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Poultry feeding, nutrition, and quality in the post-antibiotic era

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Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-8325-4649-9
DOI 10.3389/978-2-8325-4649-9

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Poultry feeding, nutrition, and quality in the post-antibiotic era

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Citation

Abdel-Moneim, A.-M. E., Biasato, I., Shehata, A. M., Mesalam, N., eds. (2024). *Poultry feeding, nutrition, and quality in the post-antibiotic era*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4649-9

Table of contents

05 Editorial: Poultry feeding, nutrition, and quality in the post-antibiotic era
Abdel-Moneim Eid Abdel-Moneim, Ilaria Biasato, Noura M. Mesalam and Abdelrazeq M. Shehata

08 Effect of purple corn extract on performance, antioxidant activity, egg quality, egg amino acid, and fatty acid profiles of laying hen
Jiaxuan Li, Di Zhou, Hui Li, Qingyuan Luo, Xu Wang, Jixiao Qin, Yiqing Xu, Qi Lu and Xingzhou Tian

19 Beta-glucans induce cellular immune training and changes in intestinal morphology in poultry
Hadar Bar-Dagan, Ofer Gover, Natalie Avital Cohen, Vaclav Vetvicka, Israel Rozenboim and Betty Schwartz

34 Effects of *Clostridium butyricum* on growth performance, meat quality, and intestinal health of broilers
Zhen Li, Lingbo Long, Xu Jin, Yang Li, Qiong Wu, Xingyong Chen, Zhaoyu Geng and Cheng Zhang

42 Effects of dietary *Chinese yam* polysaccharide copper complex on growth performance, immunity, and antioxidant capacity of broilers
Jinzhou Zhang, Yan Jin, Mengmeng Cao, Jiahua Deng, Yadi Chang, Mingyan Shi and Zhiguo Miao

51 Effect of sea buckthorn extract on production performance, serum biochemical indexes, egg quality, and cholesterol deposition of laying ducks
Bing-nong Yao, Fu-you Liao, Jiao-yi Yang, Ai Liu, Jiao Wang, Bao-guo Zhu, Gang Feng and Sheng-lin Yang

62 Betaine and nano-emulsified vegetable oil supplementation for improving carcass and meat quality characteristics of broiler chickens under heat stress conditions
Gamaleldin M. Suliman, Elsayed O. S. Hussein, Abdullah N. Al-Owaimer, Rashed A. Alhotan, Maged A. Al-Garadi, Jameel M. H. Mahdi, Hani A. Ba-Awadh, Mohammed M. Qaid and Ayman Abdel-Aziz Swelum

73 The impact of *Bacillus subtilis* DSM32315 and L-Threonine supplementation on the amino acid composition of eggs and early post-hatch performance of ducklings
Mahmoud Mostafa Azzam, Wei Chen, Weiguang Xia, Shuang Wang, Yanan Zhang, HebatAllah Kasem El-Senousey and Chuntian Zheng

81 Effect of fenugreek seeds and *Bacillus*-based direct-fed microbials on the growth performance, blood biochemicals, and intestinal histomorphology of broiler chickens
Deependra Paneru, Guillermo Tellez-Isaias, Margarita A. Arreguin-Nava, Nicholas Romano, Walter G. Bottje, Emmanuel Asiamah, Ahmed A. A. Abdel-Wareth and Jayant Lohakare

92 Effects of dietary *Astragalus membranaceus* and *Codonopsis pilosula* extracts on growth performance, antioxidant capacity, immune status, and intestinal health in broilers
Shun Liu, Gengsheng Xiao, Qi Wang, Jinpeng Tian, Xin Feng, Qingyang Zhang and Li Gong

104 Effects of dietary Chinese herbal mixtures on productive performance, egg quality, immune status, caecal and offspring meconial microbiota of Wenchang breeder hens
Mengjie Liu, Jieyi Huang, Ming Ma, Gengxiong Huang, Yingwen Zhang, Yiqing Ding, Qian Qu, Weijie Lv and Shining Guo

117 Can tree leaves be used as an alternative source of synthetic antioxidants? Use of jujube leaf extract in laying hens
Gözde Kılınç

125 Effects of silybin supplementation on growth performance, serum indexes and liver transcriptome of Peking ducks
Ziyue Zhang, Bozhi Shi, Xueze Lv, Yingchao Dong, Lei Li and Zhaofei Xia

136 Feeding spray-dried plasma to broilers early in life improved their intestinal development, immunity and performance irrespective of mycotoxins in feed
Gabriela Gómez-Verduzco, José Arce-Menocal, Carlos López-Coello, Ernesto Avila-González, Claudia C. Márquez-Mota, Javier Polo and Luis Rangel

frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 11 March 2024
DOI 10.3389/fvets.2024.1386278

OPEN ACCESS

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RECEIVED 14 February 2024
ACCEPTED 01 March 2024
PUBLISHED 11 March 2024

CITATION
Abdel-Moneim A-ME, Biasato I, Mesalam NM and Shehata AM (2024) Editorial: Poultry feeding, nutrition, and quality in the post-antibiotic era.
Front. Vet. Sci. 11:1386278.
doi: 10.3389/fvets.2024.1386278

Editorial: Poultry feeding, nutrition, and quality in the post-antibiotic era

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KEYWORDS

antibiotic alternatives, probiotics, Chinese herbal medicine, plant-derived polysaccharides, direct-fed microbes, phytochemicals, spray-dried plasma powder, poultry

Editorial on the Research Topic
Poultry feeding, nutrition, and quality in the post-antibiotic era

For the majority of the past 50 years, farmers have employed antibiotics to enhance the growth of their livestock. The utilization of antibiotic growth promoters (AGPs) has proven beneficial for animals in terms of their health and performance. However, the presence of AGPs in human-consumed meat may have an impact on normal human immunological function and metabolism. The extensive use of subtherapeutic antibiotics, however, has resulted in the emergence of antibiotic-resistant bacteria. Consequently, the prohibition of AGPs in chicken farms has become a top priority. Nevertheless, removing AGPs from chicken feed represents a significant and impactful change with far-reaching consequences. Poultry farmers require assistance in navigating this transition as they grapple with the challenges of reducing antibiotic usage (1, 2). Therefore, there is an urgent need for innovative solutions to address the aforementioned challenges and enhance the productivity of broiler chickens through the utilization of antibiotic alternatives and unconventional nutritional strategies.

The objective of this Research Topic is to compile articles that can enhance our understanding of the use of non-traditional nutritional techniques and the incorporation of eco-friendly feed additives. These efforts aim to improve poultry welfare and foster sustainable production practices.

Gómez-Verduzco et al. identified in feeding spray-dried plasma (SDP) a promising strategy to counteract mycotoxicosis in broiler chickens. The authors observed improved growth performance (in terms of weight gain and feed efficiency) and immune response (in terms of higher thymus relative weight, increased serum levels of macrophage colony-stimulating factor, and higher IgA concentrations in gut and tracheal washes) when birds were supplemented with 2% SPD.

Plant-derived bioactives and herbal extracts show great potential as environmentally friendly feed additives that can serve as alternatives to antibiotics (3–5). Numerous investigations have demonstrated that including anthocyanin plant extract in laying hen diets can enhance both the production and quality of eggs. Li, Zhou et al. examined the impact of anthocyanin-rich purple corn extract on the performance, antioxidant capacity,

egg quality, and amino acid and fatty acid profiles of laying hens in the late stages of egg production. The researchers discovered that consuming purple corn extract, which is high in anthocyanins, can increase the antioxidant capacity of the blood, promote egg production, and elevate the levels of amino acids and fatty acids in hen eggs during the late stages of laying. Another study was conducted by Yao et al. to investigate the impact of supplementing sea buckthorn extract on the production performance, serum biochemical metrics, egg quality, and cholesterol deposition in laying ducks. The researchers realized that incorporating sea buckthorn extract into diets can enhance nutrient utilization, enhance egg weight, optimize egg quality and amino acid composition in eggs, decrease blood lipids, improve the fatty acid profile and yolk cholesterol levels in eggs, and boost antioxidant capacity and immunity in laying ducks. Liu, Huang, et al. investigated the possibility of using a mixture of Chinese herbs as a feed additive in autochthonous (Wenchang) breeder hens, observing that it could improve bird productive parameters (laying rate egg weight, feed efficiency, and egg quality) by enhancing the immune status and anti-apoptosis capacity and reducing potential pathogens in the caecal microbiota, as well as maintaining the intestinal health of the offspring chicks. Another herbal-derived feed additive (jujube leaf extract) was also tested by Kilinç in laying hens, resulting in improved bird productive performance (in terms of increased feed efficiency and egg production), egg quality traits (in terms of higher albumen index, Haugh unit, shell weight, and shell thickness), and slowed down yolk lipid oxidation (in terms of reduced thiobarbituric acid reactive substances levels).

Recent years have seen a rise in interest in the nutritional and therapeutic properties of Chinese yam polysaccharide. The yam's bioactive components were complex, but the water extract's main component, non-starch polysaccharides, gained a lot of interest due to its many potential biological activities (6). Incorporating 0.10 g/kg of Chinese yam polysaccharide copper complex into broiler diets was suggested as an effective way to enhance growth, immunity, and antioxidant capacity. These findings point to the possibility of Chinese yam polysaccharide copper complex as an ecologically friendly feed additive for poultry farms (Zhang J. et al.). *Astragalus membranaceus* is a popular tonic herb in numerous Asian areas. It is known to include components that exhibit diverse biological actions, such as antioxidative, anti-inflammatory, and antiviral characteristics. *Codonopsis pilosula* is recognized for its capacity to improve spleen functionality, support liver wellbeing, and provide anti-tumor, antioxidant, and antibacterial properties. Liu, Xiao et al. looked at how a combination of *Astragalus membranaceus* and *Codonopsis pilosula* extracts affected the health of broiler chickens' intestines, immune system, antioxidant activity, and overall growth performance. The results suggested that broiler chickens' gut microbiota changed in response to dietary CHM, which in turn improved feed utilization, increased mRNA expression of pro-inflammatory cytokines in the jejunal mucosa, and decreased serum endotoxin levels and activities of diamine oxidase and lactate dehydrogenase.

The transcriptome serves as a crucial tool in investigating the kinetics of gene expression, assessing and detecting illness indicators, and expediting the exploration of novel targets. The liver serves as a crucial immunological organ and plays a major role in numerous physiological processes. Hence, employing transcriptomics to investigate the hepatic gene expression in Peking ducks fed silybin-containing diets is valuable for enhancing comprehension of the impact of silybin on the growth and development of Peking ducks. Additionally, it may yield additional insights into the utilization of silybin in animal diets. Zhang Z. et al. found that supplementing Peking ducks with 1,600 mg/kg of silybin significantly improved their growth performance, enhanced their immune capabilities, and reduced their inflammatory responses. The transcriptome analysis indicates that achieving this may involve the regulation of antigen processing and presentation, amino acid metabolism and synthesis, as well as JAK–STAT pathways.

In a study conducted by Suliman et al., the researchers investigated the impact of adding water-based betaine and/or nano-emulsified vegetable oil on the features of carcass and meat quality in broilers that were grown in conditions of heat stress. Water enriched with betaine significantly enhanced carcass dressing weight, breast weight, and meat quality concerning water-holding capability and tenderness during heat stress conditions.

Probiotics, due to their environmentally favorable and non-polluting nature, are commonly administered directly to broilers to suppress the growth of infections and regulate their digestive function (7–9). A common probiotic strain, *Clostridium butyricum,* has remarkable intestinal environment adaptation due to its spores can withstand extreme conditions of temperature, humidity, stomach acid, and bile salts (9). Digestive enzymes found in *C. butyricum* metabolites can reduce the molecular size of nutrients, making them easier for the host to absorb. This improves the nutrients' utilization efficiency, which in turn boosts the host's growth performance (10). Li, Long et al. demonstrated that the incorporation of *C. butyricum* CBM 588 to the diets of broilers enhanced their growth performance and improved the quality of their meat. *C. butyricum* could improve gastrointestinal wellbeing, potentially through the stimulation of the Nrf2 signaling pathway and suppression of the NF-κB signaling pathway. However, further investigation is required to ascertain the precise mechanism by which *C. butyricum* operates.

Paneru et al. suggested that fenugreek seeds, which contain significant amounts of dietary fiber, soluble fiber, and biologically active phytochemicals, could potentially function as a prebiotic. Additionally, they speculated that Fenugreek seeds may have a synergistic effect when combined with *Bacillus*-based direct-fed microbials, leading to health-enhancing advantages and supporting the growth performance of broiler chickens. Results showed that fenugreek seeds and *Bacillus*-based direct-fed microbials affected broiler health and productivity differently at different stages of broiler age. While fenugreek seeds and *Bacillus*-based direct-fed microbials worked together to improve growth performance in the finisher phase, they had opposite effects on gut morphology and blood parameters. To better understand how fenugreek seeds and *Bacillus*-based direct-fed microbials work together, and to find the optimal dose for broiler health and production, the authors suggested the need for more research. Another study was conducted by Azzam et al. to assess the optimal performance-enhancing effects of supplementing laying duck breeders with L-Threonine and *B. subtilis* DSM32315. This study found that

supplementing the diet with a combination of L-Threonine and *B. subtilis* DSM 32315 at specific rates (0.7 g/kg for L-Threonine and 0.5 g/kg for *B. subtilis* DSM 32315) had beneficial effects on the eggshell percentage, hatchability, and body weights of newly hatched ducklings. Furthermore, the inclusion of L-Threonine as a separate component at a dosage of 0.7 g/kg has the potential to enhance the egg production of duck breeders.

The shift toward sustainable and eco-friendly practices in poultry feeding, nutrition, and quality is imperative in the post-antibiotic era. This Research Topic explores unconventional solutions, such as the utilization of alternative nutritional strategies, plant extracts, herbal-derived feed additives, and probiotics, showcasing promising avenues to enhance poultry welfare, mitigate antibiotic resistance, and promote environmentally conscious production methods. These innovative approaches not only address the challenges posed by the reduction of antibiotic usage but also pave the way for a more sustainable and resilient future in poultry farming.

Author contributions

A-MA-M: Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. IB: Writing – original draft. NM: Writing – original draft. AS: Writing – original draft.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 06 January 2023
DOI 10.3389/fvets.2022.1083842

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Animal Nutrition and Metabolism,
a section of the journal
Frontiers in Veterinary Science

RECEIVED 29 October 2022
ACCEPTED 12 December 2022
PUBLISHED 06 January 2023

CITATION
Li J, Zhou D, Li H, Luo Q, Wang X, Qin J, Xu Y, Lu Q and Tian X (2023) Effect of purple corn extract on performance, antioxidant activity, egg quality, egg amino acid, and fatty acid profiles of laying hen.
Front. Vet. Sci. 9:1083842.
doi: 10.3389/fvets.2022.1083842

Effect of purple corn extract on performance, antioxidant activity, egg quality, egg amino acid, and fatty acid profiles of laying hen

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The objective of this study was to investigate the effects of anthocyanin-rich purple corn extract (PCE) on performance, antioxidant potential, egg quality, egg amino acid and fatty acid profiles of laying hens during the late laying period. A total of 360 88-wk-old laying hens were randomly divided into 4 groups, and fed a basal diet (CON) or a basal diet supplemented with 120 (LP), 240 (MP), and 360 mg/kg (HP) PCE, respectively. No significant difference ($P > 0.05$) was observed in the ADFI or average egg weight among the groups. However, the mean feed to egg ratio was quadratically decreased ($P < 0.05$) in the LP and HP treatments. The mean TAC was linearly and quadratically increased ($P < 0.05$) in all PCE supplemented treatments. The mean SOD was linearly and quadratically increased ($P < 0.05$) in the HP treatment compared with CON and MP groups. The GPX was linearly and quadratically lower in the HP treatment compared to the CON and LP groups. Differently, the MDA was linearly and quadratically lower ($P < 0.05$) in the PCE treatments compared with the CON. The eggshell thickness value in MP and HP treatments were linearly and quadratically higher ($P < 0.05$) than that of the CON and LP groups. Hens fed PCE was linearly and quadratically increased ($P < 0.05$) most individual amino acids, essential amino acid and umami amino acid profiles in egg. The PCE treatments showed linearly and quadratic ($P < 0.05$) effect on the myristoleate, heptadecenoic acid, elaidic acid, eicosenoic acid, heneicosanoic acid, and eicosatrienoic acid concentrations. Moreover, dietary supplementation of PCE was quadratically increased egg stearic acid, oleic acid, arachidic acid, linolenic acid methyl ester, arachidonic acid, diphenylamine, docosahexaenoic acid, monounsaturated fatty acid, and polyunsaturated fatty acid compared to the CON. Therefore, dietary anthocyanin-rich PCE can enhance plasma antioxidant potential, is beneficial to egg production, and improves amino acids and fatty acids in hen eggs during the late laying period.

KEYWORDS

anthocyanin, antioxidant activity, egg quality, amino acid, fatty acid, laying hen

1. Introduction

Currently, the residues of antibiotics and drug resistance are becoming increasingly concerning, and green and safe poultry products have been increasingly welcomed by consumers (1). In recent years, many countries including China prohibited the use of antibiotics as feed additives. Thus, finding a safe substitute has been imperative. The plant extract is an excellent natural substitution for antibiotics because its addition to the diet could improve the growth, gut health, and improve performance and egg quality of chickens (2, 3). These reports have indicated that the secondary metabolites of plants can promote the growth of animals, enhance immunity, resist bacteria and disease, and improve the quality of animal products (4). Therefore, plant extracts are promising green feed additives that can replace antibiotics.

Anthocyanins are plant secondary metabolites that have excellent antioxidative capacity and antibacterial properties, which as natural antioxidants, have potential benefits for livestock and poultry production (5). Anthocyanin has strong antioxidant, immune regulation and anti-inflammation effects, which may improve the immune function and antioxidant effects of the animal, alleviating the breeding process of a variety of stress responses (6). This is because anthocyanins can provide hydrogen atoms for phenols and trap free radicals, thereby stabilizing compounds through various resonant forms, and showing high antioxidant activity (7). Hence, anthocyanins have strong antioxidative and radical scavenging activities and are a source of an attractive natural antioxidant (8). Kaya et al. (9) found that the addition of anthocyanin-rich grape seed extract could improve egg haugh units of laying hens. Sabet et al. (10) found that anthocyanin-rich sour tea (*Hibiscus Sabdariffa*) plant extract did not affect functional or immune parameters but did improve the plasma antioxidant status in laying hens.

Currently, eggs are an important component of human food, and thus can be enriched with antioxidants through manipulation of poultry feed (11). Various studies have showed that dietary supplementation with anthocyanin plant extract can improve the quality of egg production in poultry (12, 13). However, in the late laying period, laying hens are prone to convert excess nutrients into fat, causing imbalance of lipid oxidation and reducing antioxidant function, and leading to the decrease of performance (14). Additionally, purple corn is rich in anthocyanin, which may be an important source of anthocyanin extraction and has wide development and application prospects (15). However, reports about the role and mechanism of anthocyanins in laying hens during the late laying period are relatively rare. We hypothesized that the feeding of purple corn extract (PCE) can increase antioxidant activities and improve egg production, amino acids and fatty acids in hen eggs. Accordingly, the current study observed the effects of anthocyanins from PCE on the performance, plasma antioxidant activity, egg production, amino acids and fatty acids in egg of Chishui black-bone laying hens during the late laying period.

2. Materials and methods

2.1. Animals, diets, and experimental design

Chishui black-bone hen is one of the famous local poultry breeds in the Guizhou Province of China (16). Moreover, the feeding trial was carried out at the Guizhou Zhuxiang Chicken Breeding Co. Ltd., Chishui, China (28.590337 N, 105.697472 E). Amer et al. (17) demonstrated that the inclusion of 200 and 400 mg/kg anthocyanin-rich roselle, *Hibiscus sabdariffa* L. extract could improve antioxidant activity in chicken. In this study, 360 healthy and similar body weight (BW 1,728 ± 147 g, mean±standard deviation). Chishui black bone hens from 88 weeks during the late laying period were used. All hens were randomly allocated into four groups consisting of a basal diet (CON) or the basal diet supplemented with 120 (LP), 240 (MP), and 360 mg/kg (HP) anthocyanin-rich PCE. Each group had six replicates, and each replicate had 15 laying hens. The hen housed was kept at a cage, and each cage (0.076 m^3) was kept at 3 hens. All experimental hens were under the same conditions with 16 h/d light, 21°C temperature and 45∼65% humidity.

The PCE (Nanjing Herd Source Biotechnology Co., Ltd., Nanjing, China), a commercial extract, had a total anthocyanin concentration of 2,619 μg/g according to our previous study (18). The PCE was first mixed with chopped concentrate, then mixed with roughage to prepare basal diet. There was 14 d a preparation period prior to a 60 d formal experimental period of the feeding trial. Feed and water were provided *ad libitum* during the whole period. The nutritional requirements of laying hens were determined according to the farm requirements and the Chinese Standard for the Feeding Standard of chickens (NY/T33-2004) (19). The chemical compositions for dry matter (930.15), crude protein (988.05), calcium (927.02), and phosphorus (964.06) of feed were analyzed as per the method of the AOAC (20). The chemical composition and nutrition composition of basal diet as shown in Table 1.

2.2. Performance

During the entire experimental period, the daily feed amount and surplus were collected to calculate average daily feed intake (ADFI). The daily egg yield and egg weight were recorded to calculate average egg weight (g) = total daily weight/total egg weight; moreover, laying rate per hen-day (HD, %) = (laying number/layer number) × 100, and feed to egg ratio = total feed intake (g)/total egg weight (g).

TABLE 1 The chemical composition and nutrient levels of basal diet.

Ingredients, % of fed basis	Content	Nutrient levels, % of DM	Content
Corn	61.50	Dry matter, % of the as-fed diet	92.59
Soybean meal	26.19	Crude protein	16.26
Soybean oil	1.05	Metabolizable energy, MJ/kg	12.22
Limestone	7.86	Calcium	3.23
Fishmeal	0.10	Total phosphorus	0.45
NaCl	0.30	Available phosphorus	0.19
Premix	3.00	Lysine	0.87
Total	100	Methionine	0.42
		Methionin+cysteine	0.71

Provided per kilogram: vitamin A, 330000 IU; vitamin D_3, 133500 IU; vitamin E, 850 IU; vitamin B_1, 70 mg; vitamin B_2, 200 mg; vitamin B_6, 135 mg; vitamin B_{12}, 0.8 mg; vitamin K_3, 85 mg; nicotinamide, 1,200 mg; pantothenic acid, 350 mg; biotin, 9 mg; choline chloride, 12,000 mg; Cu, 340 mg; Fe, 2,000 mg; Mn, 700 mg; Zn, 2,700 mg; Se, 12 mg. Metabolizable energy was calculated value according to the feed database in China (2018).

2.3. Plasma antioxidant activity parameters

Two hens from each replicate for a total of twelve hens per group at 20, 40, and 60 d before feeding were randomly selected, blood (about 5 mL) was obtained from the inferior wing vein by a negative pressure vacuum tube with 12.5 IU/mL heparin sodium (Nanchang Ganda Medical Instrument Co., Ltd., Nanchang, China). Blood samples were centrifuged (KJH80-2, Jiangsu KangJianhua Medical Supplies Co., Ltd., Jiangsu, China) at 4,000 $\times g$ for 15 min, and the plasma was collected and kept at a −80°C fridge for further analysis. Total antioxidant capacity (TAC, A015-1), superoxide dismutase (SOD, A001-3), glutathione peroxidase (GPX, A005), catalase (CAT, A007-1), and malondialdehyde (MDA, A003-1) were determined, and all kids purchased from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China).

2.4. Egg quality

Two hens from each replicate for a total of twelve hens per group at 20 d, 40 d and 60 d were randomly selected, and egg weight, albumen height, yolk weight, yolk color, and haugh unit were analyzed by an egg quality meter (EA-01, ORKA, Israel). Moreover, eggshell strength was analyzed by an eggshell strength tester (KQ-1A, Nannong Animal Husbandry Technology Co., Ltd., Beijing, China). Moreover, the egg shape index and the thickness of the eggshell were determined using cursor calipers (3 V, MASTERPROOF professional TOOL, Germany).

2.5. Egg amino acids and fatty acids

Two eggs from each replicate and a total of twelve eggs per group at the end of experimental period were randomly selected, and transferred into a vacuum freeze dryer (LYOQUEST-85 Plus, Bole Technology, Spain). The condenser was set at −80°C, and the vacuum pump was set to 0.000 m Bar. Next, the egg samples were ground by a high-speed grinder after 72 freeze-drying cycles. Amino acids and fatty acids were detected according to our previous study (16). Briefly, amino acid was analyzed using an automatic amino acid analyser (Biochrom 30, Biochrom Ltd., Cambridge, the United Kingdom). The amino acid analyser conditions were as follows: the chromatographic column was a sulfonic acid cation resin; detection wavelengths were 570 and 440 nm, respectively; individual amino acid was determined using peak area according to external standard method. For fatty acid, approximately 1 g of sample was weighed for hydrolysis; then, the fat extract was obtained, fat saponification and fatty acid esterification were processed, and the upper solution was collected and kept in a sample bottle. The individual fatty acids were detected by an Agilent 6,890 gas chromatograph (Agilent Technologies Co. Ltd., Palo Alto, USA). The gas chromatograph conditions were as follows: the injection volume was 1.0 μL; capillary column was a polar stationary phase of 100 m×0.25 mm×0.2 μm polydicyanide siloxane; 270°C of injector temperature; 280°C of detector temperature. The individual fatty acids were calculated using the peak area normalization method.

