Illnskaya et al., 2017). The increased abundance of this genus in the diet group may be a key to the stable health and physiological response of the host. However, the Firmicutes group was enriched in the control group compared to the treated groups. Firmicutes are linked with polysaccharide degradation and energy utilization in the gut due to genes encoding non-starch polysaccharide degrading enzymes. The FSBM lower the Firmicutes level in the treatment groups may diminish the bacterial species that can extract energy from the diet, which is favorable to the host. Additionally, the abundance of WPS-2 phyla in the cecum was significantly higher, similar to findings on laying hens fed fermented phytogenic feed (Zhu et al., 2022). The function of this phyla in the cecum of laying hens remains unclear. Nevertheless, the *Anaerostipes* genus is associated with intestinal inflammation (Guo et al., 2021). Thus, the reduced abundance in the FSBM groups would mean improved gut health for better performance. Taken together, a healthy gastrointestinal microbiome is critical for normal physiological functions of the animal, probably because gut microbiota provides nutrients from diet and regulates digestive and immune systems.

5. Conclusion

The microbial fermentation of SBM improved the nutritional value, possibly via reducing the level of trypsin inhibitors, increasing the CP content, and enhancing the bioavailability of amino acids and minerals. Fermentation of SBM improved gut morphology, amino acid digestibility, immune and antioxidant function for increased laying performance and high-quality eggs. Furthermore, the balance of the gut microbiota was noticeably in favor of the beneficial microbes, i.e., the abundance of *Lachnospiraceae, E. coprostanoligenes, Weissella and Staphylococcus* was improved while *Anaerostipes* was reduced. Our findings are an indication that the fermentation of soybeans may be an effective method for improving the nutritional quality of the corn–soybean diet and providing probiotics for animal health. Therefore, FSBM could be used as a feed ingredient in the diet of laying hens at an inclusion level of 2.5% or 5.0%.

Author contributions

Uchechukwu Obianwuna: Methodology, Investigation, Writing-Original draft. **Kai Qiu:** Conceptualization, Supervision. **Haijun Zhang:** Writing-Review and Editing, Data curation. **Jing Wang:** Writing-review and Editing. **Guanghai Qi:** Funding. **Shugeng Wu:** Funding, Project administration and Supervision. All authors reviewed and accepted this final version of the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal

interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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