2.6. Statistical analysis

All raw data were recorded with Microsoft Excel 2010. All observations were analyzed by the general linear models procedure using in Statistical Product and Service Solutions 20.0 software (SPSS Inc., Chicago, Illinois, USA) with the least square mean noted by Tukey's test. All variables were tested for linear and quadratic effects, and the level of significance was assessed at $P < 0.05$.

3. Results

3.1. Performance

The mortality of hens did not differ ($P > 0.05$) throughout the feeding experimental period (data not shown; Table 2). No significant difference ($P > 0.05$) was observed in the ADFI or average egg weight among the groups during the entire experimental period. Compared to the CON, dietary supplementation of PCE exhibited linear and quadratic effect ($P < 0.05$) on the HD, with the highest values noted at HP treatment. Moreover, the feed to egg ratio was not significantly

TABLE 2 Effect of purple corn extract on performance in laying hen.

Items	PCE supplemental levels (mg/kg)				SEM	*P*-Value	
	0	120	240	360		Linear	Quadratic
90–93 weeks							
ADFI, g	95.22	96.14	93.67	95.96	1.99	0.976	0.942
Average egg weight, g	52.75	53.26	53.42	53.26	0.35	0.280	0.350
Laying rate per hen-day, %	31.32	38.23	33.95	41.65	0.03	0.001	0.005
Feed to egg ratio	6.23	4.95	5.68	4.55	0.30	0.651	0.694
93–96 weeks							
ADFI, g	101.12	100.71	97.72	99.13	2.01	0.018	0.035
Average egg weight, g	55.39	55.13	55.32	54.61	0.41	0.039	0.074
Laying rate per hen-day, %	41.02	44.19	42.83	51.79	0.02	0.000	0.000
Feed to egg ratio	4.53	4.26	4.30	3.67	0.12	0.229	0.177
96–99 weeks							
ADFI, g	97.73	97.82	93.67	97.76	2.02	0.336	0.061
Average egg weight, g	56.61	57.4	57.63	55.37	0.47	0.421	0.209
Laying rate per hen-day, %	43.25	42.59	41.61	48.89	0.02	0.004	0.000
Feed to egg ratio	4.20	4.21	4.09	3.71	0.15	0.795	0.040
Mean							
ADFI, g	98.02	98.22	96.56	97.62	0.75	0.224	0.161
Average egg weight, g	54.76	54.62	54.42	53.68	0.49	0.458	0.164
Laying rate per hen-day, %	38.53	41.67	39.46	47.45	0.08	0.000	0.000
Feed to egg ratio	4.99	4.47	4.69	3.98	0.14	0.574	0.040

ADFI, average daily feed intake; SEM, standard error of mean.

($P > 0.05$) different in all groups from d 21 to 40 and d 21 to 40 except for the d 41 to 60, which was quadratically decreased relative to the CON group. In addition, the mean feed to egg ratio was quadratically decreased in the LP and HP treatments.

3.2. Antioxidant activity parameters

For day 20, there was no differences ($P > 0.05$) among groups in terms of TAC, SOD, and CAT concentrations (Table 3). The GPX was linearly and quadratically decreased ($P < 0.05$) in the HP treatment. For day 40, the TAC was linearly and quadratically increased ($P < 0.05$) in MP and HP treatments. The SOD was not significantly ($P > 0.05$) different between all groups. The GPX was linearly and quadratically decreased ($P < 0.05$) in the HP treatment. For day 60, the TAC and SOD were linearly and quadratically increased ($P < 0.05$) in the HP treatment. The mean TAC was linearly and quadratically increased ($P < 0.05$) in all PCE supplemented treatments. The mean SOD was linearly and quadratically increased ($P < 0.05$) in the HP treatment compared with CON and MP groups. The mean GPX was linearly and quadratically lower in the HP compared to the CON and LP groups. During the whole experimental period, no significant ($P > 0.05$) differences were found in the plasma CAT among the four groups; whereas the MDA was linearly and quadratically lower ($P < 0.05$) in the PCE treatments compared with the CON group.

3.3. Egg quality

No significant difference ($P > 0.05$) was detected in egg shape index, eggshell strength, yolk weight, albumen height, yolk color, and haugh unit among the four groups during the entire experimental period (Table 4). The eggshell thickness was quadratically decreased ($P < 0.05$) in the LP and MP treatments at day 40. The eggshell thickness was linearly and quadratically increased ($P < 0.05$) in all PCE supplemented treatments at day 60. Moreover, the eggshell thickness value in MP and HP treatments were linearly and quadratically higher ($P < 0.05$) than that of the CON and LP groups.

TABLE 3 Effect of purple corn extract on plasma antioxidant activity in laying hen.

Items	PCE supplemental levels (mg/kg)				SEM	*P*-value	
	0	120	240	360		Linear	Quadratic
93-week							
TAC, U/mL	5.49	6.43	6.08	6.24	0.57	0.409	0.538
SOD, U/mL	18.15	19.40	18.34	18.82	0.51	0.711	0.753
GPX, U/mL	224.03	237.50	209.30	197.58	5.95	0.000	0.000
CAT, U/mL	4.14	4.48	4.35	4.73	0.23	0.170	0.389
MDA, nmol/mL	11.21	7.64	6.99	7.02	0.54	0.000	0.000
96-week							
TAC, U/mL	5.54	5.75	8.77	8.31	0.56	0.000	0.000
SOD, U/mL	19.53	19.49	20.82	20.35	0.39	0.062	0.064
GPX, U/mL	162.90	165.39	157.75	144.57	1.74	0.000	0.000
CAT, U/mL	6.22	6.23	5.81	5.65	0.25	0.060	0.114
MDA, nmol/mL	9.00	7.32	7.09	7.34	0.48	0.009	0.003
99-week							
TAC, U/mL	3.38	7.47	5.19	8.11	0.74	0.034	0.100
SOD, U/mL	19.04	18.92	18.58	20.90	0.49	0.022	0.002
GPX, U/mL	187.21	200.78	183.04	188.76	4.07	0.482	0.500
CAT, U/mL	4.26	4.26	4.08	4.64	0.22	0.364	0.315
MDA, nmol/mL	10.66	7.38	7.92	7.72	0.60	0.013	0.005
Mean							
TAC, U/mL	4.88	6.41	6.60	7.46	0.39	0.000	0.000
SOD, U/mL	19.01	19.27	19.00	20.04	0.29	0.007	0.019
GPX, U/mL	192.19	202.51	185.69	176.97	3.45	0.000	0.000
CAT, U/mL	4.86	4.98	4.90	5.02	0.16	0.956	0.902
MDA, nmol/mL	10.11	7.40	7.26	7.28	0.31	0.000	0.000

TAC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde; GPX, glutathione peroxidase; CAT, catalase; SEM, standard error of mean.

3.4. Egg amino acids

The inclusion of PCE was linearly and quadratically increased ($P < 0.05$) most individual amino acid (including aspartate, threonine, serine, glutamate, glycine, alanine, valine, isoleucine, leucine, threonine, histidine, lysine (Lys), arginine, and proline) concentrations in eggs compared to the without PCE group (Table 5). Dietary PCE addition had no significant effect ($P > 0.05$) on the egg methionine content among four groups. The phenylalanine was quadratically increased ($P < 0.05$) in the PCE treatments relative to the CON group. In addition, hens fed PCE was linearly and quadratically increased in essential amino acid (EAA) and umami amino acid (UAA) profiles in egg.

3.5. Egg fatty acids

There were no significant differences ($P > 0.05$) for lauric acid (C12:0), myristic acid (C14:0), myristoleate (C14:1), palmitate (C16:0), palmitoleate (C16:1), margaric acid (C17:0), linolelaidic acid (C18:2n6t), linoleic acid (C18:2n6c), methyl linolenate (C18:3n6), eicosadienoic acid (C20:2), behenic acid (C22:0), erucic acid (C22:1n9), and SFA in egg among all

TABLE 4 Effect of purple corn extract on egg quality in laying hen.

Items	PCE supplemental levels (mg/kg)				SEM	*P*-value	
	0	120	240	360		Linear	Quadratic
90–93 weeks							
Egg-shaped index	1.31	1.30	1.31	1.32	0.02	0.454	0.688
Eggshell strength, kgf/cm²	42.83	41.14	38.49	38.21	2.63	0.116	0.281
Eggshell thickness, mm	0.27	0.25	0.29	0.27	0.01	0.197	0.378
Yolk weight, g	17.36	17.55	16.91	17.35	0.34	0.646	0.835
Albumen height, mm	4.03	4.16	4.11	4.41	0.21	0.243	0.471
Yolk color	6.92	7.92	7.82	7.33	0.40	0.525	0.144
Haugh unit	57.46	61.41	64.01	60.43	2.66	0.334	0.227
93–96 weeks							
Egg-shaped index	1.36	1.36	1.33	1.32	0.01	0.083	0.094
Eggshell strength, kgf/cm²	48.27	43.06	42.64	44.36	2.82	0.293	0.231
Eggshell thickness, mm	0.33	0.29	0.29	0.30	0.01	0.053	0.029
Yolk weight, g	17.64	18.72	18.38	17.59	0.39	0.770	0.135
Albumen height, mm	3.76	4.20	3.76	3.88	0.18	0.905	0.663
Yolk color	6.33	6.64	6.18	7.67	0.44	0.077	0.086
Haugh unit	58.72	62.61	57.78	59.00	1.52	0.534	0.535
96–99 weeks							
Egg-shaped index	1.33	1.31	1.30	1.31	0.01	0.192	0.183
Eggshell strength, kgf/cm²	45.71	42.73	48.31	42.61	1.90	0.643	0.674
Eggshell thickness, mm	0.18	0.25	0.28	0.29	0.01	0.000	0.000
Yolk weight, g	17.30	18.46	18.29	18.01	0.43	0.310	0.142
Albumen height, mm	4.06	4.92	3.93	4.44	0.26	0.901	0.812
Yolk color	6.00	5.75	6.42	6.17	0.48	0.583	0.583
Haugh unit	57.64	68.31	54.40	64.25	3.44	0.724	0.935
Mean							
Egg-shaped index	1.33	1.32	1.31	1.32	0.01	0.187	0.330
Eggshell strength, kgf/cm²	45.68	42.38	42.68	41.90	1.46	0.071	0.154
Eggshell thickness, mm	0.26	0.26	0.29	0.29	0.01	0.002	0.009
Yolk weight, g	17.43	18.22	17.82	17.64	0.23	0.794	0.072
Albumen height, mm	3.95	4.44	3.93	4.24	0.13	0.513	0.657
Yolk color	6.42	6.77	6.79	7.06	0.27	0.106	0.267
Haugh unit	57.89	64.25	58.65	61.29	1.59	0.527	0.417

SEM, standard error of mean.

the groups (Table 6). The PCE treatments showed linearly and quadratic ($P < 0.05$) effect on the myristoleate (C15:0), heptadecenoic acid (C17:1), elaidic acid (C18:1n9t), eicosenoic acid (C20:1n9), heneicosanoic acid (C21:0), eicosatrienoic acid (C20:3n6) concentrations. Moreover, dietary supplementation of PCE was quadratically increased egg stearic acid (C18:0), oleic acid (18:1n9c), arachidic acid (C20:0), linolenic acid methyl ester (C18:3n3), arachidonic acid (C20:4n6), diphenylamine (DPA), docosahexaenoic acid (DHA), monounsaturated fatty acid, and polyunsaturated fatty acid (PUFA) compared to the CON group.

TABLE 5 Effect of purple corn extract on egg amino acid profiles of 99-week-old laying hens.

Items (%)	PCE supplemental levels (mg/kg)				SEM	*P*-value	
	0	120	240	360		Linear	Quadratic
Asparagine	4.62	4.85	4.93	4.88	0.05	0.011	0.002
Threonine	2.36	2.44	2.52	2.48	0.03	0.011	0.005
Serine	3.48	3.59	3.68	3.66	0.04	0.005	0.004
Glutamate	5.69	5.99	6.05	5.97	0.06	0.031	0.002
Glycine	1.54	1.61	1.65	1.62	0.02	0.017	0.002
Alanine	2.50	2.61	2.66	2.62	0.02	0.019	0.003
Valine	2.91	3.07	3.13	3.09	0.03	0.010	0.001
Methionine	1.40	1.47	0.91	1.39	0.02	0.341	0.197
Isoleucine	2.38	2.41	2.46	2.44	0.03	0.008	0.003
Leucine	3.87	4.05	4.15	4.12	0.04	0.004	0.001
Threonine	1.99	2.09	2.13	2.11	0.03	0.016	0.009
Phenylalanine	2.99	3.13	3.17	3.10	0.03	0.074	0.004
Histidine	1.01	1.07	1.09	1.07	0.01	0.018	0.003
Lysine	3.32	3.48	3.50	3.51	0.03	0.009	0.005
Argnine	2.97	3.12	3.18	3.16	0.03	0.005	0.001
Proline	1.67	1.76	1.83	1.80	0.02	0.004	0.001
EAA	18.77	19.66	20.02	19.81	0.19	0.027	0.040
UAA	19.33	20.30	20.58	20.31	0.20	0.022	0.003

EAA, essential amino acids; UAA, umami amino acids; SEM, standard error of mean. EAA, threonine+valine+methionine+isoleucine+leucine+phenylalanine+lysine; UAA, asparagine+glutamate+glycine+alanine+threonine+phenylalanine.

4. Discussion

The free radicals in poultry are usually in homeostasis and have important biological functions under normal circumstances (21). However, free radicals accumulated and resulted in animals entering the oxidative status, reducing the resistance and production performance of poultry (22). Plant flavonoids could improve the reproductive capacity of animals by releasing hormones, such as growth hormone, in livestock and poultry (23). Additionally, animals receiving anthocyanin extract can enhance digestive enzyme activity, thus improving feed digestibility and affecting nutrient absorption (17). Hence, we found that PCE could improve the HD values, which might be related to anthocyanin improve reproductive capacity and nutrient absorption. Consistent with our results, Tufarelli et al. (24) found that the feeding of anthocyanin-rich dried grape pomace in a laying hen diet did not affect the ADFI, whereas it could improve egg production. Duenas et al. (25) suggested that broiler chickens receiving anthocyanin-rich cranberry extract had an improved feed conversion ratio relative to the control group.

Radical reactions in the body must be in dynamic balance. Oxidative injury induced by excess free radicals is an etiology of several diseases. As a high-yielding animal species, laying hens have a more vigorous metabolism, producing higher levels of reactive oxygen free radicals. Generally, the activity of antioxidant enzymes decreases, and free radicals accumulate, and thus negatively affecting health and production of laying hens (26). Anthocyanin-rich plants can improve antioxidant potential by decreasing thio-barbituric acid-reactive substance values in breast meat in broilers (27). In addition, plant flavonoids can decrease the production of proliferation, T-cell activation, and proinflammatory cytokines, and thus regulating the immune system in animals (28). Saleh et al. (29) concluded that dietary supplementation with anthocyanin-rich sorghum can modulate the abundances of the mRNAs of genes related to antioxidative properties, such as GPX and SOD, which were increased in chickens. Moreover, anthocyanin plants can relieve the inflammatory reaction by the expression of genes related to interleukin-1β and interleukin-6 in chickens, thus enhancing antioxidants in broiler chickens (30). Hence, we found that the inclusion of PCE in hen diets can increase the concentration of TAC, SOD, and GPX, as well as decrease the content of MDA in blood. This might be because anthocyanins can provide excess phenolic hydrogen atoms to free radicals and neutralize free radicals in the animal body (31); and anthocyanins can

TABLE 6 Effect of purple corn extract on egg fatty acid profiles of 99-week-old laying hens.

Items (%)	PCE supplemental levels (%)				SEM	*P*-value	
	0	120	240	360		Linear	Quadratic
Lauric acid, C12:0	0.007	0.009	0.009	0.011	0.00	0.240	0.517
Myristic acid, C14:0	0.445	0.495	0.439	0.439	0.00	0.256	0.097
Myristoleate, C14:1	0.118	0.148	0.109	0.117	0.00	0.301	0.274
Myristoleate, C15:0	0.039	0.043	0.043	0.043	0.00	0.009	0.000
Palmitate, C16:0	26.533	27.411	26.020	26.603	0.03	0.406	0.649
Palmitoleate, C16:1	3.911	4.492	3.679	3.928	0.01	0.369	0.462
Margaric acid, C17:0	0.125	0.119	0.136	0.131	0.00	0.060	0.188
Heptadecenoic acid, C17:1	0.100	0.099	0.103	0.142	0.00	0.001	0.000
Stearic acid, C18:0	8.055	7.693	7.871	8.128	0.02	0.417	0.000
Elaidic acid, C18:1n9t	0.039	0.045	0.099	0.145	0.02	0.002	0.008
Oleic acid, C18:1n9c	47.972	46.231	47.197	47.871	0.08	0.746	0.002
Linolelaidic acid, C18:2n6t	0.013	0.028	0.017	0.011	0.01	0.554	0.215
Linoleic acid, C18:2n6c	10.882	10.897	12.038	10.337	0.01	0.782	0.050
Arachidic acid, C20:0	0.037	0.025	0.027	0.029	0.00	0.087	0.000
Methyl linolenate, C18:3n6	0.052	0.098	0.073	0.061	0.01	0.977	0.226
Eicosenoic acid, C20:1n9	0.014	0.303	0.287	0.272	0.01	0.011	0.000
Linolenic acid methyl ester, C18:3n3	0.219	0.256	0.248	0.234	0.00	0.350	0.000
Heneicosanoic acid, C21:0	0.016	0.017	0.047	0.041	0.00	0.001	0.003
Eicosadienoic acid, C20:2	0.113	0.109	0.121	0.104	0.00	0.389	0.128
Behenic acid, C22:0	0.084	0.093	0.070	0.080	0.00	0.110	0.295
Erucic acid, C22:1n9	0.016	0.012	0.020	0.026	0.00	0.054	0.070
Eicosatrienoic acid, C20:3n6	0.113	0.116	0.112	0.108	0.00	0.009	0.000
Arachidonic acid, C20:4n6	0.886	1.008	1.007	0.925	0.00	0.438	0.000
Diphenylamine, DPA	0.041	0.046	0.045	0.039	0.00	0.515	0.000
Docosahexaenoic acid, DHA, C22:6n3	0.154	0.207	0.182	0.176	0.00	0.456	0.000
SFA	35.341	35.906	34.661	35.504	0.04	0.563	0.764
MUFA	52.171	51.330	51.495	52.501	0.07	0.406	0.000
PUFA	12.473	12.764	13.844	11.994	0.02	0.856	0.012

SFA, total saturated fatty acid profiles; MUFA, total monounsaturated fatty acid profiles; PUFA, total polyunsaturated fatty acid profiles; SEM, standard error of mean.

participate in the metabolism of phospholipids, arachidonic acid and protein phosphorylation to protect lipids from oxidative damage (32). Consistent with our findings, Wang et al. (33) showed that the addition of 100 mg/kg anthocyanin-rich bilberry extract could increase SOD and GPX, but it could decrease MDA of yellow-feathered chickens. Additionally, Reis (34) demonstrated that laying hens receiving anthocyanin-rich grape pomace flour could increase TAC, SOD and GPX in the serum compared to the control.

Generally, with the increase in the daily age of laying layers, the eggshell quality of eggs produced decreases (35). Free radicals accumulate in the body due to their long oxidative metabolism, which may also damage the formation of egg protein and reduce the protein haugh unit of the laying hens at the later laying period (36). Moreover, the decline in eggshell quality were decreases of estrogen levels, calcium absorption and metabolic capacity in late egg laying (37). Notably, the inclusion of phenolic-rich plant extract may regulate the intestinal microflora profile in chickens, and improving health condition (38). Of interest, anthocyanins and their metabolites can reduce pathogenic bacteria and improve beneficial bacteria (*Lactobacillus* and E*nterococcus*), improving animal health and

increasing production performance in poultry (39). Thus, we found that laying hens fed a diet containing anthocyanin-rich PCE showed higher value of eggshell thickness in eggs, perhaps due to anthocyanin can improve the intestinal environment and liver SOD concentration in laying hens (40, 41). Furthermore, Kara et al. (42) demonstrated that supplementing laying hen diets with 4% anthocyanin-rich grape pomace has the potential to increase egg weight, improving the egg quality of 80-week-old 96 Bovans laying hens. In short, anthocyanin-rich PCE improved the nutritional value of eggs, which has a higher market value and is more conducive to future application in production.

The amino acid content of eggs is balanced, which is easily absorbed and utilized by consumers, and it is the most ideal source of high-quality protein in natural food (43). Dietary supplementation with natural antioxidants in animal diets could improve UAA concentrations because it can increase antioxidant potential and regulate related umami gene expression in the body (44). In the current research, we found that the feeding of anthocyanins from PCE could improve UAA concentrations in eggs compared to the CON group. This possibly due to purple corn anthocyanins might take part in the UAA signaling pathway in laying hens, resulting in the higher EAA and UAA content in eggs in Chishui black bone hens. The exact reasons remain unclear, and further observations are needed. Our observations are in agreement with Omar et al. (45), who showed that phenolic-rich onion extract could improve the amino acid ileal digestibility of amino acids in broiler chicken. Mishra et al. (46), who found that anthocyanins could enhance higher levels of antioxidant activity, and improve the concentrations of Lys and tryptophan in eggs in white leghorn layer chickens.

The PUFAs are important to the human body, and the intake of a certain amount of unsaturated fatty acids has a good regulatory effect on blood fat, vision and immune function. However, PUFA molecules could transform into hydroperoxides, conjugated dienes, and peroxy radicals (47). Anthocyanins have antioxidant properties, which can prevent unsaturated fatty acids on the cell membrane from being oxidized. Crescenti et al. (48) showed that anthocyanin-rich grape seed can promote related fatty acid absorption and beta-oxidation genes overexpression, which can subsequently ameliorate obesity and metabolic disorders. Thus, MP and HP treatments increased UFA concentration in eggs, perhaps because anthocyanins not only exhibit a higher oxygen radical absorbance capacity; but also can provide hydrogen donors to the lipid free radicals, and thus resulting in the inhibition of lipid peroxidation (49–51). Consistent with our observations, Untea et al. (52) found that anthocyanin-rich bilberry leaves could improve egg UFA profiles (C18:2n6, C18:3n6, C20:3n6 and total n-6 fatty acids) in laying hens.

5. Conclusions

The results of the current research suggest that dietary supplementation with PCE can enhance plasma antioxidation ability, increase egg production, and improve egg amino acid and fatty acid profiles during the late laying period of laying hens.

Data availability statement

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

Ethics statement

The animal study was reviewed and all experimental animal care procedures were approved by the Rules of Animal Welfare and Experimental Animal Ethics of Guizhou University (EAE-GZU-2021-P017), Guizhou, China.

Author contributions

JL conducted writing–original draft, data curation, methodology, and software. DZ and HL conducted resources, investigation, and supervision. QLuo, XW, JQ, and YX conducted resources and validation. QLu contributed by investigation. XT contributed by resources, writing–review and editing, software, and project administration. All authors contributed to the article and approved the submitted version.

Funding

This study has gained the support of the Joint Research Project of Local Poultry Industry of Guizhou Province (Qiannongcai (2020)175), the National Natural Science Foundation of China (32260849), the Science and Technology Project of Guizhou Province (Qiankehe foundation-ZK [2021] General 164), the Youth Science and Technology Talent Development Project of Guizhou Province (Qianjiaohe KY [2022] 150), and the Cultivating Project of Guizhou University (2019-33).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 09 January 2023
DOI 10.3389/fvets.2022.1092812

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to
Animal Nutrition and Metabolism,
a section of the journal
Frontiers in Veterinary Science

RECEIVED 08 November 2022
ACCEPTED 15 December 2022
PUBLISHED 09 January 2023

CITATION
Bar-Dagan H, Gover O, Cohen NA,
Vetvicka V, Rozenboim I and
Schwartz B (2023) Beta-glucans
induce cellular immune training and
changes in intestinal morphology in
poultry. *Front. Vet. Sci.* 9:1092812.
doi: 10.3389/fvets.2022.1092812

Beta-glucans induce cellular immune training and changes in intestinal morphology in poultry

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Introduction: Beta-glucans are known as biological response modifiers due to their ability to activate the immune system. This research aimed to determine the efficacy and safety of feeding beta-glucans from various sources on the immune status and intestinal morphology of chickens.

Methods: To this end we used *in vitro* and *in vivo* set-ups. In the *in vitro* set-up the chicken macrophage cell line HD-11 was used to measure the response of the chicken immune cells to beta-glucans extracted from algae and mushrooms on immune-related gene expression and associated activities. Additionally, we conducted two *in vivo* experiments using either beta-glucans extracted from yeast or mix of yeast and mushrooms beta-glucans as part of the chicks feed in order to test their effects on the chick intestinal morphology.

Results: In the *in vitro* set-up exposure of HD-11 cells to a concentration of 1 mg/ml of algae and mushroom beta-glucans resulted in significantly higher expression of 6 genes (TNFα, IL4, IL6, IL8, IL10, and $iNOS_2$) compared to control. The release of nitrite oxide (NO) to the medium after exposure of HD-11 cells to mushrooms or algae beta-glucans was significantly increased compared to control. Additionally, significantly increased phagocytosis activity was found after exposure of the cells to algae and mushroom beta-glucans. In the *in vivo* set-up we observed that the length of the villi and the number of goblet cells in the ileum and the jejunum in the beta-glucan fed chicks were significantly augmented compared to control, when the chicks were fed with either yeast or yeast and mushroom beta-glucans mix.

Discussion: In conclusion, dietary supplementation of poultry with beta-glucan exerts significant and positive effects on immune activity and the intestinal morphology in poultry.

KEYWORDS

beta-glucans, HD-11 cells, immune training, biological response modifiers, goblet cells

1. Introduction

Bacterial infections in poultry represent an essential issue of farming that affects animal welfare, health, and productivity. Infections due to bacteria in poultry are an important concern for the bird's health and efficient production. So far, farmers have used antibiotics to control diseases involving bacterial infections in poultry. Chickens have also been fed with low concentrations of antibiotics to improve daily weight gain and feed efficiency through alterations in disease suppression (1). Due to the massive use of antibiotics, a surge in the development and spread of antibiotic-resistant bacteria has become a major cause for concern. Antimicrobial resistance is a natural event that takes place when bacteria no longer respond to antibiotics to which they were previously susceptible and that were previously active in treating infections caused by these microorganisms (2). Over the past few decades, no major new types of antibiotics have been produced. Almost all known antibiotics are increasingly losing their effectiveness against pathogenic microorganisms, especially those used in poultry farming (3). As the incidence of antibiotic resistance has become a serious problem, there is increased pressure on producers to reduce the use of antibiotics in the poultry farming industry (2).

With the reduced availability of antibiotics, poultry producers work to reduce the use of antibiotics and look for feed additives to stimulate the immune system of chickens to resist microbial infection (4). Moreover, there has been increased interest in "natural" feed additives that can stimulate the immune system of poultry. In addition, a significant push to produce antibiotic-free poultry is gaining popularity among the general public (5).

Several studies show that beta-glucans may play a role in replacing antibiotics and stimulate the immune system (6). Glucans are carbohydrates made of complex glucose polymers that provide the primary structure found in the cell wall of yeast, fungi, algae, and cereal grains such as oats and barley (7). The structures of glucans vary depending on their source and the type of linkages on the glucose polymers (8). The nature of these linkages will affect the functionality of the molecules.

Beta-glucans are transported to the small intestine, then pass through the Peyer's patches in the gut-associated lymphoid tissue (GALT), and subsequently, they are transported around the body (9). The effect of beta-glucans is mediated by its binding to specific receptors. It stimulates macrophages, secretion of antibodies, and increases the activity of natural killer cells. Similar to mammals, avian macrophages synthesize cytokines and chemokines such as Tumor Necrosis Factor-alpha (TNFα), Interleukin 4 (IL4), Interleukin 6 (IL6), Interleukin 8 (IL8), Interleukin 10 (IL10), and interferon gamma (IFN-γ) (10, 11). In addition to the direct stimulation of specific and non-specific immunity, glucans can also influence the expression of immune-related genes and proteins. The stimulation in immune functioning serves to combat the adverse effects of enteric infection or immune suppression due to high-stress rearing conditions (9).

Beta-glucans were reported to be effective in promoting the growth of broiler chickens and improving their meat quality (12). Beta-glucans have been shown to improve gut health in poultry subjected to a bacterial challenge and to increase the flow of new immunocytes into the various lymphoid organs (13). Beta-glucans can increase macrophage functionality (14), affect intestinal morphology, and function as anti-inflammatory immunomodulators (7). Dietary Mannan-Oligosaccharide (MOS) Yeast Extract containing MOS and beta-glucans supplementation to turkey poults can enhance the number of goblet cells and upregulate mucin-2 production from goblet cells (15), improves villus height and crypt depth and their ratio in the duodenum and ileum (16). Moreover, consumers can more easily accept feed enriched with beta-glucans with more confidentiality than antibiotics-treated poultry (5). We propose that when using a declaration "glucans introduced into the poultry feed" will be always associated with "antibiotic free poultry," and this information can be easily transferred to the consciousness of the consumer. Beta-glucan mixtures may be implemented to obtain optimal anti-inflammatory, immunomodulatory, growth performance and optimized intestinal morphology and histology responses in poultry (17, 18).

The specific activity of each beta-glucan subtype is unknown, and no direct comparison of beta-glucans derived from different sources has been made in poultry. Despite numerous glucans isolated from various sources, the decision about the most active glucans in avian or any other animal model has not been reached, sometimes leading to contradictory results. Because there is extensive variation in beta-glucan structure based on their origin, their effectiveness in modulating the immune system can vary (8). Nonetheless, it is generally accepted that 1,3 and 1,6-beta-glucans, derived from yeast and mushroom, are considered the most effective form available due to their complex network of carbohydrate branching (19, 20). Therefore, we focused on using beta-glucans, originating from yeast, mushroom, and algae, as putative immunomodulators in poultry. Our goal was to find the optimal beta-glucan type and concentration to exert the optimal immunological training and adaptations on intestinal morphology activities in poultry using *in vitro* and *in vivo* setups. The results of our study may contribute to the general goal of reducing or even eliminating antibiotics which may be exceptionally beneficial to farmers to prevent economic losses and to consumers of chicken that seek antibiotic-free meat (21). The results may contribute commercially necessary knowledge on this critically not adequately explored agricultural field.

2. Materials and methods

2.1. Beta-glucans

(1) An algae glucan extract, used in *in-vitro* assays, was kindly provided by Quegen Biotech (S. Korea). (2) Yeast (Saccharomyces cerevisiae) glucans (Glucan #300) used in *in-vivo* assays were kindly provided by Transfer Point (Columbia, SC, USA). Algae beta-glucans were used for the *in-vitro* assays since these beta glucans are easily soluble in water. Yeast (Saccharomyces cerevisiae) glucans are completely insoluble in water or any other solvents thus we could not use them in the *in vitro* assays. Moreover, the algae beta glucans cost is extremely high thus they are not feasible for using for feeding purposes, thus we elected for the feeding experiments to use yeast glucans (the ratio of alpha to beta glucans in both preparations is similar) and algae beta glucans for the *in vitro* assays. For *in-vitro* assays glucans were dissolved in double distilled water (DDW) to a final concentration of 50 mg/ml of the extract in a water bath for 1 h at 80°C. The solution was sterilized in an autoclave by heating at 121°C for 30 min. The solution was then diluted in Dulbecco's Modified Eagle Medium (DMEM) to a final concentration of 5, 1, 0.5, and 0.05 mg/ml. (3) Mushroom glucans were extracted from King oyster mushrooms (kindly provided by Tekoa farms, Israel). Mushrooms were ground to fine powder in dry ice. Mushroom powder was dissolved in distilled water, in a concentration of 1 g of mushrooms powder in 10 ml and autoclaved at 121°C for 30 min. The solution was then pelleted at a speed of 13,000×*g* at 10°C for 15 min; the liquid phase was transferred to ethanol to a final concentration of 1:2. The solution was then stored at a temperature of −20°C overnight. After about 24 h, a float is formed. The float was dried in a fume hood for 24 h. The dry float was frozen at −80°C overnight and lyophilized to obtain a uniform powder (22). For *in-vitro* experiments the mushrooms extracted glucans were dissolved in the solvent dimethyl sulfoxide (DMSO) to a final concentration of 5 mg/ml of the beta glucan extract in a water bath for 1 h at 90°C and diluted in DMEM at the final concentration of 5, 1, 0.5, and 0.05 mg/ml. α and β glucans were quantified using Megazyme beta-glucan assay kit (Megazyme, Ireland) according to manufacturer protocol.

2.2. Cell culture

The chicken HD11 macrophage cells were kindly provided by Dr. Elisabeth Kowenz-Leutz (Max Delbrueck Center, Berlin, Germany) and grown in 75 mm^2 flasks with 18 ml DMEM growth medium supplemented with 8% fetal bovine serum (FBS), 2% chicken serum, 1% penicillin, and streptomycin. Then the cells were incubated at 37°C and 5% CO_2 until they attained 95% confluency. For gene expression and Nitric Oxide analysis cells were grown in 6-well plates (1×10^6 cells/ml). After 48 h, the medium was removed, and fresh DMEM medium with Lipopolysaccharide (LPS) (from *E. Coli*, O111:B4; Sigma L4130 (used at specified concentrations as indicated below) or glucans were added and used as a pro-inflammatory stimulant. Cells were harvested for RNA and supernatants were collected and stored at −80°C.

2.2.1. Gene expression

RNA was extracted from HD11 cells using NucleoSpin RNA, Mini kit (MN, Germany), according to manufacturer instructions. RNA was quantified using Nanodrop 2000 (ThermoFisher USA). 2 μg of RNA was used to synthesize cDNA using qScript cDNA Synthesis Kit (Quanta bio, USA).

Real-time qPCR was performed using a fast SYBR green master mix (ABI, USA) on Quant studio 1 machine (ABI, USA). For normalization of gene expression in all reactions, we used the GAPDH gene. Primers for relative gene expression are presented in Table 1. Normalization and quantification were performed using either the std curve of serial 1:5 dilutions or the ddCt method (23).

2.2.2. Nitric oxide colorimetric assay

Nitrate/Nitrite colorimetric assay kit (Cayman Chemical, USA) was used to quantify the amount of released nitric oxide to the medium. The process was done according to the manufacturer's protocol. Briefly, 200 μL of Assay Buffer was added to empty 96-wells, followed by 80 μL of the sample medium. 10 μL of Enzyme Cofactor was added to each well, followed by 10 μL of Nitrate Reductase Mixture for 1 h at room temperature. The wells were supplemented with Griess Reagent for 10 min of incubation and transferred to a plate reader to read the absorbance at 540 nm (BioTek synergy H1, Agilent technologies CA, USA).

2.2.3. Phagocytosis assay

The macrophage phagocytosis activity was assessed using the kit pHrodo™ Red Zymosan BioParticles ("Zymosan," Life Technologies, USA) according to the manufacturer's protocol. Briefly, HD11 cells were grown in 96-well plates (1×10^6 cells/ml) for all experiments. After 48 h, the medium was removed, and a fresh DMEM medium with either algae beta-glucans (1 mg/ml), mushroom beta-glucans (1 mg/ml), or LPS (100 ng/ml, prepared in sterilized DDW) was added and used as a stimulant activator for 1 h. The BioParticles provided by the kit were vortexed and resuspended homogeneously in clear DMEM (pH = 7.4) and then sonicated for 10 min (Bioruptor; Diagenode, Denville, NJ) in order to homogeneously disperse the particles. The medium was then removed from the cultures, replaced with the dispersed "Zymosan particles" 100 μL/well, and incubated at 37°C with 5% CO_2 for another 1–1.5 h.

TABLE 1 List of primers tested for relative gene expression.

Gene	Primer F	Primer R
GAPDH	CCTAGGATACACAGAGGACCAGGTT	GGTGGAGGAATGGCTGTCA
TNFa	CGCTCAGAACGACGTCAA	GTCGTCCACACCAACGAG
IL-4	GCTCTCAGTGCCGCTGATG	GAAACCTCTCCCTGGATGTCAT
IL-6	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT
IL-8	TCCTGGTTTCAGCTGCTCTG	TGGCGTCAGCTTCACATCTT
IL-10	ACAAAGCCATGGGGGAGTTC	GTTAAGCTGCCATTGAGCCG
IFN-γ	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC
Cytochrome C	ACGCAAAACAGGACAAGCTG	TCAGAGTATCCTCACCCCAAGT
BAX	TCCTCATCGCCATGCTCAT	CCTTGGTCTGGAAGCAGAAGA
BCL-2	GATGACCGAGTACCTGAACC	CAGGAGAAATCGAACAAAGGC
Caspase-9	CGAAGGAGCAAGCACGACAG	CCGCAGCCCTCATCTAGCAT
iNOS	AACTCTCACAAAAACACGAAGCA	TTGTGTGATGTGGGAACGCT

Phagocytosis activity in the attached cells was measured using a plate reader using excitation of 560 nm and emission of 590 nm (BioTek synergy H1, Agilent technologies CA, USA).

2.3. *In vivo* experiments

All experimental procedures were carried out under the approval of the ethics committee of the Hebrew University of Jerusalem. Two experiments were conducted at the poultry unit and research laboratories at the Faculty of Agriculture, Food and Environmental Sciences, Israel. All animal experiments were done under the care and supervision of Prof. Israel Rozenboim's. One of the *in vivo* experiments tested the effects of treatment with beta-glucans extracted from yeast at various concentrations; on the growth and development of broilers. The second *in vivo* experiment tested the effects of treatment with two types of beta-glucans isolated from yeast and mushrooms, at various concentrations. The experiments tested the growth and development of broilers and their gut health. Briefly, 1-day-old Fertile Ross 308 broilers were randomly allotted by sex (male and female) into a complete randomized block design experiment in a 3 × 2 factorial design for a 5-week feeding trial. Broiler chickens were subjected to a standard commercial diet supplemented with different levels of beta-glucan: No beta-glucans (control), Beta-glucans at a ratio of 250 mg/kg, and 1 g/kg feed were mixed with a standard commercial feed (basic feed). The first *in vivo* experiment was on February 2021 at the Faculty of Agriculture experimental hen house in Rehovot Israel. 150 chickens were divided according to gender (75 male and 75 females) and subdivided to three treatment groups. Control, 250 mg/kg feed beta glucans (Glucan #300, 89% beta-glucans), 1 g/kg feed glucans. The second *in vivo* experiment was conducted on April 2022. 60 chickens were divided according to gender (30 male and 30 females) and subdivided to three treatment groups. (1) Control (2) 250 mg/kg beta-glucans from yeast and cultivated mushrooms, in a 3:1 yeast-to-mushroom ratio into the feed; (3) 1 g/kg beta-glucans from yeast and cultivated mushroom in a 3:1 yeast-to-mushroom ratio into the feed. This feeding regime was elected based on preliminary results in a small number of birds (not shown) demonstrating positive effects on parameters measured for yeast beta-glucans. At the age of 35 days birds were sacrificed and paraffin slides were created as described below. During the whole period of the experiment, all birds had free access to feed and water. Birds were raised on two littered floor rearing rooms partitioned into 15 pens, each at a density of 10 birds per pen for each sex. The room temperature was centrally regulated and ventilated. All broilers were exposed to a 23 L:1D photoperiod. At 35 days of age, birds were sacrificed by CO_2 asphyxiation, and an autopsy was conducted. Segments of the small intestine were obtained and fixed in a 4% buffered formalin solution. Serial 4 μm transverse sections from the ileum and jejunum were cut and stained for morphological evaluation (H&E stain). The villi length and the number of goblet cells were measured and counted using ImageJ software (National Institutes of Health, Bethesda, version 1.53s).

2.3.1. Alcian blue-periodic acid: Schiff (AB-PAS) staining

AB-PAS staining of paraffin-embedded sections and formalin-fixed slides was performed according to the manufacturer's instructions [Periodic Acid-Schiff (PAS) Stain Kit (Scytek Laboratories)]. Briefly, 3% acetic acid solution

was added dropwise onto tissue sections, followed by 5–10 drops of Alcian blue solution (pH 2.5). The slides were stained for 25–30 min and then added 5–10 drops of the periodic acid solution for 5 min. Slides were rinsed and added with Schiff's solution for 20 min, followed by Hematoxylin staining. Sections were mounted and visualized under the microscope and goblet cells were counted manually.

3. Statistics

All statistics were performed on JMP pro 14 (SAS Institute Inc., Cary, Nc, 1989-2019); data are expressed as mean ± SE. Comparison between means of more than two groups was analyzed using means ANOVA and Tukey HSD.

4. Results

4.1. Incubation of HD11 macrophages with algae β-glucans

Out of 11 genes assayed 6 showed differential expression in response to exposure to LPS. Expression was tested at different time points. TNFα, IL4, IL6, INFγ, Cytochrome C and BAX exibited maximal expression after 4 h of incubation with 100 ng/ml LPS (Supplementary Figure 1). IL8 showed maximal expression at 8 h with 100 ng/ml LPS (Supplementary Figure 1D), anti-inflamatory cytokine IL10 peaked after 2 h with 200 ng/ml LPS (Supplementary Figure 2A), BCL-2 peaked after 24 with 200 ng/ml LPS and Caspase 9 after 4 h with 200 ng/ml as well (Supplementary Figures 3A, B). $iNOS_2$ significatly increased after 8 h and 50 ng/ml LPS (Supplementary Figure 3C).

Algae beta-glucans stimulated dose-dependently gene expression of TNFα and IL4. The maximal expression obtained for TNF-α (Figure 1A) was detected at 5 mg/ml after 8 h incubation and 1 and 5 mg/ml for 24 h incubation. For IL4, the highest expression was reached at 5 mg/ml for 8 h followed by 1 and 5 mg/ml for 24 h (Figure 1B).

A similar expression pattern was shown for IL6 and IL10 (Figures 2A, B), where the expression peaked at 8 h and 5 mg/ml whereas for IL8 and $iNOS_2$ the highest levels were detected at 24 h (Figures 2C, D). The results indicate upregulation of cytokine gene expression resulting from incubation with algae beta-glucans.

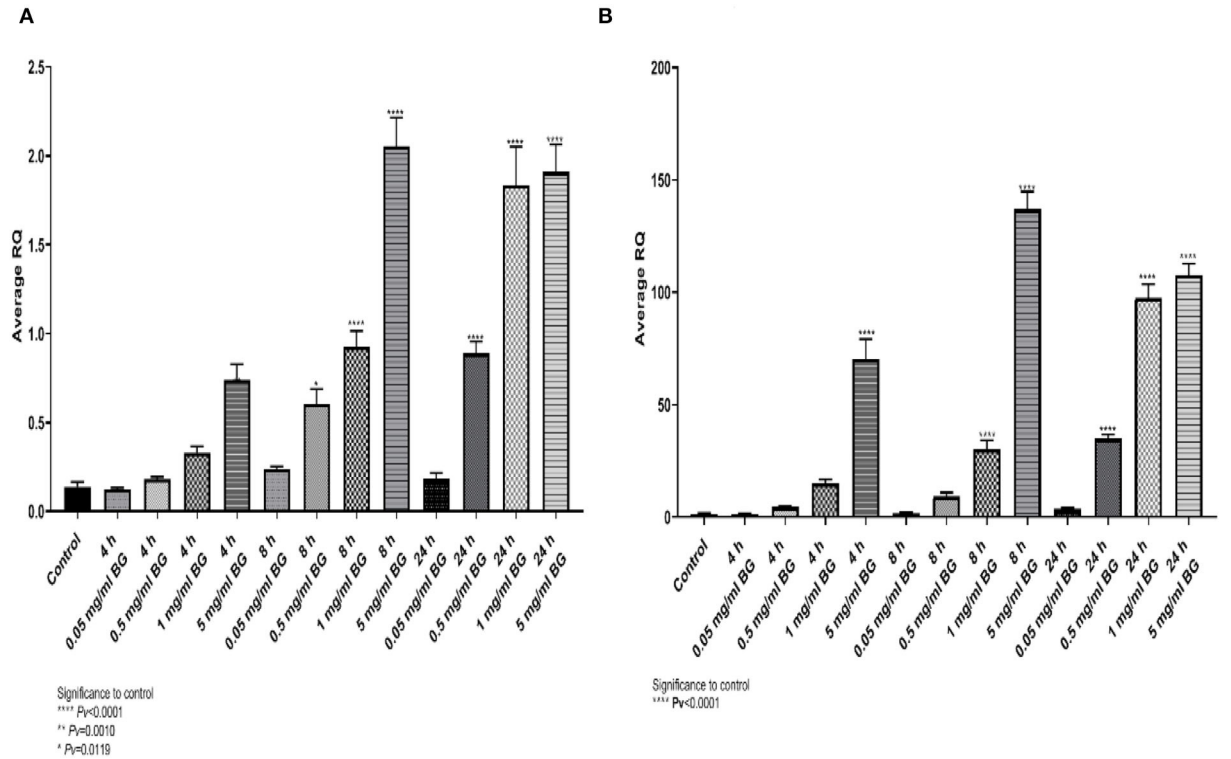

FIGURE 1
(A) Average relative expression for the gene TNFα, **(B)** the relative fold gene expression (DDCT) for the gene IL4, after exposure to algae beta-glucans at 3-time points and 4 concentrations (n = 6). ****Pv < 0.000.1, **Pv = 0.0010, *Pv = 0.0119—compared to control.

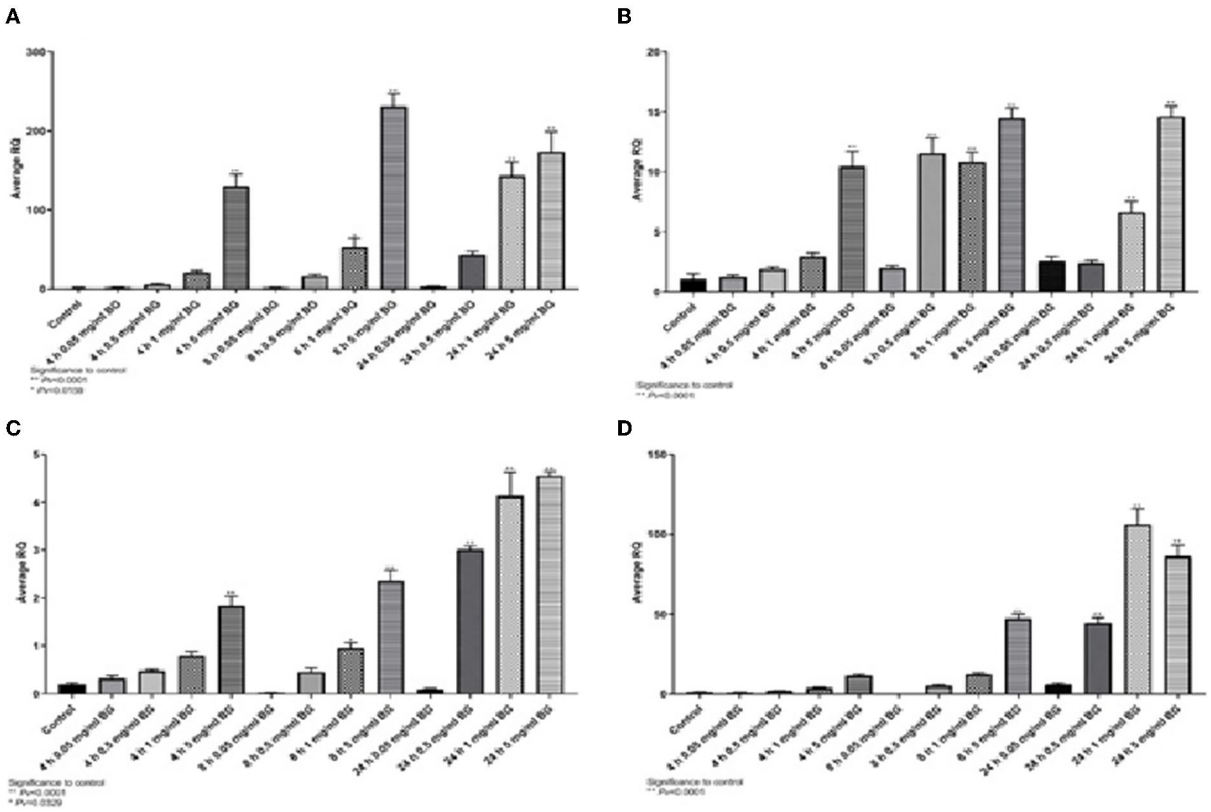

FIGURE 2
Expression of cytokine genes at different algae β-glucan concentrations and incubation times. **(A)** IL6, **(B)** IL10, **(C)** IL8 and **(D)** $iNOS_2$.

4.1.1. Release of nitric oxide

Nitric oxide is is an important molecule related to the antimicrobial effects of macrophages and is a chemical indicator of inflammatory response. The release of NO was significantly different from the control levels after exposure to 1 mg/ml of algae beta-glucans for 24 h. The release of NO after stimulus with LPS at a concentration of 100 ng/ml for 4 h of stimulation showed no significant difference from the control; however, after 8 h was significantly different from control. The release of NO to the medium after exposure to algae beta-glucans at a concentration of 1 mg/ml for 24 h was significantly higher than even after the exposure to LPS for 4 h as well as for 8 h (Figure 3).

4.2. Comparison of beta-glucans extracted from algea and mushrooms

Analysis of glucan content revealed that algae extracts consisted of 59.4% beta-glucans whereas mushroom extracts contain 8.5% beta-glucans. HD11 macrophages were incubated with 1 mg/mL of each extract for 24 h.

4.2.1. Gene expression

TNFα expression was significantly higher than control after exposure to algae beta-glucans, but no significant expression was measured when exposed to beta-glucans extracted from mushrooms (Figure 4A). The expression of IL4, IL6, and IL8 was significantly different from the control after 24 h of exposure to algae beta-glucans and mushrooms beta-glucans (Figures 4B–D). IL10 expression was significantly different from the control after exposure to mushrooms beta-glucans, but no significant expression was measured when exposed to algae beta-glucans (Figure 4E). iNOS, similarly to IL4, IL6, and IL8, expressed significantly from the control after 24 h of exposure to both algae and mushrooms beta-glucans (Figure 4F).

4.2.2. Nitric oxide secreation

The release of NO to the medium after exposure to either mushrooms or algae beta-glucans for 24 h, and 1 mg/ml beta-glucans, was significantly greater compared to the control group. Algae beta-glucans induced a release of more NO than the groups exposed to LPS for either 4 or 8 h. These results are consistent with our previous results related to NO gene expression. In contrast, the amount of NO released after

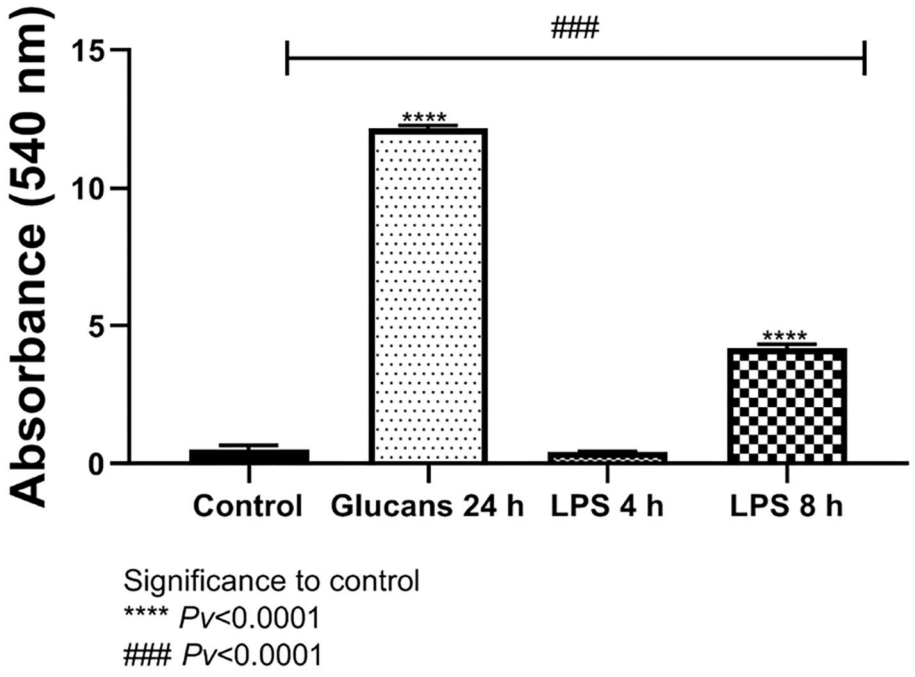

FIGURE 3
Release of NO to the medium after exposure to algae beta-glucans glucans for 24 h, or LPS. ****Pv < 0.0001 compared to the control group. ###Pv < 0.0001 compared the exposure to beta-glucans in contrast to LPS for 4 and 8 h (n = 5).

exposure to mushroom beta-glucans was significantly different from the group exposed to 4 h of LPS but not the one exposed to 8 h of LPS (Figure 5).

4.2.3. Phagocytosis

To examine phagocytosis activity, HD11 cells were incubated for 1 h with 1 mg/ml of either mushroom or algae beta-glucans or 100 ng/ml of LPS. Phagocytosis was measured using pHrodo™ Red Zymosan BioParticles ("Zymosan," Life Technologies, USA) after 60 min (Figure 6). Algae and mushroom beta-glucans increased phagocytosis activity significantly compared to control at both time points. LPS increased phagocytosis activity significantly from the control only after 60 min (Figure 6).

4.2.4. Vili size

The increase of villi height directly affects the nutrient absorption capability in the intestine as it would increase the absorptive and surface area (24). The villi height was defined as the distance from the villus tip to the crypt junction. Measurements of the villi taken from the female ileum showed no differences between treatment with glucan #300 beta-glucans to control (Figure 7A, Supplementary Figure 4). The length of the male villi from the ileum was significantly longer in both treatment groups compared to the control (Figure 7B). Measurements of the female and male villi from the jejunum showed significant differences between treatment with glucan #300 beta-glucans to control (Figures 7C, D).

4.2.5. Goblet cell count

The number of goblet cells from the ileum and the jejunum of the females showed significant differences between both treatments, 1 g/kg and 250 mg/kg, with beta-glucans to control (Figures 8A, C, Supplementary Figure 5). These results were also evident in the male group (Figures 8B, D).

4.3. Effect of a combination of yeast and mushroon glucans

Measurements of the villi taken from the female jejunum showed significant differences between treatment with beta-glucans to control. The length of the female and male villi from the jejunum was significantly longer in both treatment groups compared to the control (Figures 9A, B). The number of goblet cells found in the jejunum of the females showed significant differences between both treatments, 1 g/kg and 250 mg/kg beta-glucans treatments as compared to control (Figure 9C). These results were also observed in the male group (Figure 9D). Similar

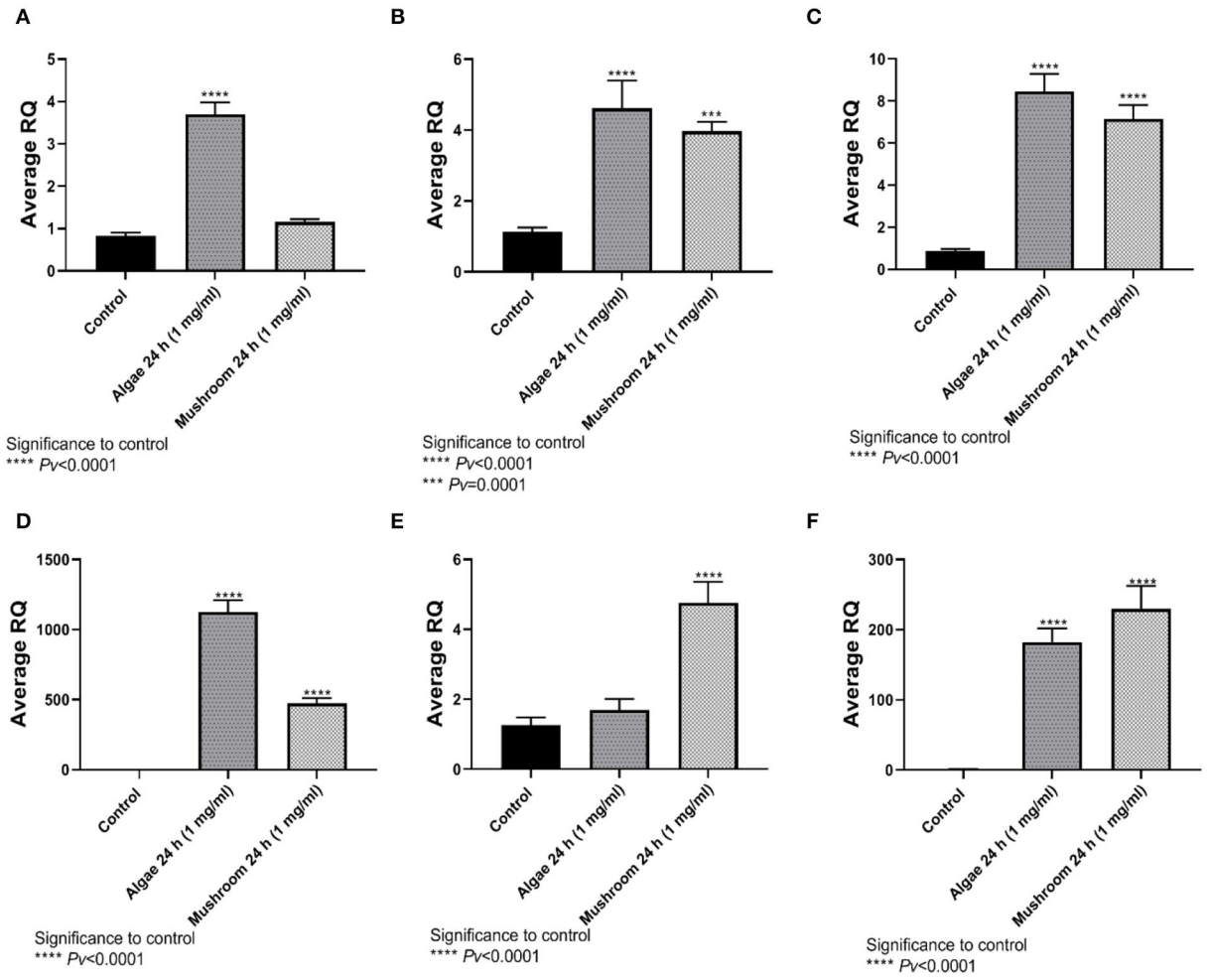

FIGURE 4
Gene expression after 24 h incubation with Algea or Mushroom glucans. **(A)** Tnfα, **(B)** IL4, **(C)** IL6, **(D)** IL8, **(E)** IL10, **(F)** $iNOS_2$ compared to the control group using the relative fold gene expression (DDCT) (n = 5).

results were obtained for ileum length and goblet cell number measurements (not shown).

5. Discussion

Bacterial infections in poultry are an essential concern for both animal health and productivity. The current treatment in poultry farming relies heavily on antibiotics to prevent disease outbreaks (2). It is known that over 60% of all antibiotics produced worldwide, find their use in animal production for both therapeutic and non-therapeutic purposes (25). The massive use of antibiotics led to an increased risk for pathogen resistance. The result has been the withdrawal of several antibiotics from the toolbox available to poultry producers (9). The potential risk of antibiotic resistance resulted in the ban of some antibiotics as feed additives by the European Union as of January 1, 2006. Unfortunately, this ban has led to a decline in animal health and more significant illness variability (26).

Beta-glucans are polymers of glucose that can be derived from the cell walls of yeast, bacteria, fungi, and cereals (27). Considerable variation exists in the structure of beta-glucans from these different sources, which ultimately results in differences in their physiological functions (28). The effects of beta-glucans as biologically active immunomodulators have been well noted in mammalian species; while the mammalian and avian immune systems are similar, they are different enough that it is difficult to extrapolate mammal research to poultry. Recent research shows that beta-glucans may play a role in replacing antibiotics and stimulating the immune system in the poultry industry (16).

Dietary supplementation of yeast cell wall beta-glucans induced a moderate weight gain in the spleen and the bursa of Fabricius (12). In contrast, Cox et al. (16) found no significant

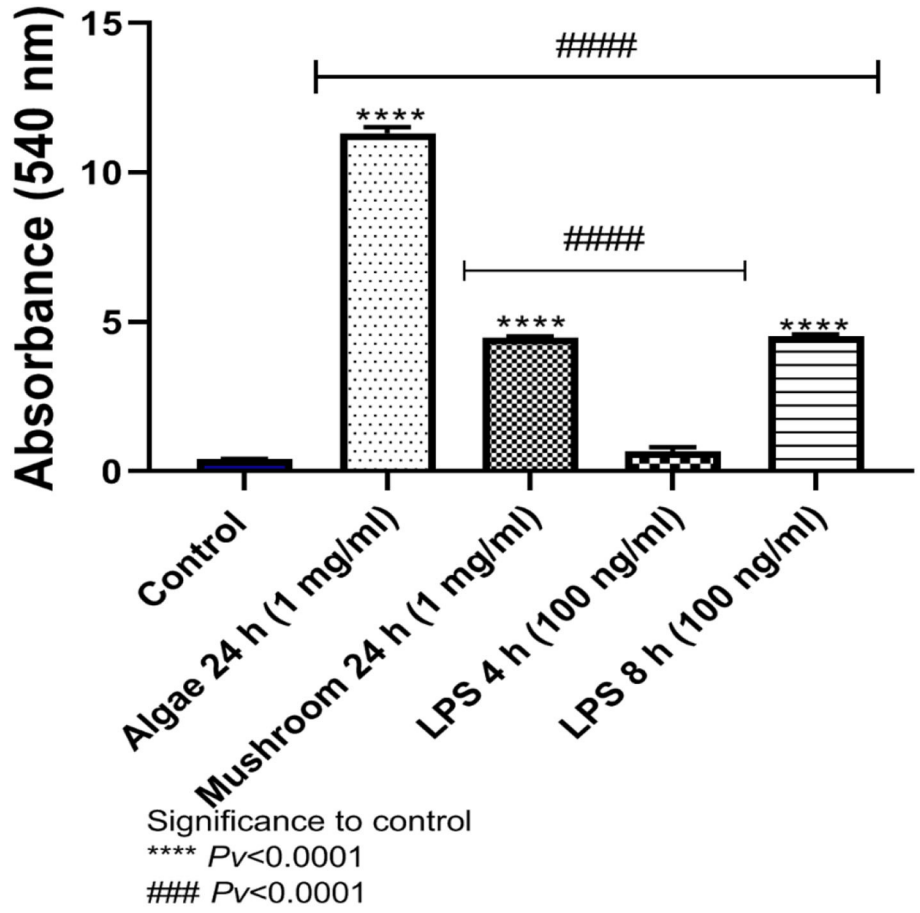

FIGURE 5
Release of NO to the medium after exposure to algae or mushrooms beta-glucans glucans for 24 h, or LPS. ****Pv < 0.0001 significance to control group. ###Pv < 0.0001 significance between beta-glucans to LPS for 4 and 8 h (n = 5).

differences in total body weight and in body weight gain among the treatment groups with beta-glucans. These inconsistent results could be attributed to various reasons, such as differences in the source of the beta-glucan, the presence and type of challenge used, or both. As such, it emphasizes the importance of further research to pinpoint the optimal dosage of beta-glucan supplementation for consistently favorable results in poultry.

The results of the present study suggest that incorporating beta-glucans into the chickens' diet significantly improves gut health by priming intestinal macrophage function and thus affecting intestinal morphology and function, resulting in effective anti-inflammatory immunomodulators. Beta-glucan supplementation benefits the number of goblet cells and the villi height in both the jejunum and the ileum. Moreover, beta-glucans supplementation increased the phagocytic function of macrophages and enhanced the immune response by altering the cytokine profiles of chickens. As alluded to earlier, during ingestion beta-glucans reach the intestine and from their pass through the Peyer's patches into the GALT, and subsequently, they are transported around the body (9). In this way, beta glucans from food can directly stimulate macrophages and affect their secretion of immune-related molecules.

Chicken macrophages are the most potent antigen-presenting cells capable of resistance to exogenous pathogenic microorganisms (14). Dietary supplementation with beta-glucans to poultry has been demonstrated to stimulate phagocytosis, which eventually suppresses the invasion of the pathogen into organs (29). Additionally, beta-glucan supplementation increased the phagocytic function of macrophages as we show herein and as previously published (30).

Macrophages have been extensively studied in several mammalian species, but little attention has been given to the study of avian macrophages. Our *in vitro* experiment was conducted using macrophages originated from a transformed chicken macrophage cell line, HD-11. To our knowledge, no previous research was conducted using the HD-11 macrophage cell line to test the effect of beta-glucans. Other cell lines were

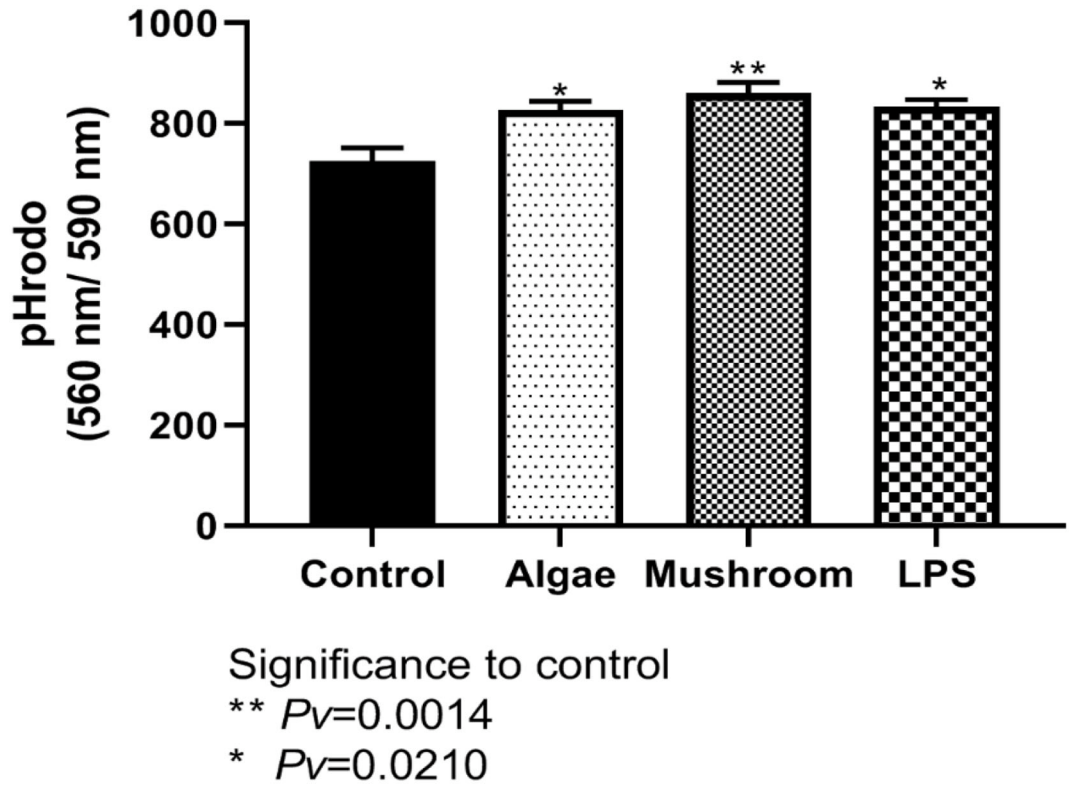

FIGURE 6
Measured phagocytosis activity after 60 min. HD11 cell line were incubated for 1 h with 1 mg/ml of either mushrooms or algae beta-glucans (n = 6), or 100 ng/ml of LPS (n = 3) and compared to control (n = 3).

assayed for the effect of beta-glucans, mainly chicken MQ-NCSU macrophage cells (30).

To understand the immunomodulatory effect of beta-glucans HD-11 chicken macrophage cell line was used. We measured the expression of 6 genes at different time points and concentrations. Significant expression from control was measured with all 6 genes (TNFα, IL4, IL6, IL8, IL10, and $iNOS_2$). To stimulate the maximal response of secretion, we choose to use a concentration of 1 g/ml of beta-glucans for 24 h of incubation (Figures 1, 2). When comparing yeast beta-glucans to mushroom β-glucans expression of IL4, IL6, IL8, and iNOS were significantly higher than control after 24 h of exposure to both algae and mushrooms beta-glucans (Figure 4). IL10 expression was significantly different from the control after exposure to mushrooms beta-glucans, but no significant expression was measured when exposed to algae beta-glucans (Figure 4F). Zhang et al. (31) observed a quadratic increase in the cytokines levels of TNFα and IFN-γ in the blood serum of chicken after being fed with either 50 or 75 mg/kg/feed beta-glucans. Supplementation with beta-glucan for 7-days post hatch up-regulated IL4 expression in the duodenum, jejunum, and ileum. By day 14, IL4 expression was up-regulated in the duodenum and ileum (16). Queiroz et al. (32) reported that a concentration of 50 mg/kg/fed beta-glucan from mushrooms increased the level of IL10 concomitant with a reduction of IFN-γ, in poultry chickens.

In contrast, when compared with un-supplemented controls, broiler chickens who received beta-glucan from yeast were shown to down-regulate IL8. The down-regulation of the IL8 gene with beta-glucan supplementation suggests that the beta-glucan functions as an anti-inflammatory immunomodulator (16). Qureshi et al. (30) reported that no detectable IL6 bioactivity was observed in macrophage culture supernatants with or without beta-glucan exposure. This result may be explained since their experiment was conducted using the MQ-NCSU macrophage cell line, which is derived from the spleen and may not express IL6, in contrast to HD11, who is derived from chicken bone marrow and does express IL6. We suggest that care should be undertaken in regard to the different data published in regards to the various effects caused by beta-glucans. Since many products may be called beta-glucans, the composition and analytical conformation is likely to differ from source to source, which may influence the vast variety of responses observed.

NO is an important molecule in the anti-inflammatory and antimicrobial effects of macrophages and is a chemical indicator

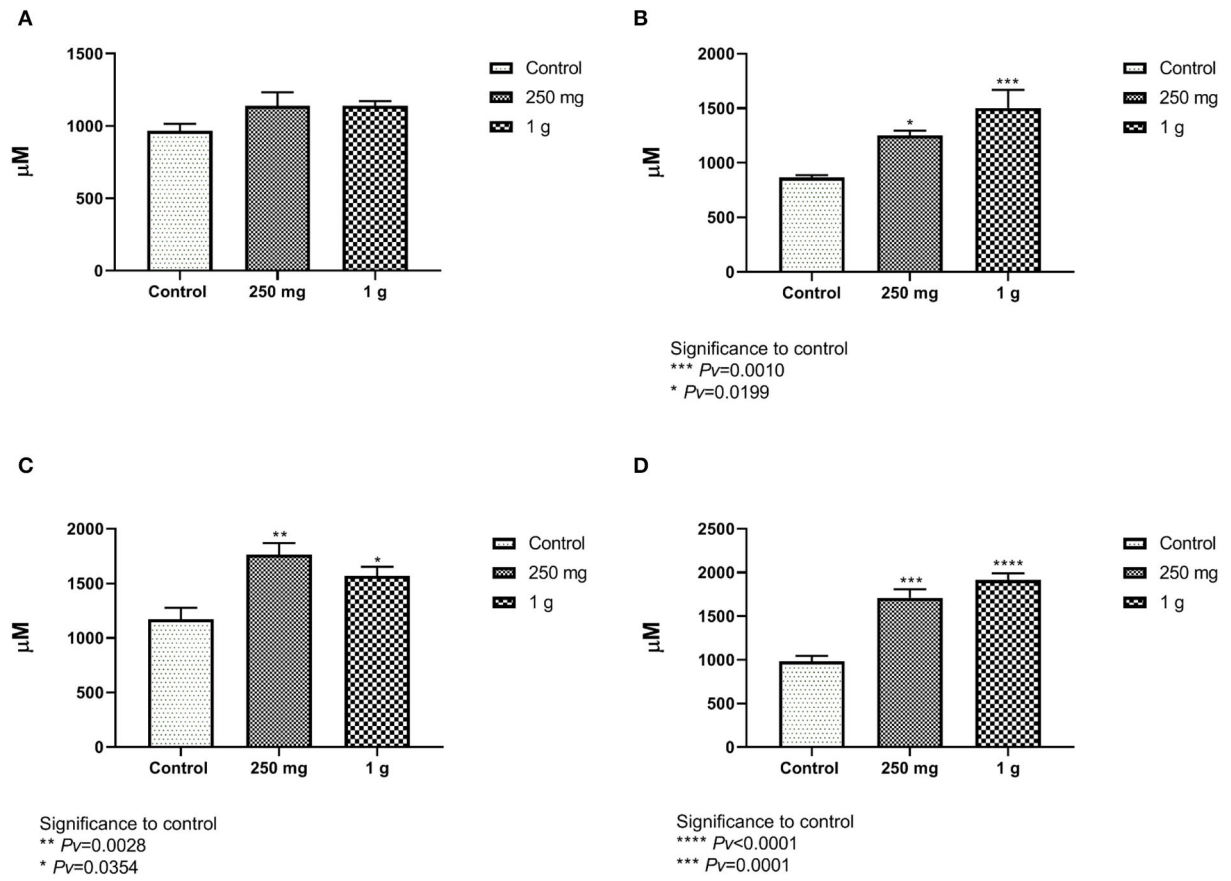

FIGURE 7
(A, B) The length of the villi from the ileum compared with the beta-glucans treatment groups to the control. **(A)** The females and figure. **(B)** The males (n = 5 per group). **(C, D)** The length of the villi from the jejunum compared with the beta-glucans treatment groups to the control. **(C)** Represent the females, and **(D)** for the males (n = 5 per group).

of inflammation and inflammatory diseases. iNOS is induced in inflamed tissues and generates relatively large amounts of NO (33).

When exposed to antigens or chemotactic agents, macrophages will begin to express iNOS. This enzyme leads to the production of NO, which will subsequently react with superoxide anions to generate toxic derivatives, allowing macrophages to proficiently kill several types of pathogens (34). The upregulation in iNOS implies an enhanced capability of macrophages to kill invading pathogens, allowing the host to eliminate infectious threats more efficiently (16). Beta-glucan exposure increased NO release into the supernatant fraction of broiler macrophage culture, suggesting that beta-glucans may induce nitric oxide synthase activity similar to other known macrophage activators such as LPS (35). The release of NO to the medium after exposure to 1 mg/ml of either mushrooms or algae beta-glucans for 24 h or 100 ng/ml of LPS for 4 and 8 h was measured. The results showed a significantly greater NO release for all treatment groups than the amount released from the untreated control group. Exposure to algae beta-glucans induced the released of more NO than the groups exposed to LPS for either 4 or 8 h (Figure 3). These results were consistent when comparing algae and mushroom beta-glucan NO release (Figure 5) and the significant expression of the nitric oxide synthase (iNOS) gene after exposure to beta-glucans for 24 h (Figures 2D, 4F).

Macrophages are one of the first responder innate immune cells upon a new infection, as seen in infection models that show the infection causes a quick increase in the number of macrophages (36). They can phagocytize bacteria and subsequently produce multifunctional compounds, including reactive oxygen species (ROS), nitric oxide (NO), and cytokines, to kill the infectious microorganisms and signal to other immune cells to establish an appropriate response to the infection (37). Moreover, activated macrophages can produce NO, which plays an important role in the host defense against microbial infection, and act as effector molecules to kill invading pathogens (38). The release of NO to the medium after exposure

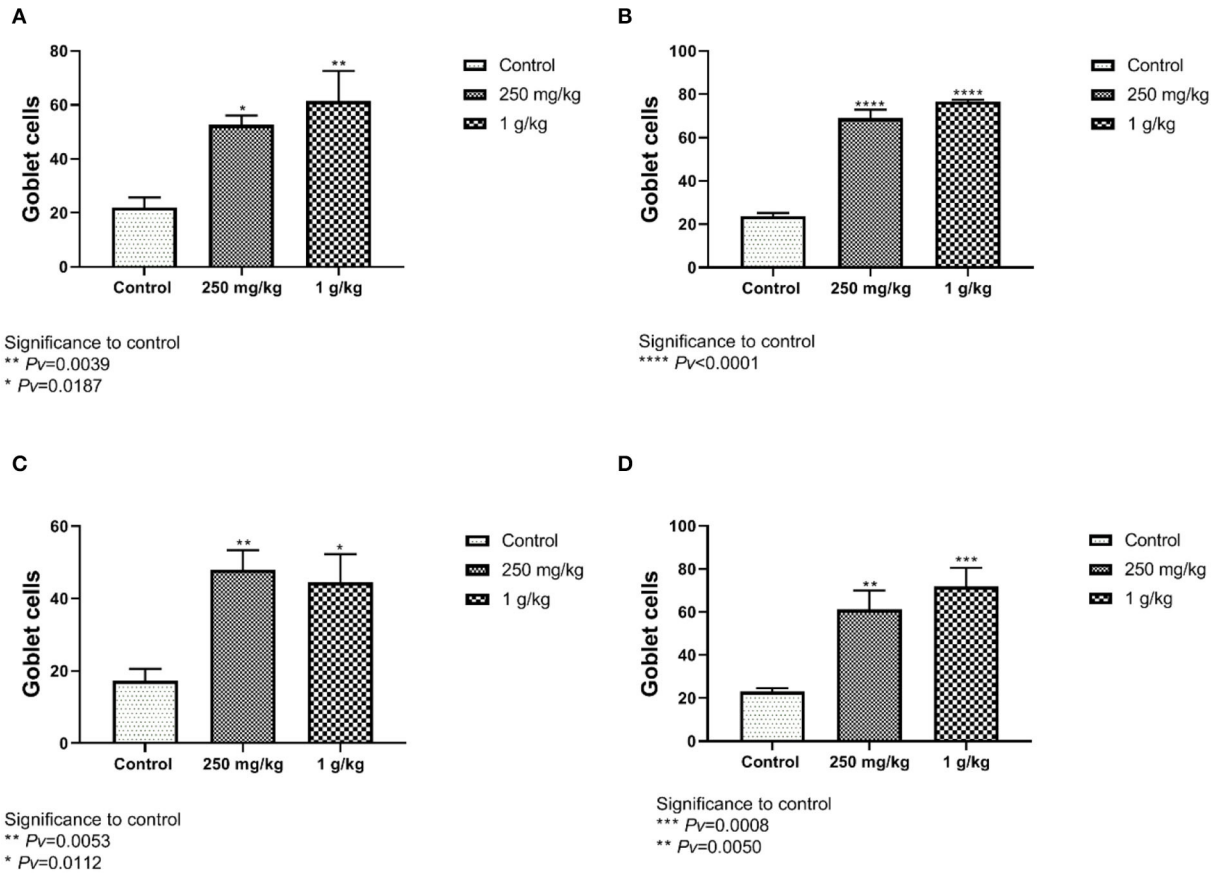

FIGURE 8
The number of goblet cells counted from the jejunum and ileum sections. Beta-glucans treatment groups compared to the control. **(A)** Females ileum, **(B)** male ileum, **(C)** female jejunum, **(D)** male jejunum (n = 5 per group). Pv is compared to control.

to beta-glucans was observed in our research and can be explained by the increase of phagocytosis activity. Many studies on phagocytic activity have been described for mammalian macrophages, but the available data for chicken macrophages is limited.

Besides the intracellular killing of pathogens, professional phagocytes play an important role in modulating the immune response by expressing cytokines and chemokines. For example, induced pro-inflammatory cytokines IL8 as well as inflammatory cytokine IL6 (38), which was also observed in our research. Phagocytosis is a complex process involving a diverse set of receptors that can stimulate phagocytosis. Cells were incubated for 1 h with 1 mg/ml of either mushroom or algae beta-glucans or 100 ng/ml of LPS. Algae and mushroom beta-glucans increased phagocytosis activity significantly from the control (Figure 6). From the following data it can be concluded that β-glucans from both algae and mushroom can elicit a proinflammatory response in HD-11 avian macrophages. This mild proinflammatory response can prime macrophages to be active for subsequent putative infections.

Longer intestinal villi indicate an improved ability to absorb nutrients in the intestine and may account for increased body weight (39). The crypts of the villus contain several specialized cells, including absorptive cells, goblet cells, and regenerative cells, that are responsible for the production of mucus and the replacement of old cells (15). Assessment of intestinal villi morphology is important since the small intestine is the primary site for nutrient assimilation and is therefore sensitive to diet changes (40). Measurements of the villi taken from the female ileum showed no differences between treatment with beta-glucans to control (Figure 7A). The length of the male villi from the ileum was significantly longer compared to the control (Figure 7B). Measurements of the female and male villi from the jejunum showed significant differences between treatment groups to control (Figures 7C, D, 9A, B). Correlating with our findings, several studies have also observed that dietary yeast beta-glucan administration to chickens resulted in higher villi height in the jejunum compared to chickens fed with no beta-glucan (40–42). Confirming our studies Solis de Los Santos et al. (15) found that the villi height of the ileum, the surface area,

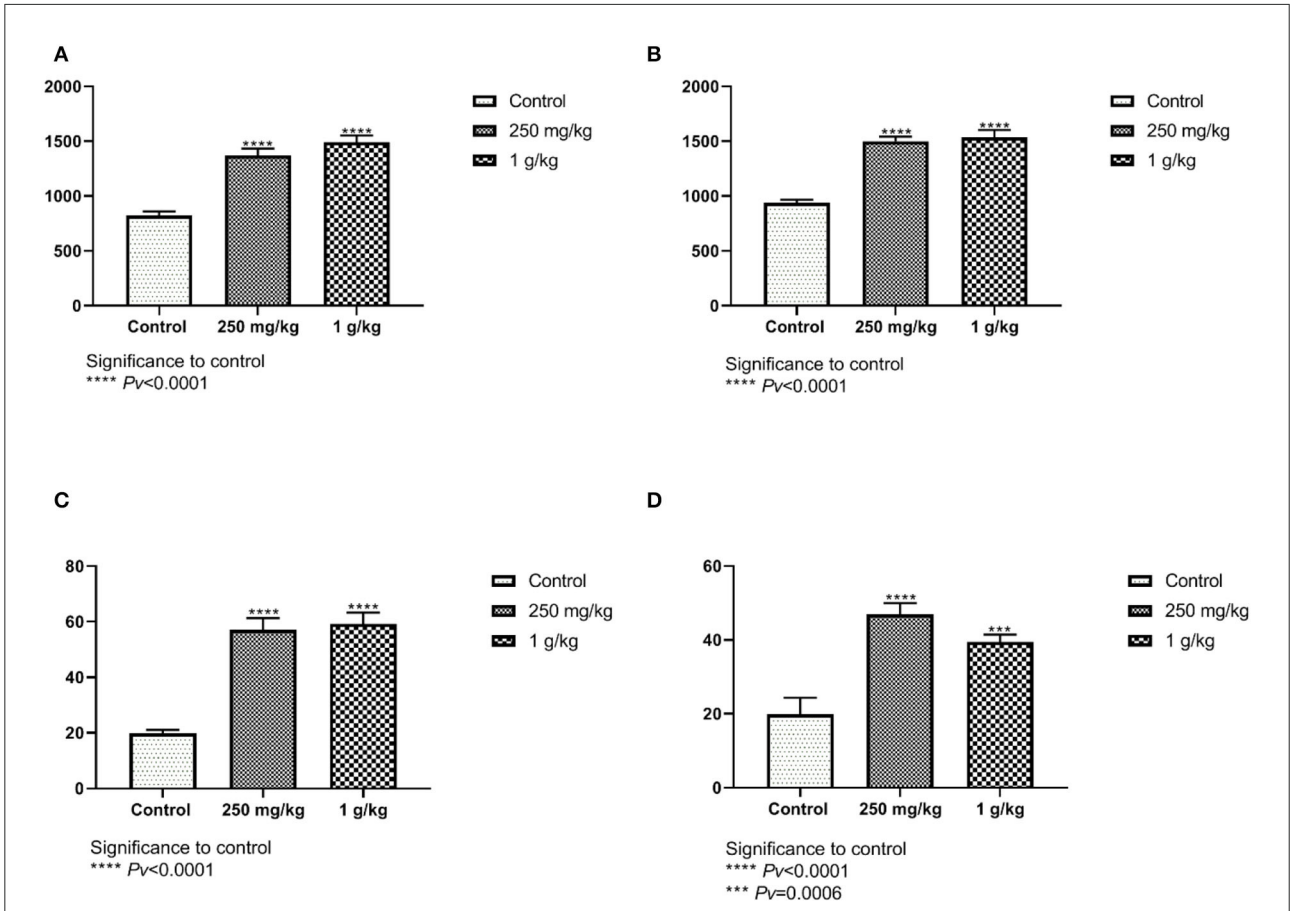

FIGURE 9
(A, B) The length of the villi from the jejunum compared with the beta-glucans treatment groups to the control. **(A)** Females, and **(B)** males (n = 10 per group). **(C, D)** The number of goblet cells in the jejunum. Beta-glucans treatment groups compared to the control. **(C)** Females, and **(D)** males (n = 10 per group). Pv are compared to control.

the lamina propria thickness, the crypt depth and the goblet cell density were enhanced due to the consumption of yeast extract beta-glucans as a dietary supplement.

The intestinal lining provides the innate defense barrier against most intestinal pathogens. Goblet cells in the intestinal mucosa secrete mucus which provides the first line of defense against intestinal injury (9). The mucus layer protects the intestinal surface from the invasion of enteric bacteria, bacterial and environmental toxins, and some dietary components that may damage the mucosa (15, 42, 43). When the mucus layer is disturbed, the adhesion of microbes to the intestinal epithelial surface may increase epithelial permeability and reduce the absorption of nutrients (44, 45). We measured herein whether the number of goblet cells was affected by the beta-glucan treatment. Goblet cell number from the ileum and the jejunum of the males and females showed significant differences between treatments with beta-glucans to control (Figures 8A–D, 9C, D). Dietary beta-glucan administration significantly increased the number of goblet cells in the jejunum and ileum. Goblet cells are a type of intestinal mucosal epithelial cells whose primary function is to synthesize and secrete mucus. Mucin production and secretion are important in maintaining the mucus barrier. A wide range of factors, including feeding, microbes, microbial products, toxins, and cytokines, has been shown to regulate these processes, thus affecting the mucus barrier (44–46). Correlating with our findings, previous studies also found that supplementation with beta-glucan significantly increased the number of goblet cells in the jejunum of chickens (15, 41). This suggests that beta-glucan supplementation of poultry feed plays a significant role in improving their gut health during a bacterial challenge.

6. Conclusions

Cumulatively, our results indicate that beta-glucans can influence cytokine expression profiles indicating that avian macrophages may express beta-glucans receptors. We conclude that algae and mushrooms beta-glucans modulate differently the expression of cytokines-associated with immune response suggesting that the use of the two beta-glucans types may result in a broader immune response.

Our findings are important since a healthier gut indicates better digestion and absorption of nutrients, and therefore a healthy functioning immune system. Similarly extrapolating our findings to human set-ups, the rationale of using adjuvants in vaccine formulations in order to improve the efficacy of adaptive immunity is widely accepted, however little attention has been given to the direct effects of adjuvants on innate immunity and early protection against infection (15, 44). As such, beta-glucans may enhance resistance to acute or chronic human infections and reduce the wide use of antibiotics.

Institutional Review Board Statement: "The study was conducted in accordance with the Dec-laration of Helsinki, and approved by the ethics committee of the Hebrew University of Jerusalem. The experiment was conducted at the poultry unit and research laboratories at the Faculty of Agriculture, Food and Environmental Sciences, Israel. All animal experiments were done under the care and supervision of Prof. Israel Rozenboim. Protocol code AG-20-16348-4, NIH approval number OPRR-A01-5011, date of approval, 28/10/2020.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the study was conducted in accordance with the Dec-laration of Helsinki, and approved by the Ethics Committee of the Hebrew University of Jerusalem. The experiment was conducted at the poultry unit and research laboratories at the Faculty of Agriculture, Food and Environmental Sciences, Israel. All animal experiments were done under the care and supervision of Prof. Israel Rozenboim. Protocol code AG-20-16348-4, NIH approval number OPRR-A01-5011, date of approval, 28/10/2020.

Author contributions

Conceptualization: BS, IR, and VV. Methodology: HB-D, OG, and NC. Writing-original draft preparation: OG, HB-D, and BS. Writing-review and editing, supervision, and funding acquisition: BS and IR. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by a grant of BARD (BINATIONAL AGRICULTURAL RESEARCH AND DEVELOPMENT FUND) No. IS-5241-20 to BS, IR, and VV.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1092812/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 24 January 2023
DOI 10.3389/fvets.2023.1107798

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

RECEIVED 25 November 2022
ACCEPTED 09 January 2023
PUBLISHED 24 January 2023

CITATION
Li Z, Long L, Jin X, Li Y, Wu Q, Chen X, Geng Z and Zhang C (2023) Effects of *Clostridium butyricum* on growth performance, meat quality, and intestinal health of broilers. *Front. Vet. Sci.* 10:1107798. doi: 10.3389/fvets.2023.1107798

Effects of *Clostridium butyricum* on growth performance, meat quality, and intestinal health of broilers

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This study investigated the effects of *Clostridium butyricum* on the growth performance, meat quality and intestinal health of broilers. A total of 800 one-day-old male Arbor Acres broilers were randomly assigned to two groups with 16 replicates of 25 broilers per group and fed with a basal diet (CON) or a basal diet supplemented with 1.5×10^9 cfu/kg *C. butyricum* and 5×10^8 cfu/kg *C. butyricum* at 1–21 d and 22–42 d, respectively (CB). The results indicated that *C. butyricum* significantly increased the final body weight, average daily gain at 1–42 d in the growth performance of broilers ($P < 0.05$). Moreover, *C. butyricum* significantly increased a^*_{24h} value and pH_{24h} value of breast meat but reduced the drip loss and shear force ($P < 0.05$). Regarding serum antioxidant indices, *C. butyricum* significantly increased the total superoxide dismutase (T-SOD) and total antioxidative capacity activities and reduced the malondialdehyde content ($P < 0.05$). Furthermore, the broilers in the CB demonstrated an increase in jejunal lipase and trypsin activities, villus height (VH) and VH-to-crypt depth ratio at 42 d compared with those in the CON ($P < 0.05$). *C. butyricum* also upregulated the intestinal mRNA levels of zonula occludens-1, nuclear factor erythroid 2-related factor 2 (*Nrf2*), *SOD1* and interleukin-10 in the jejunal mucosa ($P < 0.05$), but it downregulated the mRNA levels of nuclear factor kappa B (*NF-κB*) and tumor necrosis factor-α ($P < 0.05$). These results indicate that *C. butyricum* can improve the growth performance and meat quality of broilers. In particular, *C. butyricum* can improve the intestinal health of broilers, which is likely to be related to the activation of the Nrf2 signaling pathway and inhibition of the NF-κB signaling pathway.

KEYWORDS

broilers, *Clostridium butyricum*, growth performance, meat quality, intestinal health

1. Introduction

Generally, the growth performance of broilers is highly dependent on their intestinal health, which is influenced by a variety of factors, such as diet, stress and infection. These factors may increase the intestinal permeability of broilers and compromise the structural integrity of their intestinal epithelium, resulting in an increase in inflammatory responses and ultimately the restriction of normal growth (1). In recent years, because of the ban on antibiotics in poultry feed, unspecified intestinal diseases have emerged in poultry production (2). Therefore, new alternatives are urgently required to reduce the incidence of intestinal disease and increase the production efficiency of broilers. Among these potential alternatives, probiotics, owing to their ecofriendly and pollution-free characteristics, are typically fed directly to inhibit the growth of pathogens and regulate their intestinal function of broilers (2, 3). Studies have demonstrated that probiotics could prevent pathogenic bacteria from adhering to the intestinal mucosa and hence improve the microecological environment of the gastrointestinal tract in order to optimize

the intestinal function of broilers, thereby enhancing their growth performance (4, 5). Hence, dietary supplementation with probiotics is regarded as an effective method for enhancing the growth performance and enteric diseases resistance of broilers.

Clostridium butyricum CBM 588 is a typical probiotic strain that produces spores that are resistant to high temperature, humidity, stomach acid and bile salts, indicating its high adaptability to the intestinal environment (6). *C. butyricum* metabolites contain digestive enzymes that can break down large molecules of nutrients into small molecules that can be easily absorbed, thereby enhancing the utilization efficiency of these nutrient substances and ultimately promoting the growth performance of the host (6). As a source of energy, short-chain fatty acids (SCFA), another metabolite of *C. butyricum*, not only retard the growth of pathogenic enteric bacteria, but also promote intestinal development. Studies have indicated that the addition of *C. butyricum* to the diet of broilers could increase their intestinal digestive capacity and improve their intestinal morphology, which in turn enhances their growth performance (7, 8). Liao et al. (9) reported that *C. butyricum* treatment increased the concentrations of polyunsaturated fatty acids in the meat and improved the meat quality of broilers. In addition, *C. butyricum* was found to positively affect the immune response of broilers, which could promote the secretion of anti-inflammatory factors and immunoglobulins (10, 11). A recent study on Pekin ducks revealed that dietary *C. butyricum* supplementation increased the SCFA content in the gut and optimized the intestinal flora (12). Although the effects of *C. butyricum* treatment on the growth performance and intestinal health have been extensively documented in the literature on poultry production, the results remain inconsistent. Therefore, the further research on more targeted indicators is necessary to accurately evaluate the benefits of *C. butyricum* and explore the mechanism of through which *C. butyricum* regulates intestinal health. In addition, because of *C. butyricum* and its metabolites showing certain immuno–antioxidant properties in poultry (9, 13), we hypothesize that *C. butyricum* could regulate the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway (a key signaling pathway for regulating antioxidant capacity) and nuclear factor-κB (NF-κB) signaling pathway (a key signaling pathway for regulating inflammatory response). Accordingly, the present study investigated the effects of *C. butyricum* on the growth performance, meat quality and intestinal health of broilers to elucidate the potential mechanism though which *C. butyricum* regulates intestinal health.

2. Materials and methods

2.1. Experimental design and diets

The experimental procedures performed on the animals included in this study were approved by the Animal Ethics Committee of Anhui Agricultural University. A total of 800 one-day-old male Arbor Acres (AA) broilers from the same batch were randomly assigned to two groups with 16 replicates of 25 broilers per group and fed with a basal diet (CON) or a basal diet supplemented with 1.5×10^9 cfu/kg *C. butyricum* and 5×10^8 cfu/kg *C. butyricum* at 1–21 d and 22–42 d, respectively (CB). The *C. butyricum* additive contained at least 5×10^9 cfu/g powder. The addition level of *C. butyricum* used in this study was based on an unpublished gradient addition trial which found that *C. butyricum* supplementation at 1.5×10^9 cfu/kg from 1 to 21 d and 5×10^8 cfu/kg from 22 to 42 d could maximize the growth performance of broilers. The broilers were provided with mash feed and fresh water *ad libitum* from 1 d until 42 d. The temperature was gradually reduced from 34°C ± 1°C to 23°C ± 1°C at a rate of 2°C or 3°C per week and then maintained at 23°C ± 1°C until the end of the experiment, and the lighting program was set to produce 1 h of darkness and 23 h of light. The coop was longitudinally ventilated using an exhaust fan, and the humidity maintained between 55 and 65%. The basal diets are formulated according to the requirements for broilers by NRC (1994), and their composition and nutritional level are displayed in Supplementary Table 1. Body weight (BW) at 21 d and 42 d and daily feed consumption were documented on a replicate basis to calculate the average daily BW gain (ADG), average daily feed intake (ADFI) and feed to gain ratio (F/G). These indexes of growth performance were calculated using the following formulas.

$$\text{ADFI} = \Sigma[(\text{Feed amount} - \text{Residual amount}) / \text{Number of broilers}]/\text{Days}$$

$$\text{ADG} = (\text{Final average weight} - \text{Initial average weight})/\text{Days}$$

$$\text{F/G} = \text{Total feed consumption}/\text{Total weight gain}$$

2.2. Sample collection

At 21 and 42 d, one broiler of the average pen BW was selected from each replicate and euthanized by electrical stunning, and the remaining broilers meeting the market requirement were sold after the end of the experiment. Blood samples were then collected from the wing vein after 12 h of fasting, placed in polypropylene tubes, and centrifuged at 1500 × *g* for 15 min at 4°C to obtain the serum, which was then stored at −20°C until the analysis of antioxidant-related indicators. After the slaughter and dissection processes, the right breast muscle was removed for the analysis of cooking loss and shear force, and the left breast muscle was removed and kept at 4°C for the analysis of pH, meat color and drip loss. To evaluate the digestive enzyme activity, duodenal, jejunal, and ileal digesta were collected in sterile centrifuge tubes, speedily removed in liquid nitrogen and kept at −80°C. Subsequently, segments measuring 2 cm were cut from the middle of the duodenum, jejunum and ileum and fixed in 10% buffered formalin for morphological evaluation. Mucosal samples were then obtained by gently scraping the jejunal wall using sterile slides, immediately frozen in liquid nitrogen, and stored at −80°C until gene expression analysis.

2.3. Meat quality

The color (L*, lightness; a*, redness; b*, yellowness) and pH of breast muscle were measured in triplicate using a chroma meter (CR-300, Minolta Camera, Osaka, Japan) and a pH meter (pH-STAR, SFK-Technology, Copenhagen, Denmark) at 45 min and 24 h postmortem, respectively. Subsequently, drip loss, cooking loss, and shear force were measured using our previously described methods (14).

2.4. Metabolite contents and enzyme activities

The intestinal contents were first homogenized in precooled phosphate-buffered saline and centrifuged at 1000 × *g* for

10 min at 4°C, and then the supernatants were obtained. Subsequently, malondialdehyde (MDA) level and the activities of catalase (CAT), total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px) in serum, as well as intestinal amylase, lipase, and trypsin activities were determined by commercially available kits (Nanjing Jiancheng Biochemistry Institute, Nanjing, China) according to the manufacturer's instructions.

2.5. Intestinal morphology

Intestinal samples were dehydrated, embedded in paraffin, and stained with hematoxylin and eosin. Subsequently, under an inverted fluorescence microscope, villus height (VH) and crypt depth (CD) were measured and recorded using an image processing and analysis system (Leica Imaging Systems, Cambridge, UK) to evaluate the ratio of VH to CD (VH/CD).

2.6. Quantitative reverse transcription PCR

Total RNA was separated from jejunal mucosa using TRIzol (Yeasen, Shanghai, China). The RNA samples were then reverse-transcribed into cDNA for next analysis using a Hifair® II 1st Strand cDNA Synthesis Kit (Yeasen, Shanghai, China), and gene expression levels were quantified using real-time PCR with a Hieff® qPCR SYBR Green Master Mix (Yeasen, Shanghai, China) and a real-time PCR system (Thermo Fisher Scientific, MA, USA). All primers (Supplementary Table 2) were synthesized by Hefei Qingke Biotechnology Co., Ltd. The reaction conditions and parameter settings followed our previous research (15). The relative expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method based on the expression of *β-actin*.

2.7. Statistical analysis

Data were analyzed using SPSS 18.0 (SPSS, Chicago, IL, USA) and are expressed as mean ± standard error. Student's *t*-test was performed to analyze and compare the CON and CB groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Growth performance

As shown in Table 1, the addition of *C. butyricum* significantly increased the BW and ADG of broilers at 42 d and 1–42 d, respectively ($P < 0.05$), and tended to improve their ADG at 22–42 d ($P = 0.057$). However, *C. butyricum* did not have significant effects on the ADFI or F/G at each stage in either group ($P > 0.05$).

TABLE 1 Effect of *C. butyricum* on the growth performance of broiler chickens.

Items	CON	CB	*P*-value
Initial BW (g)	53.09 ± 0.43	53.78 ± 0.30	0.200
BW at 21 d (g)	724.4 ± 5.62	740.2 ± 7.73	0.109
BW at 42 d (g)	2305 ± 10.71	2365 ± 18.76	0.010
ADG (g)			
1–21 d	31.97 ± 0.27	32.69 ± 0.37	0.126
22–42 d	75.25 ± 0.57	77.36 ± 0.90	0.057
1–42 d	53.61 ± 0.26	55.02 ± 0.45	0.012
ADFI (g)			
1–21 d	52.40 ± 0.51	53.15 ± 0.60	0.346
22–42 d	141.8 ± 1.31	142.4 ± 1.50	0.779
1–42 d	97.10 ± 0.67	97.76 ± 0.90	0.561
F/G			
1–21 d	1.64 ± 0.02	1.63 ± 0.03	0.778
22–42 d	1.89 ± 0.01	1.84 ± 0.02	0.138
1–42 d	1.81 ± 0.01	1.78 ± 0.02	0.139

CON, basal diet; CB, basal diet + Clostridium butyricum. BW, Body weight; ADFI, average daily feed intake; ADG, average daily gain; F/G, feed to gain ratio.

TABLE 2 Effects of *C. butyricum* on the meat quality of broiler.

Items	CON	CB	*P*-value
L^*_{45min}	46.13 ± 0.89	45.24 ± 1.08	0.528
a^*_{45min}	1.64 ± 0.10	1.98 ± 0.16	0.077
b^*_{45min}	12.00 ± 0.52	11.93 ± 0.41	0.911
L^*_{24h}	53.36 ± 0.39	52.64 ± 0.28	0.156
a^*_{24h}	2.77 ± 0.14	3.48 ± 0.31	0.044
b^*_{24h}	14.13 ± 0.45	13.85 ± 0.28	0.617
pH_{45min}	6.21 ± 0.06	6.28 ± 0.03	0.308
pH_{24h}	5.70 ± 0.06	5.86 ± 0.04	0.048
Drip loss (%)	2.90 ± 0.11	2.60 ± 0.07	0.045
Shear force (*N*)	27.92 ± 1.12	24.32 ± 1.29	0.049
Cooking loss (%)	25.44 ± 1.37	25.18 ± 1.16	0.887

CON, basal diet; CB, basal diet + Clostridium butyricum. L*, lightness; a*, redness; b*, yellowness.

3.2. Meat quality

As listed in Table 2, the broilers in the CB group had higher a^*_{24h} and pH_{24h} values and a lower drip loss and shear force than did those in the CON group ($P < 0.05$). In addition, treatment with *C. butyricum* tended to increase their a^*_{45min} value ($P = 0.077$).

3.3. Serum antioxidant statues

As presented in Table 3, the broilers in the CB group recorded the lower ($P < 0.05$) content of MDA at 42 d and higher ($P < 0.05$) activities of T-AOC at 42 d and T-SOD at d 21 and d 42 than did those in the CON group. Furthermore, *C. butyricum* treatment tended to decrease the MDA content at 21 d ($P = 0.083$). However,

TABLE 3 Effects of *C. butyricum* on serum antioxidant capacity of broilers.

Age	Items	CON	CB	*P*-value
21 d	MDA (nmol/mL)	3.45 ± 0.14	3.13 ± 0.10	0.083
	GSH-Px (U/mL)	655.2 ± 24.97	723.8 ± 32.39	0.119
	T-SOD (U/mL)	83.85 ± 2.00	90.90 ± 2.55	0.047
	CAT (U/mL)	36.46 ± 0.71	37.21 ± 0.61	0.440
	T-AOC (U/mL)	0.52 ± 0.02	0.55 ± 0.01	0.101
42 d	MDA (nmol/mL)	4.15 ± 0.12	3.67 ± 0.14	0.021
	GSH-Px (U/mL)	837.2 ± 35.40	864.0 ± 18.54	0.518
	T-SOD (U/mL)	117.2 ± 3.00	128.1 ± 2.41	0.015
	CAT (U/mL)	38.77 ± 0.67	39.30 ± 0.39	0.499
	T-AOC (U/mL)	0.68 ± 0.01	0.71 ± 0.01	0.013

CON, basal diet; CB, basal diet + Clostridium butyricum. CAT, catalase; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; T-AOC, total antioxidative capacity, MDA: malondialdehyde.

TABLE 4 Effects of *C. butyricum* on intestinal morphology of broilers.

Age	Items	CON	CB	*P*-value
21 d	Duodenum			
	VH (μm)	1279 ± 16.17	1329 ± 11.94	0.023
	CD (μm)	204.3 ± 8.61	192.6 ± 4.34	0.227
	VH/CD	6.52 ± 0.30	6.92 ± 0.13	0.211
	Jejunum			
	VH (μm)	1224 ± 24.15	1246 ± 13.65	0.435
	CD (μm)	170.5 ± 5.11	166.2 ± 3.45	0.502
	VH/CD	7.20 ± 0.10	7.52 ± 0.15	0.090
	Ileum			
	VH (μm)	853.9 ± 7.14	858.3 ± 10.64	0.726
	CD (μm)	135.8 ± 4.92	129.8 ± 4.72	0.395
	VH/CD	6.38 ± 0.23	6.73 ± 0.32	0.375
42 d	Duodenum			
	VH (μm)	1473.3 ± 36.37	1524.3 ± 46.83	0.402
	CD (μm)	244.1 ± 7.69	223.0 ± 3.53	0.119
	VH/CD	6.11 ± 0.28	6.66 ± 0.27	0.174
	Jejunum			
	VH (μm)	1443 ± 14.95	1500 ± 21.05	0.036
	CD (μm)	229.6 ± 10.11	213.1 ± 4.22	0.150
	VH/CD	6.37 ± 0.29	7.05 ± 0.10	0.039
	Ileum			
	VH (μm)	1234 ± 14.52	1245 ± 22.09	0.676
	CD (μm)	212.9 ± 5.73	206.9 ± 6.12	0.487
	VH/CD	5.83 ± 0.15	6.06 ± 0.18	0.335

CON, basal diet; CB, basal diet + Clostridium butyricum. VH, villus height; CD, crypt depth; VH/CD: the ratio of villus height to crypt depth.

no marked difference was observed in the activities of GSH-Px and CAT ($P > 0.05$).

TABLE 5 Effect of *C. butyricum* on digestive enzymes activities of broilers.

Age	Items	CON	CB	*P*-value
21 d	Duodenum			
	Amylase (U/mg protein)	0.72 ± 0.12	0.75 ± 0.05	0.784
	Lipase (U/g protein)	37.38 ± 1.82	43.20 ± 3.08	0.141
	Trypsin (U/g protein)	3916 ± 227.4	3986 ± 199.7	0.821
	Jejunum			
	Amylase (U/mg protein)	0.86 ± 0.08	0.94 ± 0.10	0.544
	Lipase (U/g protein)	27.16 ± 1.45	33.87 ± 2.49	0.040
	Trypsin (U/g protein)	4911 ± 691.0	5034 ± 310.1	0.868
	Ileum			
	Amylase (U/mg protein)	1.60 ± 0.17	1.63 ± 0.19	0.905
	Lipase (U/g protein)	35.38 ± 3.94	40.27 ± 4.17	0.413
	Trypsin (U/g protein)	7607 ± 241.1	7744 ± 301.4	0.729
42 d	Duodenum			
	Amylase (U/mg protein)	0.85 ± 0.03	0.92 ± 0.04	0.175
	Lipase (U/g protein)	106.0 ± 12.84	118.9 ± 5.60	0.415
	Trypsin (U/g protein)	8956 ± 432.6	9650 ± 737.0	0.501
	Jejunum			
	Amylase (U/mg protein)	1.39 ± 0.09	1.49 ± 0.12	0.498
	Lipase (U/g protein)	100.3 ± 3.90	120.0 ± 7.08	0.048
	Trypsin (U/g protein)	10581 ± 1239	13335 ± 537	0.046
	Ileum			
	Amylase (U/mg protein)	1.73 ± 0.18	1.75 ± 0.12	0.917
	Lipase (U/g protein)	100.2 ± 6.11	110.5 ± 9.34	0.366
	Trypsin (U/g protein)	10060 ± 607.9	11356 ± 460.7	0.112

CON, basal diet; CB, basal diet + Clostridium butyricum.

3.4. Intestinal morphology

As listed in Table 4, no significant difference was observed in CD between the CON and CB groups ($P > 0.05$). However, the addition of *C. butyricum* significantly increased the jejunal VH and VH/CD at 42 d ($P < 0.05$). Similarly, *C. butyricum* supplementation significantly improved the duodenal VH at 21 d ($P < 0.05$). However, no remarkable influence in ileal morphology was observed between the CON and CB groups ($P > 0.05$).

3.5. Digestive enzyme activity

As displayed in Table 5, no remarkable difference was observed in the digestive enzyme activity in the duodenum or ileum between the CON and CB groups ($P > 0.05$). Compared with

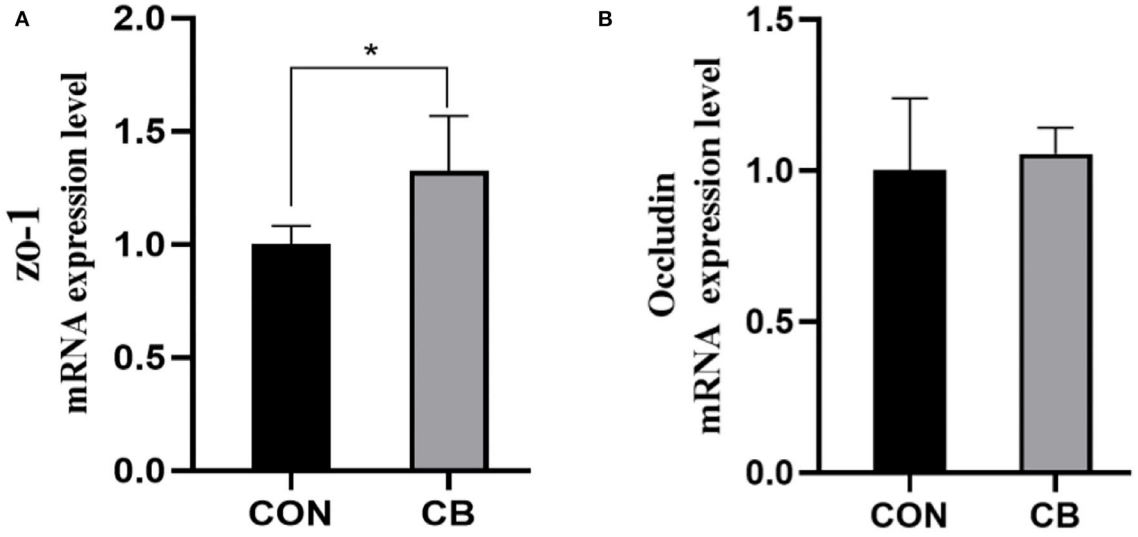

FIGURE 1
(A, B) Effect of *C. butyricum* on the mRNA expression levels of jejunal barrier genes of broilers. CON, basal diet; CB, basal diet + *Clostridium butyricum*. *Significant difference between group ($P < 0.05$). *ZO-1*, zonula occludin protein 1.

the CON group, the CB group demonstrated higher lipase activity in the jejunum at 21 and 42 d ($P < 0.05$). Similarly, *C. butyricum* dramatically increased jejunal trypsin activity at 42 d ($P < 0.05$).

3.6. mRNA expression levels of intestinal barrier genes

As illustrated in Figure 1, the CB group had a higher expression level of the tight-junction-protein-related gene *ZO-1* than did the CON group ($P < 0.05$). However, occludin level was similar in both groups ($P > 0.05$).

3.7. mRNA expression levels of antioxidant-related genes

As presented in Table 6, *C. butyricum* addition effectively increased the jejunal mRNA levels of *Nrf2* and *SOD1* in the broilers ($P < 0.05$). However, no significant differences were observed in the mRNA levels of *CAT* or *GSH-Px* ($P > 0.05$).

3.8. mRNA expression levels of inflammation-related genes

As listed in Table 7, *C. butyricum* effectively downregulated jejunal mRNA expression of *NF-κB* and tumor necrosis factor-α (*TNF-α*) ($P < 0.05$), but it upregulated interleukin (*IL*)*-10* mRNA expression ($P < 0.05$). However, no significant difference was observed in the mRNA expression levels of *IL-1β* or *IL-6* between the CON and CB groups ($P > 0.05$).

4. Discussion

The development of intensive production has predisposed poultry to diseases, which in turn inhibits their growth and development. Including probiotics such as *C. butyricum* in their diets is therefore regarded as an effective solution to this problem (16, 17). As the most reliable indicator of broiler growth and development, growth performance is the key to increase economic efficiency. According to a previous study, AA broilers fed with 1×10^9 cfu/kg *C. butyricum* exhibited an increase in ADG and a decrease in F/G at 1–21 and 1–42 days, respectively (18). Nevertheless, Yang et al. (11) and Liao et al. (16) discovered that different doses of *C. butyricum* addition significantly increased the ADG of broilers at 22–42 d but did not considerably affect their feed conversion ratio. In the present study, we investigated the effect of *C. butyricum* on the growth performance of AA broilers. Our results indicate that although *C. butyricum* did not considerably affect the growth performance of the broilers at 1–21 days, it significantly improved their final BW and ADG at 42 and 1–42 days, respectively, which is consistent with the results of Yang et al. (11). These results suggest that *C. butyricum* treatment improves the growth performance of broilers, which may be due to the production of butyric acid, vitamin B, and amylase by *C. butyricum* to enhance the utilization of feed nutrients (19). In addition, different effects of *C. butyricum* supplementation have been observed in various studies, which could be attributed to some essential factors such as additive dosage, broiler breed and physiological stage, diet composition, and feeding management.

With the rise in living standards, consumers have become increasingly concerned with meat quality, especially in China. Meat quality is an accurate indicator of the physical and chemical properties of meat. In general, L*, a*, and b* are used to evaluate the color of meat, which is a crucial indicator of the meat's freshness and quality (20). After broilers are slaughtered, the lactic acid is produced by glycolysis accumulates in the muscle tissue, resulting in a decrease in the pH value, protein denaturation, and a decline in the ability of

TABLE 6 Effect of *C. butyricum* on the mRNA expression levels of jejunal antioxidant-related genes of broilers.

Items	CON	CB	*P*-value
Nrf2	1.00 ± 0.08	1.32 ± 0.09	0.031
CAT	1.00 ± 0.19	1.12 ± 0.10	0.631
SOD1	1.00 ± 0.10	1.37 ± 0.11	0.029
GSH-Px	1.00 ± 0.18	1.10 ± 0.09	0.621

CON, basal diet; CB, basal diet + Clostridium butyricum. Nrf2, nuclear factor erythroid 2-related factor 2; CAT, catalase; SOD1, superoxide dismutase 1; GSH-Px, glutathione peroxidase.

TABLE 7 Effect of *C. butyricum* on the mRNA expression levels of jejunal inflammation-related genes of broilers.

Items	CON	CB	*P*-value
NF-κB	1.00 ± 0.05	0.81 ± 0.07	0.033
TNF-α	1.00 ± 0.07	0.78 ± 0.05	0.027
IL-1β	1.00 ± 0.13	0.96 ± 0.05	0.843
IL-6	1.00 ± 0.09	0.87 ± 0.04	0.219
IL-10	1.00 ± 0.08	1.34 ± 0.11	0.023

CON, basal diet; CB, basal diet + Clostridium butyricum. NF-κB, nuclear factor kappa B; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α.

muscle protein to bind water (21). Thus, the final pH and rate of pH decline in meat have a direct effect on the drip loss and ultimately on the water retention capacity and tenderness of the meat. Liu et al. (20) reported that *C. butyricum* treatment increased the pH_{45min} and a* values but reduced the L* value, drip loss and shear force of in Peking duck breast meat. In another study, Li et al. (22) dicovered that Partridge shank chickens fed with a synbiotic diet including *C. butyricum* exhibited lower breast muscle drip loss and cooking loss and higher a^*_{24h} and pH_{24h} values. In the present study, we discovered that *C. butyricum* increased the a^*_{24h} and pH_{24h} values but reduced the drip loss and shear force of breast meat, indicating an improvement of the freshness, tenderness and water retention capacity of the meat. These favorable results indicate that *C. butyricum* can enhance the meat quality of broilers.

Antioxidant enzyme activity and oxidation product concentration are reliable biomarkers for assessing the antioxidant status of animals. T-SOD is a crucial component of the enzyme system, which effectively scavenges free radicals and inhibits peroxidation. MDA is one of the final peroxidation products derived from membrane lipids. Its level is regarded as a crucial index for quantifying the degree of peroxidation (14). According to previous studies, *C. butyricum* could mitigate peroxidation and improve the antioxidant enzyme activity *in vivo* (13, 16, 23). Li et al. (18) reported that the addition of *C. butyricum* to the diets of broilers enhanced the activities of their serum T-AOC, GSH-Px and SOD. Zhan et al. (13) discovered that *C. butyricum* supplementation resulted in the improvement of T-SOD and GSH?Px activities in the serum of laying hens. Similarly, Han et al. (23) found that *C. butyricum* addition enhanced serum GSH-Px activity in weaned piglets. In the present study, we discovered that *C. butyricum* supplementation significantly decreased the serum MDA content in broilers at 42 d, indicating the lower level of lipid peroxidation. In addition, *C. butyricum* addition significantly increased the activities of T-SOD and T-AOC at 42 d, indicating an improvement of antioxidant capacity. Generally, an improvement in antioxidant capacity may reduce the degree of protein and lipid oxidative damage in muscles and prolong the shelf life of meat (14), further confirming that *C. butyricum* improves the quality of meat.

Digestive enzymes are important for the digestion of nutrient materials and their activities have a substantial effect on the feed conversion and growth performance of broilers. Previous studies have recorded that probiotics can produce digestive enzymes and enhance their activities in the intestine. According to a previous study, Cobb broilers fed with *C. butyricum* exhibited elevated jejunal amylase, lipase, and protease activity (10). Wang and Gu (24) discovered that the addition of *Bacillus coagulans NJ0516* to the diets of broilers resulted in a significant increase in their duodenal protease and amylase activities. However, Rodjan et al. (25) reported that the supplementation of *Bacillus* spores did not significantly affect the digestive enzyme activity of Ross 308 broilers. Similar to the findings of Zhang et al. (10), we found that *C. butyricum* increased the jejunal lipase and trypsin activities in the broilers. These favorable results suggest that *C. butyricum* significantly improves the digestive function of broilers; this is attributable to their metabolism of SCFA, which may stimulate the secretion of digestive enzymes by digestive glands. However, the currently available information is inconsistent and limited, necessitating further evaluation of the underlying mechanism.

The intestine is an essential conduit for the exchange of materials between the external environment and the host environment. Intestinal morphology is a major factor in the evaluation of the intestinal status and is closely linked with the development of the intestinal epithelial structure, which is typically evaluated though the VH, CD and VH/CD. A decrease in CD and an increase in VH and VH/CD indicate a natural increase in epithelial cell turnover and a well-differentiated intestinal mucosa, suggesting an enhanced digestive and absorptive capacity (26). A previous study reported that *C. butyricum* treatment significantly improved the duodenal VH and VH/CD and reduced the duodenal CD in broilers (27). Cao et al. (28) found that broilers fed with *C. butyricum* exhibited an increased VH and a decreased CD in the ileum. Furthermore, a recent study revealed that the addition of *C. butyricum* to the diet of broilers infected with *Clostridium perfringens* increased their intestinal VH/CD and ameliorated the degree of their intestinal damage (29). In the present study, we discovered that *C. butyricum* considerably improved the duodenal VH and jejunal VH and VH/CD of the broilers, indicating an improvement in intestinal morphology. These positive results suggest that *C. butyricum* has a promotive effect on the development of the intestinal epithelial structure and thereby improves the intestinal morphology.

The intestinal barrier is crucial for protecting the host against infection and other diseases and maintaining normal physiological function. In addition to preventing the entry of luminal bacteria and dietary allergens into the mucosa, an intact intestinal barrier selectively regulates the entry of nutrients, ions, and water into the body. ZO-1 and occludin, the main tight junction proteins of the intestinal barrier, play a major role in regulating the integrity and permeability of the gut barrier by binding to the actin cytoskeleton and contributing to the formation of a tight junction structure. According to Li et al. (18), the addition of *C. butyricum* to the diets of broilers increased the relative expression levels of claudin-1 and *ZO-1* in the gut. Liu et al. (30) reported that treatment with *C. butyricum* significantly increased the expression of occludin and *ZO-1* in the

jejunum of broilers at 42 d. A similar result was observed in our study; that is, after 42 d of feeding broilers with a diet containing *C. butyricum*, we discovered that the jejunal mRNA levels of *ZO-1* increased, indicating an improvement in the broilers' intestinal barrier integrity.

With the exception of the intestinal barrier, intestinal health is inseparable from intestinal antioxidant status and immune function. As a major regulator, Nrf2 is a basic leucine zipper protein that regulates the expression of genes related to antioxidants or detoxifying enzymes and protects the body against oxidative stress (31, 32). In general, Nrf2 exists in an inactive form in the cytoplasm binding to its inhibitor: Kelch-like epichlorohydrin-related protein 1 (Keap1). In response to oxidative stress, the Keap1–Nrf2 complex dissociates, allowing Nrf2 to translocate into the nucleus and bind to the antioxidant response element. This results in the transcription of antioxidant genes such as heme oxygenase-1, *SOD* and *CAT*. Hence, activating the Nrf2 signaling pathway may increase the expression levels of antioxidant genes (33). In our work, we first found that *C. butyricum* supplementation in broilers resulted in a significant increase in the expression levels of *Nrf2* and *SOD1* in their jejunal mucosa, indicating an improvement in their intestinal antioxidant capacity. This positive finding may be attributable to the activation of the Nrf2 signaling pathway, which effectively protects the intestinal mucosa against oxidative stress damage (33). Moreover, NF-κB, a key transcription factor involved in immunity, can recognize, rapidly respond to, and transcribe a variety of proinflammatory cytokines and inflammatory mediators, such as *TNF-α* and *ILs*, resulting in an inflammatory crisis (34). Reduced expression of genes encoding proinflammatory cytokines, such as interferon-γ, *IL-1β* and *TNF-α*, may retard the activation of the NF-κB signaling pathway. Li et al. (18) demonstrated that *C. butyricum* increased the expression levels of *IL-1β* and *TNF-α* in the ileum of broilers. Besides, a study on weaned piglets challenged with lipopolysaccharides revealed that *C. butyricum* downregulated the expression of *IL-1β*, *TNF-α* and *NF-κB*, thereby mitigating inflammatory damage (35). Similarly, in the present study, we discovered that *C. butyricum* treatment significantly downregulated the mRNA expression of *NF-κB* and *TNF-α* in the jejunal mucosa of the broilers. Moreover, we first observed that *C. butyricum* significantly increased the mRNA expression of *IL-10* in their mucosa. These positive results indicate the lower inflammation and the improved immune status, which is by likely inhibiting NF-κB signaling pathway. Indeed, a potential cross-link exists between the Nrf2 and NF-κB signaling pathways (36), and inhibiting the Nrf2 signaling pathway reduces the antioxidant capacity and increases the likelihood of oxidative stress damage to the body, ultimately leading to inflammation through NF-κB signaling pathway activation (37, 38). Therefore, improvements in intestinal health, including the intestinal morphology, intestinal barrier, antioxidant capacity and immune status, induced by *C. butyricum* may be closely related to the activation of the Nrf2 signaling pathway and inhibition of the NF-κB signaling pathway.

5. Conclusions

The addition of *C. butyricum* to the diets of broilers can improve their growth performance and meat quality. *C. butyricum* can also improve intestinal health, which is likely to be linked with the activation of the Nrf2 signaling pathway and inhibition of the NF-κB signaling pathway. However, additional research is necessary to determine the exact mechanism of action of *C. butyricum*.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of Anhui Agricultural University.

Author contributions

Conceptualization: ZL and XJ. Methodology: LL and YL. Investigation and writing—original draft preparation: ZL and LL. Data curation: QW and YL. Formal analysis: XJ and QW. Writing—review and editing: CZ and XC. Supervision: ZG and CZ. Funding acquisition: CZ. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Major Science and Technology Project of Huaibei City (Z2020013), the Technical Service Project of Poultry Breed Industry and Efficient Production Technology Innovation Center in Xuanzhou District of Anhui Province (21339032), and the Innovation and Entrepreneurship Cultivation Project for Graduate Students of Anhui Agricultural University.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1107798/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 February 2023
DOI 10.3389/fvets.2023.1123002

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

RECEIVED 13 December 2022
ACCEPTED 30 January 2023
PUBLISHED 16 February 2023

CITATION
Zhang J, Jin Y, Cao M, Deng J, Chang Y, Shi M and Miao Z (2023) Effects of dietary *Chinese yam* polysaccharide copper complex on growth performance, immunity, and antioxidant capacity of broilers. *Front. Vet. Sci.* 10:1123002. doi: 10.3389/fvets.2023.1123002

Effects of dietary *Chinese yam* polysaccharide copper complex on growth performance, immunity, and antioxidant capacity of broilers

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Chinese yam polysaccharide (CYP) has received attention in recent years owing to its positive nutritional and medicinal characteristics. Copper is an essential trace metal in animals, which plays an important role in iron absorption and hemoglobin synthesis. However, no published study has evaluated *Chinese yam* polysaccharide copper complex (CYP-Cu) as a dietary additive in broilers. This study was conducted to investigate the effects of dietary CYP-Cu on growth performance, immunity, and oxidative resistance in broilers. A total of 360 1-day-old 817 broiler chickens were randomly divided into 4 groups, with 3 replicates of 30 birds each and were fed a basal diet with the addition of 0 (control group), 0.02, 0.10, and 0.50 g/kg CYP-Cu. The feeding trial lasted 48 days. On day 28 and day 48, 6 broilers in each group were slaughtered, respectively. Then the parameters of growth and carcass, serum biochemistry, immunity, and antioxidation, and the expression level of hepatic antioxidative genes were investigated. The results showed that compared with the control group, the supplementation of dietary CYP-Cu could improve the indexes of the growth, carcass, serum biochemistry, immunity and oxidation resistance in broilers, such as average daily gain (ADG), the slaughter percentage (SP), semi-evisceration weight percentage (SEWP), eviscerated carcass weight percentage (EWP), breast muscle percentage (BMP), leg muscle percentage (LMP), serum albumin (ALB), high density lipoprotein (HDL), insulin-like growth factor I (IGF-I), triiodothyronine (T3), thyroxine (T4), growth hormone (GH), insulin (INS), immunoglobulin M (IgM), immunoglobulin G (IgG), immunoglobulin A (IgA), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), complement 3 (C3), complement 4 (C4), total superoxide dismutase (T-SOD), total antioxidative capacity (T-AOC), glutathione peroxidase (GSH-Px), and glutathione s-transferase (GSH-ST); these parameters in the 0.10 g/kg CYP-Cu treated group were significantly increased ($P < 0.05$) in the total trial period, with the exceptions that feed conversion ratio (FCR) and serum low density lipoprotein (LDL), malondialdehyde (MDA) were decreased in the total trial period. In addition, the antioxidative gene mRNA expression of Nuclear factor E2-related factor 2 (Nrf_2), Superoxide dismutase 1 (SOD_1), Superoxide dismutase 2 (SOD_2), and Catalase (CAT) were upregulated in the liver ($P < 0.05$). These results indicated that the supplementation of dietary CYP-Cu improved the growth, immunity, and oxidation resistance of broilers, and the addition of 0.10 g/kg CYP-Cu in broiler diets is recommended, which suggests that CYP-Cu may be a promising green feed additive in the poultry industry.

KEYWORDS

***Chinese yam* polysaccharide copper complex, growth performance, immunity, oxidation resistance, broilers**

Introduction

Chinese yam polysaccharide (CYP) extracted from the *Chinese yam*, a very popular tuber crop for food and medical purposes in China (1), has received attention in recent years, owing to its positive nutritional and medicinal characteristics (2–5). Previous studies have shown that the pharmacological effects of CYP include promoting growth performance, lowering blood glucose and blood lipid, and enhancing anti-oxidation and immunity in animal (6–9). It is known that CYP has good antioxidant activity, and removed hydroxyl radical capacity (4). CYP could enhance the cellular and humoral immune activities in chickens induced by Newcastle disease virus vaccine, and promote proliferation of peripheral blood lymphocytes induced by concanavalin A (9). Another study has shown that mice in a CYP group had heavier body weights than controls, and that CYP could regulate inflammatory responses and oxidative stress in mice (9). Our previous study showed that the supplementation of dietary CYP (0.50 g/kg) promoted thymus index, serum immunoglobulin A (IgA), complement 3 (C3), complement 4 (C4), insulin-like growth factor I (IGF-I), triiodothyronine (T3), thyroxine (T4), insulin (INS), growth hormone (GH), interleukin 2 (IL-2), interleukin 4 (IL-4), and interleukin 6 (IL-6) levels in broilers (10).

An important issue is the application of trace metals in the diets of livestock and poultry. Copper is an essential trace metal in the body, it plays an important role in iron absorption and hemoglobin synthesis (11), and also has strong antimicrobial activity (12). However, inorganic copper is not well absorbed, and can lead to poisoning and environmental pollution, while organic copper is more easily absorbed and used, and reduces environmental pollution and toxic effects (12).

Recent research has shown that the addition of polysaccharides and metal complexes can be effective substances against bacteria (13–15). To date, no published study has evaluated *Chinese yam* polysaccharide copper complex (CYP-Cu) as a dietary additive in animals. In this paper, we prepared CYP-Cu, investigated the effects of its dietary inclusion upon the growth performance, serum immunity levels, and antioxidant capacity in broilers, and discussed the immune function of CYP-Cu in birds, as a strategy to replace antibiotic use and improve growth performance and immunity in poultry.

Materials and methods

Preparation of CYP-Cu

The *Chinese yam* polysaccharide for the test was purchased from Shaanxi Hana Biotechnology Co., Ltd (Xi'an city, China), with particle size passing through an 80 mesh sieve (≥95.00%), dry weight loss <5%, and polysaccharide content ≥30% (total carbohydrate ≥90.40%). The trial copper sulfate was purchased from Tianjin Fenghua Chemical Reagent Factory (analytically pure, Tianjin city, China); molecular formula: $CuSO_4$-$5H_2O$; molecular weight: 249.68, content: 99.00%. To prepare the CYP-Cu, a water bath was adjusted to 60–70°C; 4 g of CYP and 1 g of sodium citrate were placed into a beaker, and 120 mL of water was added and mixed well; stir until its temperature up to 60–70°C. Sodium hydroxide was added in a dropwise fashion to increase alkalinity; then the saturated copper sulfate solution was added dropwise (to maintain the alkaline environment) until flocculent material appeared; at this point, no more was added. The water bath was stirred for 1 h; the contents were centrifuged while hot, the supernatant was separated and 95% ethanol was added. The solution was left overnight at 4°C, and then centrifuged to produce the precipitate, which is the CYP-Cu. The content of copper in CYP-Cu was determined to be 1591.59 ± 6.07 mg/kg (16).

Experimental design

In this experiment, 360 1-day-old healthy 817 broilers (39.54 ± 0.51 g, sex balance), a commercial Chinese crossbred broiler produced by crossing a fast-growing broiler cock and layer hen (17), were selected from the same batch at Henan Fengyuan Poultry Co (Xinxiang city, China). The broilers were randomly placed into 4 groups with 3 replicates, with 30 broilers per replicate. The control group was fed only a basal diet (without CYP-Cu); the 3 treated groups were supplemented with 0.02, 0.10, and 0.50 g/kg of CYP-Cu in the basal diet, respectively. The experimental feeding lasted until the chickens were 48 days old, and was divided into two stages, 1–28 and 29–48 days.

TABLE 1 Composition and nutrition level of basic diet.

Item	Content	
	1–28 days of age	29–48 days of age
Ingredient (%)		
Corn	58.00	63.50
Soybean meal	34.00	29.00
Wheat bran	1.00	
Soybean oil	1.00	2.00
Fish meal	2.00	1.60
Calcium hydrogen phosphate	1.30	1.30
Limestone	1.40	1.30
Table salt	0.30	0.30
Premix[a]	1.00	1.00
Total	100.00	100.00
Nutrient level		
Metabolic energy ME ($MJ \cdot kg^{-1}$)[b]	13.23	13.25
Crude protein CP (%)	23.00	20.00
Calcium Ca (%)	1.00	0.90
Total phosphorus TP (%)	0.65	0.6
Available phosphorous AP (%)	0.45	0.35
Lysine met (%)	0.50	0.38
Methionine Lys (%)	1.10	1.00

[a]The premix provided the following per kg of diet: VA 3000 IU, VD_3 500 IU, VE 10 IU, VK_3 0.5 mg, VB_6 3.5 mg, VB_1 3.8 mg, D-pantothenic acid 10 mg, folic acid 0.5 mg, biotin 0.15 mg, Fe 80 mg, Cu 8 mg, Zn 75 mg, Mn 60 mg, and Se 0.15 mg.
[b]ME was a calculated value and others were measured values.

Diets and management

The broilers were fed commercial diets formulated with reference to the NRC (1994) nutritional requirement standards for broilers. The composition and nutritional levels of the basic diet for broilers are shown in Table 1.

All birds were kept under continuous lighting for 24h, and birdhouse temperature was kept at 32°C for the first 3 days, cooled 2–3°C every week, and dropped to 21°C on the 35th until end of the trial. During the experiment, broilers were caged, and had *ad-libitum* access to diet and water; in addition, routine management followed the company's procedures.

Growth and carcass parameters measured

During the feeding trial period, the amount of intake and leftovers were recorded daily, and body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) of broilers were calculated at the end of the trial, respectively. On day 28 and day 48, after collecting blood samples, 6 broilers (3 males and 3 females) in each group were slaughtered, respectively. The data of carcass weight, semi-evisceration weight, evisceration weight, breast muscle, and leg muscle were measured. Then the slaughter percentage (SP), semi-evisceration weight percentage (SEWP), eviscerated carcass weight percentage (EWP), breast muscle percentage (BMP), and leg muscle percentage (LMP) of broilers were determined. The samples of liver tissue were taken from the same position and kept at −80°C until analysis.

Blood collection

On day 28 and day 48, 6 broilers (3 males and 3 females) were randomly selected from each group for the collection of blood samples. After weighing, the blood was drawn from the wing veins of birds, and put into centrifuge tubes for overnight coagulation at 4°C. Following centrifugation (3,000 g, 10 min, at 4°C), the serum was collected into sterile tubes and kept at −80°C until analysis.

Serum biochemical, immune, and antioxidative parameters measured

Serum biochemistry parameters, such as serum albumin (ALB), low density lipoprotein (LDL), and high density lipoprotein (HDL), were obtained by using a 7,180 automatic analyzer (Hitachi High-Technologies Co., Ltd., Tokyo, Japan). The assays of serum biochemical, immune, and antioxidative profiles included insulin-like growth factor I (IGF-I), triiodothyronine (T3), thyroxine (T4), growth hormone (GH), insulin (INS), immunoglobulin M (IgM), immunoglobulin G (IgG), immunoglobulin A (IgA), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), complement 3 (C3), complement 4 (C4), total superoxide dismutase (T-SOD), total antioxidative capacity (T-AOC), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and glutathione s-transferase (GSH-ST); concentrations were analyzed by ELISA according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing city, China).

TABLE 2 Primer design for hepatic antioxidant gene assay by qRT-PCR.

Gene	Primer sequence (5′-3′)	Product length (bp)
β-actin	F:5′-CATTGAACACGGTATTGTCACCAACTG-3′	270
	R:5′-GTAACACCATCACCAGAGTCCATCAC-3′	
Nrf$_2$	F:5′-AACACACCAAAGAAAGACCCTCCTG-3′	207
	R:5′-TTCACTGAACTGCTCCTTCGACATC-3′	
SOD$_1$	F:5′-GGTCATCCACTTCCAGCAGCAG-3′	377
	R:5′-AACGAGGTCCAGCATTTCCAGTTAG-3′	
SOD$_2$	F:5′-TTCCTGACCTGCCCTACGACTATG-3′	357
	R:5′-AGCCTGATCCTTGAACACCAACTG-3′	
CAT	F:5′-CTCTCAGAAGCCAGATGCCTTGAC-3′	293
	R:5′-CAGCAACAGTGGAGAACCGTATAGC-3′	

Nrf$_2$, nuclear factor E2-related factor 2; SOD$_1$, superoxide dismutase 1; SOD$_2$, superoxide dismutase 2; CAT, catalase.

Assay of hepatic antioxidative gene expressions

Total RNAs were isolated from hepatic tissues using Trizol reagent (Invitrogen, Thermo Fisher Scientific, USA) following the manufacturer's instructions. The quality and quantity of RNA was detected with a NanoPhotometer® spectrophotometer (Implen, Westlake Village, CA, USA), and RNA integrity wasdetected with 1.0% agarose gelelectrophoresis. Total RNAs were transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The genes of Nuclear factor E2-related factor 2 (*Nrf*$_2$), Superoxide dismutase 1 (*SOD*$_1$), Superoxide dismutase 2 (*SOD*$_2$), and Catalase (*CAT*) were selected for the detection of hepatic antioxidative gene expression profiles. The primer sequences for quantitative real-time PCR (qRT-PCR) assay are shown in Table 2. The qRT-PCR was carried out in a QuantStudio 6 Flex Real-Time PCR System (ABI, Carlsbad, CA, USA) with the miScript SYBR Green PCR kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's instructions. In the present study, β-actin as a housekeeping gene, the relative expression of the target genes was calculated by $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data obtained from this experiment were analyzed using One-Way ANOVA in SPSS 26.0 for Windows (IBM Corp., Chicago, IL, USA), and presented as mean ± standard error of the means (SEM), and significant differences among all groups were determined at $P < 0.05$ by Duncan's multiple range tests.

10.3389/fvets.2023.1123002

TABLE 3 Effect of dietary CYP-Cu on the growth performance in broilers.

		CYP-Cu level					
Item	Age	0 g/kg	0.02 g/kg	0.10 g/kg	0.50 g/kg	SEM	*P*-value
BW (g)	1 day	39.71	39.86	39.67	39.70	0.437	0.972
	28 days	618.11^{b}	630.10^{ab}	638.63^{a}	640.49^{a}	5.702	0.016
	48 days	1,560.53^{c}	1,623.61^{b}	1,682.35^{a}	1,658.08^{ab}	22.067	0.003
ADFI (g/days)	1–28 days	31.93	31.34	31.25	31.59	0.528	0.597
	29–48 days	98.16	97.48	99.31	98.56	1.831	0.791
	1–48 days	60.41	61.60	62.87	62.81	1.413	0.321
ADG (g/days)	1–28 days	20.66^{b}	21.08^{ab}	21.46^{a}	21.39^{a}	0.207	0.017
	29–48 days	47.12^{b}	49.68^{a}	52.09^{a}	50.97^{a}	1.064	0.008
	1–48 days	31.68^{c}	33.00^{b}	34.22^{a}	33.72^{ab}	0.460	0.003
FCR	1–28 days	1.54	1.48	1.45	1.47	0.035	0.138
	29–48 days	2.18^{a}	1.96^{b}	1.91^{b}	1.93^{b}	0.030	0.002
	1–48 days	1.91	1.87	1.84	1.86	0.027	0.165

CYP-Cu, *Chinese yam* polysaccharide copper complex; SEM, standard error of the means (n = 6); BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.
In the same line, values with different lowercase superscripts indicate significant differences ($P < 0.05$), and values with the same lowercase superscripts indicate no significant differences ($P > 0.05$).

TABLE 4 Effect of dietary CYP-Cu on the carcass performance in broilers on day 48.

		CYP-Cu level					
Item	Age	0 g/kg	0.02 g/kg	0.10 g/kg	0.50 g/kg	SEM	*P*-value
SP (%)	48 days	88.21^{b}	89.96^{a}	90.75^{a}	90.03^{a}	0.560	0.011
SEWP (%)	48 days	80.56^{b}	81.84^{a}	82.36^{a}	81.79^{a}	0.389	0.010
EWP (%)	48 days	67.70^{b}	68.14^{ab}	69.10^{a}	68.51^{a}	0.374	0.030
BMP (%)	48 days	17.78^{b}	18.28^{ab}	18.69^{a}	18.37^{a}	0.307	0.022
LMP (%)	48 days	20.75^{c}	21.38^{b}	22.22^{a}	21.63^{b}	0.240	0.002

CYP-Cu, *Chinese yam* polysaccharide copper complex; SEM, standard error of the means (n = 6); SP, slaughter percentage; SEWP, semi-evisceration weight percentage; EWP, eviscerated carcass weight percentage; BMP, breast muscle percentage; LMP, leg muscle percentage.
In the same line, values with different lowercase superscripts indicate significant differences ($P < 0.05$), and values with the same lowercase superscripts indicate no significant differences ($P > 0.05$).

Results

Growth performances

The effect of dietary CYP-Cu on growth performances evaluated in this study is shown in Table 3. The BW of broilers on day 28 and day 48 was affected by the addition of CYP-Cu, the BW of the 0.10 and 0.50 g/kg CYP-Cu group was significantly higher than that of the control group on day 28 ($P < 0.05$), while the BW of the 0.10 g/kg group was significantly higher than that of control group on day 48 ($P < 0.05$). Compared with the control, the ADG of the treatments increased with the addition of CYP-Cu, and the 0.10 g/kg group had the highest ADG in all trial periods ($P < 0.05$). However, no difference of ADFI between the treatments and the control were observed in all trial periods ($P > 0.05$). By contrast, the FCR of the treatments in 29–48 days periods was significantly lower than that of control group ($P < 0.05$), while no difference of FCR between the treatments and the control were observed in 1–28 and 1–48 days periods ($P > 0.05$). However, the FCR of the treatments had a downward trend with the addition of CYP-Cu, and the 0.10 g/kg group had the lowest FCR in all trial periods.

Carcass performances

The supplementation of dietary CYP-Cu had a positive effect on the carcass performances of broilers on day 48 (Table 4). Compared to that of the control, SP, SEWP, EWP, BMP, and LMP of 48-day broilers showed a gradual increase with the addition of CYP-Cu. Among them, the carcass performances of the 0.10 and 0.50 g/kg CYP-Cu group showed a significantly greater increase than those of the control in 48-day broilers ($P < 0.05$).

Serum growth factors and biochemical parameters

As presented in Table 5, the concentrations of serum growth factors, IGF-I, T3, T4, GH, and INS, in the 3 treated groups expressed a higher tendency than those in the control group of broilers on day 28 and day 48. The results of the 0.10 g/kg CYP-Cu group were the most significant ($P < 0.05$), with the exception of serum GH concentration in the 0.50 g/kg group, which had the highest level on day 28 ($P < 0.05$).

10.3389/fvets.2023.1123002

TABLE 5 Effects of dietary CYP-Cu on serum growth factors and biochemical parameters in broilers.

		CYP-Cu level					
Item	Age	0 g/kg	0.02 g/kg	0.10 g/kg	0.50 g/kg	SEM	*P*-value
ALB (g/L)	28 days	22.05^{b}	24.51^{a}	25.35^{a}	24.96^{a}	0.467	0.002
	48 days	20.83^{c}	23.19^{ab}	23.60^{a}	22.57^{b}	0.356	0.001
LDL (μmol/L)	28 days	378.65	375.89	353.56	369.70	3.730	0.312
	48 days	401.15	401.74	392.64	388.22	6.873	0.216
HDL (μmol/L)	28 days	131.83^{d}	140.31^{c}	145.87^{b}	151.71^{a}	1.641	0.004
	48 days	129.05^{b}	147.04^{a}	147.50^{a}	145.99^{a}	3.023	0.001
IGF-1 (μg/L)	28 days	36.17^{c}	39.30^{b}	42.61^{a}	37.01^{c}	0.633	0.001
	48 days	34.80^{b}	39.29^{a}	40.20^{a}	37.82^{a}	1.254	0.012
T3 (pmol/L)	28 days	288.39^{d}	307.14^{c}	363.10^{a}	340.15^{b}	4.215	0.007
	48 days	297.92^{c}	316.37^{b}	346.50^{a}	307.69^{bc}	4.532	0.009
T4 (pmol/L)	28 days	928.44^{c}	950.19^{bc}	1,116.95^{a}	990.52^{b}	19.075	0.004
	48 days	1,025.39^{c}	1,041.74^{c}	1,181.25^{a}	1,093.74^{b}	13.518	0.015
GH (μg/L)	28 aysd	18.24^{d}	20.29^{c}	20.94^{b}	21.69^{a}	0.232	0.002
	48 days	20.43^{b}	24.49^{a}	25.45^{a}	24.84^{a}	0.550	0.003
INS (μg/L)	28 days	16.35^{c}	17.50^{b}	20.42^{a}	18.14^{b}	0.375	0.003
	48 days	16.78^{c}	19.53^{a}	19.85^{a}	18.90^{b}	0.268	0.005

CYP-Cu, *Chinese yam* polysaccharide copper complex; SEM, standard error of the means (n = 6); ALB, serum albumin; LDL, low density lipoprotein; HDL, high density lipoprotein; IGF-I, insulin-like growth factor I; T3, triiodothyronine; T4, thyroxine; GH, growth hormone; INS, insulin.
In the same line, values with different lowercase superscripts indicate significant differences ($P < 0.05$), and values with the same lowercase superscripts indicate no significant differences ($P > 0.05$).

According to Table 5, the densities of ALB and HDL in the 3 treated groups were significantly higher than those in the control group on day 28 and day 48 ($P < 0.05$). ALB levels in the 0.10 g/kg CYP-Cu group were highest in broilers on day 28 and day 48 ($P < 0.05$), while HDL level was highest in the 0.50 g/kg CYP-Cu group on day 28 and in the 0.10 g/kg CYP-Cu group on day 48 ($P < 0.05$). Interestingly, the densities of LDL in the 3 treated groups had a decreasing trend with increasing dietary CYP-Cu supplementation, but there were no significant differences in LDL levels between the treated and the control groups ($P > 0.05$).

Serum immune parameters

The serum immune parameters in broilers on day 28 and day 48 are shown in Table 6. Compared with the control group, the serum levels of IgM, IgA, IgG, IL-2, IL-4, IL-6, C3, and C4 in the 3 treated groups were significantly higher on day 28 ($P < 0.05$). The dietary supplementations of 0.10 and 0.50 g/kg CYP-Cu were more effective. On day 48, the serum concentrations of IgM, IgA, IgG, IL-2, IL-6, C3, and C4 in the 3 treated groups were significantly higher than those in the control group ($P < 0.05$), meanwhile, the serum levels of IL-2 in the 0.02 and 0.10 g/kg CYP-Cu groups were significantly higher than those in the control group ($P < 0.05$). The additions of 0.10 g/kg CYP-Cu in the diet of broilers on day 48 seemed to have a greater effect on serum immune parameters.

Serum antioxidative parameters

According to Table 7, the dietary supplementation of CYP-Cu played a positive role in serum antioxidative parameters in broilers on day 28 and day 48. Compared with that of the control, the serum concentrations of T-SOD, T-AOC, GSH-ST, and GSH-Px in the treated groups were higher on day 28 and day 48. Therein, the results of the 0.10 and 0.50 g/kg CYP-Cu groups were the most significant on day 28 ($P < 0.05$), while the results of the 0.50 g/kg CYP-Cu group seemed to be more effective on day 48 ($P < 0.05$). Interestingly, the serum concentration of MDA in the treated groups was downregulated in contrast to the control on day 28 and day 48. The serum concentration of MDA of the 0.10 and 0.50 g/kg CYP-Cu groups showed a greater decline ($P < 0.05$). These results indicated that the supplementation of CYP-Cu in the diet of broilers could raise oxidation resistance.

Hepatic antioxidative gene expression profiles

In this experiment, the antioxidant genes, *CAT*, Nrf_2, SOD_1, SOD_2, and mRNA expression in the livers of broilers on day 48 were investigated (Figure 1, Supplementary Table S1). According to Figure 1, the antioxidative gene mRNA expression of Nrf_2, SOD_1, SOD_2 and *CAT* were significantly upregulated in the liver in the treated groups compared to the control group ($P < 0.05$), while among all the treated groups, the antioxidative genes had the highest mRNA expression level in the 0.10 g/kg group. The results show that the dietary supplementation of 0.10 g/kg CYP-Cu could improve mRNA expression of the antioxidant genes *CAT*, Nrf_2, SOD_1, and SOD_2 in the livers of broilers on day 48, and enhance their antioxidant capacity.

TABLE 6 Effects of dietary CYP-Cu on serum immune parameters in broilers.

		CYP-Cu level					
Item	Age	0 g/kg	0.02 g/kg	0.10 g/kg	0.50 g/kg	SEM	*P*-value
IgM (ng/mL)	28 days	5,866.38^{c}	6,255.93^{b}	6,607.24^{a}	6,363.59^{b}	90.639	0.034
	48 days	5,130.01^{d}	5,774.12^{c}	6,312.90^{a}	5,954.52^{b}	77.100	0.020
IgA (ng/mL)	28 days	8,588.37^{b}	9,779.10^{a}	9,886.84^{a}	9,783.41^{a}	105.803	0.005
	48 days	8,158.15^{c}	8,533.61^{b}	9,284.54^{a}	8,617.64^{b}	155.892	0.001
IgG (μg/mL)	28 days	85.98^{c}	92.05^{b}	93.20^{b}	98.64^{a}	1.380	0.001
	48 days	79.05^{d}	84.50^{c}	88.99^{b}	92.75^{a}	1.490	0.003
IL-2 (ng/L)	28 days	143.34^{b}	167.42^{a}	168.06^{a}	170.12^{a}	2.831	0.013
	48 days	179.47^{d}	224.00^{b}	235.09^{a}	211.96^{c}	3.287	0.000
IL-4 (ng/L)	28 days	168.19^{b}	213.92^{a}	214.88^{a}	213.27^{a}	2.406	0.002
	48 days	169.74^{c}	180.27^{ab}	182.20^{a}	172.06^{bc}	3.827	0.029
IL-6 (ng/L)	28 days	40.78^{c}	52.55^{b}	52.99^{b}	56.06^{a}	0.884	0.011
	48 days	42.65^{c}	52.71^{b}	55.24^{a}	51.81^{b}	0.895	0.035
C3 (μg/mL)	28 days	713.88^{b}	802.75^{a}	824.36^{a}	814.49^{a}	14.527	0.001
	48 days	725.93^{c}	824.37^{b}	859.78^{a}	848.45^{ab}	14.146	0.006
C4 (μg/mL)	28 days	481.07^{c}	502.76^{b}	560.58^{a}	567.36^{a}	8.599	0.007
	48 days	457.13^{c}	489.59^{b}	570.52^{a}	511.79^{b}	10.197	0.001

CYP-Cu, *Chinese yam* polysaccharide copper complex; SEM, standard error of the means (n = 6); IgM, immunoglobulin M; IgA, immunoglobulin A; IgG, immunoglobulin G; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; C3, complement 3; C4, complement 4.
In the same line, values with different lowercase superscripts indicate significant differences ($P < 0.05$), and values with the same lowercase superscripts indicate no significant differences ($P > 0.05$).

TABLE 7 Effects of dietary CYP-Cu on serum antioxidative parameters in broilers.

		CYP-Cu level					
Item	Age	0 g/kg	0.02 g/kg	0.10 g/kg	0.50 g/kg	SEM	*P*-value
T-SOD (pg/mL)	28 days	41.15^{c}	46.18^{b}	49.94^{a}	47.02^{b}	0.830	0.006
	48 days	44.07^{d}	49.08^{b}	50.52^{a}	47.60^{c}	0.609	0.025
T-AOC (U/mL)	28 days	5.26^{c}	5.74^{b}	5.88^{b}	6.24^{a}	0.107	0.012
	48 days	5.20^{b}	5.93^{a}	5.94^{a}	6.19^{a}	0.123	0.014
MDA (nmol/L)	28 days	15.24^{a}	15.01^{a}	13.21^{b}	14.53^{a}	0.359	0.002
	48 days	15.88^{a}	15.28^{b}	14.70^{c}	14.98^{bc}	0.218	0.004
GSH-Px (pmol/mL)	28 days	12.72^{c}	14.13^{b}	14.66^{a}	14.73^{a}	0.214	0.001
	48 days	15.22^{b}	17.56^{a}	17.67^{a}	17.99^{a}	0.211	0.001
GSH-ST (ng/L)	28 days	462.53^{c}	466.12^{c}	497.23^{b}	520.47^{a}	7.321	0.001
	48 days	471.30^{b}	476.49^{b}	481.52^{b}	510.79^{a}	8.704	0.008

CYP-Cu, *Chinese yam* polysaccharide copper complex; SEM, standard error of the means (n = 6); T-SOD, total superoxide dismutase; T-AOC, total antioxidative capacity; MDA, malondialdehyde; g GSH-Px, lutathione peroxidase; GSH-ST, glutathione s-transferase.
In the same line, values with different lowercase superscripts indicate significant differences ($P < 0.05$), and values with the same lowercase superscripts indicate no significant differences ($P > 0.05$).

Discussion

In recent years, increasing numbers of studies have shown that polysaccharide metal complexes display diverse biological activities, such as improving growth, antioxidation, immune regulation, and antibacterial activity (14, 18–21). The objective of this paper was to determine the effect of supplementation of CYP-Cu in broiler diets on growth performance, immune function, and antioxidant capacity. The treated results showed that such supplementation improved the growth and carcass performance of broiler chickens, and a dietary supplementation of 0.10 g/kg CYP-Cu was the optimal level. Cui et al. (22) reported that the supplementation of *Enteromorpha prolifera* polysaccharide-iron (III) complex (2 mg Fe/kg body weight) by intragastric administration has a considerable effect on the growth performance and blood indexes of rats with iron deficiency anemia, indicating that *Enteromorpha prolifera* polysaccharide-iron (III) complex could be used as a new iron fortifier to replace traditional iron supplements for gastrointestinal irritation and poor absorption. Another study reported the effects of pectin oligosaccharide and zinc chelate (80 mg/kg) on growth performance, antioxidant ability, and

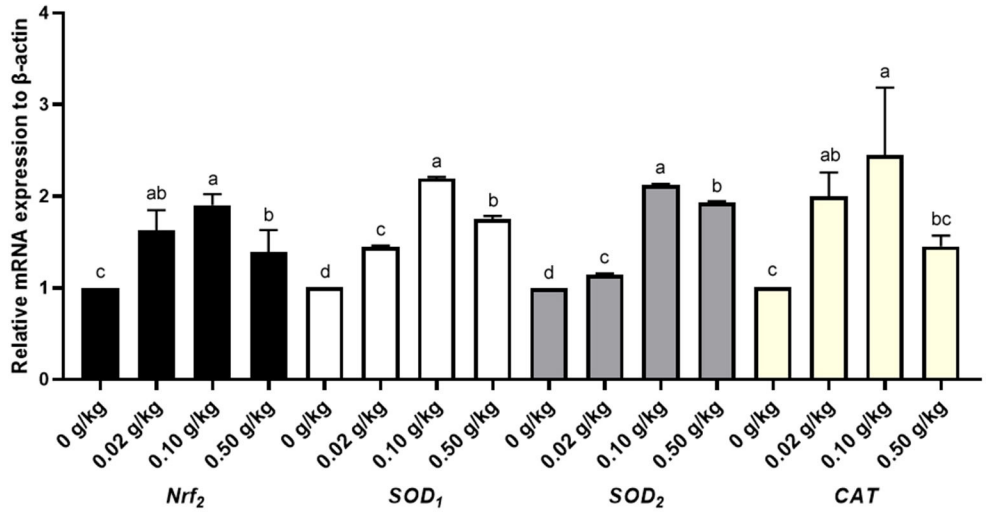

FIGURE 1
Hepatic antioxidative gene expression profiles in 48-day-old broilers. *Nrf*$_2$, nuclear factor E2-related factor 2; *SOD*$_1$, superoxide dismutase 1; *SOD*$_2$, superoxide dismutase 2; *CAT*, catalase. In the figure, antioxidative gene expression profiles of *Nrf*$_2$, *SOD*$_1$, *SOD*$_2$, and *CAT*, in the livers of 48-day-old broilers were detected by qRT-PCR. The values with different lowercase superscripts indicate significant differences ($P < 0.05$), while values with the same lowercase superscripts indicate no significant differences ($P > 0.05$).

gut function in broilers, and found that dietary supplementation with pectin oligosaccharide and zinc chelate was more effective than feeding pectin oligosaccharide and zinc sulfate alone (23). Gao et al. (20) showed that soybean polysaccharide iron complex (5.00–20.00 mg/mL) had different impacts on foodborne bacteria, promoting the growth of the beneficial bacteria, *Bacillus licheniformis*, and inhibiting the proliferation of the pathogenic bacteria, *Staphylococcus aureus*, thus improving the body's growth and immunity. These studies indicate that, compared with polysaccharide and metal alone, polysaccharide and metal complexes improve animal growth and immunity because of the synergistic interaction between polysaccharides and metals, and the enhancement of intestinal health and nutrient absorption by promoting the proliferation of beneficial bacteria to the competitive exclusion of pathogenic bacteria. Consistent with previous studies, our results showed that CYP-Cu had a positive effect on growth performance of broilers, implying a good synergy.

In the present study, the addition of CYP-Cu in diet can enhance the immunity of broilers by improving the concentrations of IgM, IgA, IgG, IL-2, IL-4, IL-6, C3, and C4 in serum at 28 days and in serum IgM, IgA, IgG, IL-2, IL-6, C3, and C4 in group 48 days, and we recommend the supplementation of 0.10 g/kg CYP-Cu in broiler diets. However, little is known about the underlying principle by which dietary CYP-Cu improves the immune performance of broilers. Gao et al. (19) demonstrated that Ulva polysaccharide iron complex (200 and 400 mg/mL) could promote the proliferation of lymphocytes, improve the activities of murine macrophages, and restore serum levels of IFN-γ, and IL-10 in immune-deficient mice to normal; furthermore, the Ulva polysaccharide iron complex exhibits excellent hematopoietic capacity. Dey et al. (24) found that chitosan conjugated green copper oxide nanoparticles (50 μg/mL) inhibited the proliferation of breast cancer and cervical cancer cells *in vivo* in a Balb/C mouse model, and increased pro-inflammatory cytokines and CD^{4+} populations; the author showed that the potential mechanism of chitosan conjugated green copper oxide nanoparticles is not only through inducing cellular immunity by activating immune cells, but they also lead to a humoral immune response through an IgG reaction. Another study reports that copper-loaded chitosan nanoparticles (100 mg/kg) in broiler diets improves the growth of poultry, and raises the serum levels of IgA, IgM, C3, and C4, suggesting that it can be used as a substitute for antibiotics (25). Although previous studies have exhibited the immune activities of polysaccharide metal complexes in animals, further research is needed to explain the underlying mechanisms by which dietary CYP-Cu improves immune performance in animals.

Free radicals, *in vivo*, are products that are inevitably generated through metabolism in a broad range of biochemical reactions; when accumulation is excessive they can lead to oxidative stress and subsequent tissue damage (26–28). Antioxidants are the substances that protect organisms against damage caused by oxidation, and they are classified into two groups, exogenous and endogenous antioxidants, according to origin. To date, increasing numbers of studies indicate that natural botanical polysaccharides are potential antioxidants (29, 30). In this paper, an evaluation about the effects of the supplementation of dietary CYP-Cu on serum antioxidative parameters of broilers demonstrated that dietary CYP-Cu significantly increased the serum concentrations of T-SOD, T-AOC, GSH-ST, GSH-Px, and decreased the serum concentrations of MDA of broilers (0.10 g/kg CYP-Cu group), which revealed that CYP-Cu had strong antioxidant activity. A previous study shown that lotus root polysaccharide iron complex could significantly increase the antioxidant capacity of mice by increasing the serum levels of CAT, SOD, and GSH-Px (31), and showed a trend of first increasing from 2 to 8 mg/mL and then decreasing. Similarly, Dong et al. (32) found that Flammulina velutipes polysacchrides and polysacchride-iron (III) complex inhibited the MDA production of health mice liver and improve its antioxidant capacity. Li et al. (33) found that novel biochanin a-chromium (III) complex enhanced the oxidative capacity of the *db*/*db* mice and improved the oxidative stress injury caused by hyperglycemia through decreasing the content of MDA,

and increasing the content of CAT, SOD, and GSH-Px in liver of mice. Our study also demonstrated that dietary CYP-Cu could improve the antioxidant capacity of broilers.

On the other hand, the current study showed that the supplementation of dietary CYP-Cu in broilers had the ability to upregulate the mRNA expression of hepatic antioxidant genes, *CAT*, Nrf_2, SOD_1, and SOD_2, and the dietary supplementation of 0.10 g/kg CYP-Cu in broilers was more effective in promoting antioxidant gene expressions. Gene expressions of antioxidant enzymes such as SOD, glutathione peroxidase, and heme oxygenase-1 are the key mechanism by which the body responds to various reactive oxygen species during inflammation, trauma, or other stressful conditions (34). SODs are a family of metalloproteins that catalyze the change of radical superoxide to oxygen and hydrogen peroxide, and protect organisms against oxidative stress. SOD_1 and SOD_2, such as Cu–Zn SOD and Mn SOD, are located in the cytoplasm and mitochondria (35, 36). Chinese wolfberry and Astragalus extract (1%) notably improved the activities of SOD_1, T-AOC, and CAT, and decreased MDA in Tibetan pig livers, and promoted the expression levels of hepatic antioxidant genes (e.g., *CAT* and SOD_1); this indicates that the extracts mechanism of regulating antioxidation is the signaling pathway of peroxisome antioxidant-oxidant stress in Tibetan pig livers (37). Another study investigated the effect of a plateau condition on oxidation in Tibetan pigs and Duroc × Landrace × Yorkshire (DLY) pigs, and found that Tibetan pigs exhibited higher SOD, GSH-Px, and T-AOC levels, but lower MDA levels in the liver and heart. Furthermore, the mRNA levels of *SOD*, *GSH-Px,* and Nrf_2 in the liver and heart of Tibetan pigs were higher than those in DLY pigs, and the authors suggest that Tibetan pigs held a stronger antioxidant activity through the AMPK/p38 MAPK/Nrf_2-ARE signaling pathways (38). Wang and Li (18) found that the Zinc-HSP (*Hohenbuehelia serotina* polysaccharides) complex (20 mg/mL) not only had superoxide anion radical scavenging ability, but also did not affect the biological activity of *Hohenbuehelia serotina* polysaccharides when combined with zinc. The dietary supplementation of *Enteromorpha prolifera* polysaccharide-zinc (EP-Zn) complex (400 mg EP-Zn/kg) in chickens upregulated the mRNA expression levels of the antioxidant genes Nrf_2, CAT_1, and SOD_1 in breast muscle and lipid metabolism genes *ACC*, $FADS_1$, *PPAR-α*, and CPT_1 in liver, thereby improving the growth performance and meat quality of chickens (39). In this paper, dietary supplementation of CYP-Cu has been shown to have effective free radical scavenging and antioxidant activities in broilers, which indicates that CYP-Cu has great potential for the application in poultry.

Conclusion

The current results showed that dietary CYP-Cu improved growth performance, serum immune function, and antioxidant capacity in broilers, and upregulated the hepatic antioxidant gene *CAT*, Nrf_2, SOD_1, and SOD_2 mRNA expression of broilers, and the supplementation of 0.10 g/kg CYP-Cu in broiler diet is recommended.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Animal Protection and Utilization Committee of Henan Institute of Science and Technology (No. 2021HIST018, Xinxiang, P. R. China).

Author contributions

JZ: design, investigation, writing—original manuscript, and editing. YJ, MC, JD, and YC: carried out the experiments and analyzed the data. MS: conceptualization, methodology, and supervision. ZM: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the Program for Innovative Research Team (in Science and Technology) in University of Henan Province (Henan Province, China; 22IRTSTHN026), and the Science and Technology Program of Henan Province (Henan Province, China; 212102110169), and the Key Scientific Research Projects of Colleges and Universities in Henan Province (Henan Province, China; 23B230003).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1123002/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 27 February 2023
DOI 10.3389/fvets.2023.1127117

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

RECEIVED 19 December 2022
ACCEPTED 06 February 2023
PUBLISHED 27 February 2023

CITATION
Yao B-n, Liao F-y, Yang J-y, Liu A, Wang J, Zhu B-g, Feng G and Yang S-l (2023) Effect of sea buckthorn extract on production performance, serum biochemical indexes, egg quality, and cholesterol deposition of laying ducks. *Front. Vet. Sci.* 10:1127117. doi: 10.3389/fvets.2023.1127117

Effect of sea buckthorn extract on production performance, serum biochemical indexes, egg quality, and cholesterol deposition of laying ducks

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The purpose of this experiment was to study the effect of sea buckthorn extract (SBE) supplementation on the production performance, serum biochemical indexes, egg quality, and cholesterol deposition of laying ducks. A total of 240 23-week-old laying ducks (female ducks) with similar body weight were randomly divided into four treatment groups with 6 replicates of 10 each. The experimental groups were fed diets supplemented with 0, 0.5, 1.0, and 1.5 g/kg of SBE, respectively. The results showed that the addition of 1.0 g/kg SBE to the diet had significant increase ($P < 0.05$) in average egg weight and feed conversion ratio. The inclusion of SBE showed the significant improvement ($P < 0.05$) in yolk weight, shell strength, egg white height and haugh unit. Ducks fed with 1.0 and 1.5 g/kg SBE displayed a significant decrease ($P < 0.05$) in yolk cholesterol. The significant improvements were observed in the contents of total amino acid essential amino acids, non-essential amino acids, umami amino acids, monounsaturated fatty acids, and docosahexenoic acids of eggs ($P < 0.05$) when supplemented with SBE. However, the contents of total saturated fatty acids, polyunsaturated fatty acids, n-3 polyunsaturated fatty acids and n-6 polyunsaturated fatty acids in eggs showed decrease when ducks fed with SBE diets ($P < 0.05$). SBE diets may reduce ($P < 0.05$) the levels of serum total cholesterol, triglyceride, and low-density lipoprotein cholesterol, while increased ($P < 0.05$) the levels of serum superoxide dismutase, total antioxidant capacity, and glutathione catalase compared to the control. The levels of serum immunoglobulin G, immunoglobulin A and immunoglobulin M were improved in SBE diets ($P < 0.05$) in comparation to the control. The addition of SBE to diets can improve feed nutrient utilization, increase egg weight, optimaze egg quality and amino acid content in eggs, reduce blood lipids, improve fatty acid profile and yolk cholesterol in eggs, and increase antioxidant capacity and immunity in laying ducks.

KEYWORDS

sea buckthorn extract, laying duck, production performance, egg quality, amino acid, fatty acid, serum biochemical indices, cholesterol deposition

1. Introduction

Meat and egg products from poultry serve as a source of food for humans, especially eggs can supply humans with high quality proteins, of which eggs are rich in many umami amino acids such as glutamic acid and glycine (1). Fatty acids in eggs are also beneficial for human health, among which n-3 polyunsaturated fatty acids in eggs are effective in preventing cardiovascular diseases (2), but the docosahexaenoic acid (DHA) content of n-3 polyunsaturated fatty acids in poultry meat and eggs is known to be very low (3). Fat and cholesterol enriched in eggs are mainly synthesized in the liver and transported *via* the blood to be deposited in eggs (4, 5). Generally, consumption of one to two eggs per day is sufficient to meet the body's dietary cholesterol requirements, and excessive intake can cause cardiovascular disease. Therefore, how to balance the nutritional structure in eggs has become a hot research topic. On the other hand, the use of antibiotics has led to the detection of antibiotic residues in a large number of livestock and poultry products, posing a safety risk to human health (6). Therefore, countries have introduced a series of regulations for the use of antibiotics, and China has explicitly prohibited the use of antibiotics in feed, and it has become a trend to find alternative antibiotic products from feed additives. It has been reported that plants and plant extracts can be used as feed additives to improve egg production performance, egg quality and immunity of chickens, and to reduce cholesterol levels (7). As everyone knows, plants are rich in many natural bioactive compounds, such as flavonoids and polysaccharides, among which mulberry leaf flavonoids can improve egg production and antioxidant capacity of chickens and improve feed conversion ratio (8).

Sea buckthorn (*Hippophae rhamnoides L.*, SBT) for the elaeagnaceae family, the genus of sea buckthorn. SBT is cold and drought tolerant and can grow in harsh environments. SBT is abundant and native to China and is distributed all over the world, including England and France (9). Feeding of SBT to broiler diets can improve feed conversion (10). SBT is a medicinal and food plant, and the flavonoids, polysaccharides, and other bioactive compounds extracted from the stems, leaves, fruits, and seeds of SBT are collectively known as Sea buckthorn extract (SBE) (11). Among them, Seabuckthorn flavonoids (SBF) has the largest proportion and is the most abundant active compound in SBT. SBE has high medicinal value and the medicinal components are isorhamnetin, quercetin, and kaempferol (12), and its medicinal functions are lipid-lowering (cholesterol, triglycerides, etc.) (13), antioxidant (14), anticancer, and immunomodulation (15), etc. It has been reported that SBT and SBE were widely used as feed additives in pig (16), cattle (17), chicken (18), and other production tests. To our knowledge, few studies on effect of SBE in ducks have been reported, especially, little is known about the study of amino acids, fatty acid profile, and egg quality in duck eggs.

Guangxi small hemp duck is a local Chinese poultry breed, produced in Guangxi, China, and is an egg-laying duck with good egg production performance. However, duck eggs contain higher levels of cholesterol and fat than chicken eggs and quail eggs, and humans are concerned about the health risks associated with high fat and cholesterol levels when consuming them. Therefore, we hypothesized that the addition of SBE to the diet could improve the production performance of laying ducks, improve egg quality, improve the amino acid and fatty acid structure of eggs, and reduce egg cholesterol. The aim of this study was to investigate the effect of SBE on production performance, serum biochemical parameters, egg quality, amino acid, fatty acid profile of egg, and cholesterol deposition during the laying period, and to provide a theoretical basis for the development of SBE as a functional feed additive for laying ducks.

2. Materials and methods

This experiment was reviewed and approved by the Guizhou University Sub-Committee of Experimental Animal Ethics (Guiyang, China; No. EAE-GZU-2021-E012).

2.1. Experiment material

The raw material of sea buckthorn extract (powdered form) used in the experiment was sea buckthorn fruit, which was produced in Shaanxi, China. The active ingredient of SBE was detected by high performance liquid chromatography-mass spectrometry, and the extractant was alcohol. 286.23 mg/g of flavonoids, 103.79 mg/g of quercetin, and 23.66 mg/g of kaempferol constituted the main components of SBE, and the purity of SBE was 40.31%. Another 20.18% was sea buckthorn polysaccharide. Produced by a company in Shanxi, China.

2.2. Experimental design

This experiment was carried out in the research duck farm of Guizhou University from September 2021 to December 2021. The experimental ducks, called Guangxi small hemp duck, were purchased from a farm in Guangxi, China, A total of two hundred and forty 23-week-old laying ducks (female ducks) with similar body weight (BW, 1,250 ± 60 g; mean ± standard deviation) were applied and randomly divided into four groups, every group contained six replicates of 10 ducks each. The experimental diets were supplemented with 0.5, 1.0, and 1.5 g/kg SBE, respectively. The basal rations were formulated according to the Criterion of Nutrients Requirements of laying ducks (SAC, GBT/41189-2021; China, 2021). The basal diet formula and nutritional level are shown in Table 1. Nutrients in the diets were tested using the AOAC method (19). The adaptation period of this experiment was 14 days, and the formal experimental period was 70 days. During the experiment, the ducks were provided with natural and artificial lighting to ensure 16 h of light each day, and the relative humidity was kept at about 65%. Feeding and drinking water freely throughout the feeding period, and the duck house was cleaned once a day.

TABLE 1 Basic diet formula and nutrition level.

Ingredients, %	Content, %
Corn	55.75
Soybean meal	27.40
Wheat bran	1.50
Rapeseed cake	4.00
$CaHPO_4$	2.75
Limestone	7.25
NaCl	0.35
Premix[a]	1.0
Total	100
Nutrient levels[b]	
Metabo lizable energy, MJ/kg	10.65
Crude protein	18.10
Crude fiber	3.07
Calcium	3.37
Total phosphorus	0.63
Lysine	0.92
Methionine	0.27
Methionine + cysteine	0.61

[a]The premix provided the following per kg of diets: retinol 4,000 IU; oryzanin 1.17 IU; pyridoxol 3.05 IU; cobalamin 0.01 IU; cholecalciferol 900 IU; tocopherol 20 IU; menadione 2 mg; biotin 0.1 mg; folic acid 1.0 mg; pantothenic acid 10 mg; nicotinic acid 50 mg; Cu 10 mg as copper sulfate; Fe 80 mg as ferrous sulfate; Mn 60 mg as manganese sulfate; Zn 60 mg as zinc sulfate; I 0.40 mg as potassium iodide; Se 0.20 mg as sodium selenite.
[b]Crude protein and crude fiber are measured values, while the others were calculated values.

2.3. Sample collection and index determination

2.3.1. Production performance

The daily feed intake was recorded according to replicates during the experiment. The ducks were fed at 7:30 and 17:00 daily, and duck eggs were collected every morning before feeding, numbered and weighed. The egg-laying ducks were weighed every Sunday at 8:00 (fasting ducks for 12 h before weighing). At the same time, the number of deaths of laying ducks was recorded for calculating the average daily feed intake [ADFI (g/d) = cumulative feed intake/(number of birds × number of days)], average egg weight (AEW = total daily egg mass/laying number), laying rate [LR (%) = (laying number/layer number) × 100], and feed conversion ratio [FCR = total feed intake/total egg weight] of the laying ducks. Used to calculate the production performance of egg ducks.

2.3.2. Serum biochemical indices

On day 70 of the experiment, 2 laying ducks with similar body condition were randomly selected from each replicate (12 laying ducks in each group) for blood collection, and the ducks drank freely and fasted for 12 h before blood collection. The blood samples were collected from the vein under the wing of common collecting blood vessel for 5 mL, left at room temperature for 2 h, centrifuged at 1,487 xg/min for 15 min, and the serum was extracted, stored at −80°C for determination of serum biochemical parameters. Serum biochemical indicators include total cholesterol (TC, COD-PAP method), triglyceride (TG, GPO-PAP enzymatic method), high-density lipoprotein cholesterol (HDL-C, direct method), low-density lipoprotein cholesterol (LDL-C, direct method), glutathione catalase (GSH-Px, colorimetric method), total superoxide dismutase (SOD, extraction method), total antioxidant capacity (T-AOC, ratio of Color method), malondialdehyde (MDA, TBA method). Determination of duck serum immunoglobulin G (IgG, Elisa method), immunoglobulin A (IgA, Elisa method), and immunoglobulin M (IgM, Elisa method) using Elisa kits. The kit was sourced from Nanjing Jiancheng Bioengineering Research Institute Co., Ltd., China, and the assay methods and steps were operated in accordance with the kit instructions. The detection instrument used in the experiment was PowerWaveXS type full-wavelength microplate reader (Bio-tek Instruments.Inc.USA).

2.3.3. Egg quality index

On the 69th day of the experiment, three fresh duck eggs with similar morphology were randomly selected in each replicate for egg quality determination (18 eggs per group), the following indicators were measured according to the method of "Performance ferms and measurement for poultry" (NY/T 823-2004) (20). The measuring instruments are listed as follows: Egg weight (DJ-A1000 electronic balance, Connecticut HZ Electronics Co., Ltd., USA), egg shell strength (EFA-01 egg shell strength tester, Orka, Israel), egg shell thickness (MNT-150T digital Vernier caliper, Shanghai Minette Industrial Co., Ltd., China), egg shape index (calculated by vernier caliper), egg yolk specific gravity (calculated by electronic balance weighing), egg white height (EA-01 egg quality tester, Orka, Israel), egg yolk Color (EA-01 egg quality tester, Orka, Israel), Haugh Units (EA-01 egg quality tester, Orka, Israel).

Three fresh duck eggs were randomly selected from each replicate for the determination of yolk cholesterol on the 69th day of the experiment. First, break the fresh duck egg to separate the yolk, weigh 1 g of egg yolk in the middle of the egg yolk and place it in a 10 mL centrifuge tube, add 10 mL of anhydrous ethanol, and mix thoroughly to obtain the sample to be tested, which is used to determine the total protein and total cholesterol of the egg yolk, and then the cholesterol content of the egg yolk was calculated using the formula. Determination formula: Yolk cholesterol (mmol/gprot) = $(A_{sample}\text{-}A_{blank})/(A_{calibration}\text{-}A_{blank})\times C_{calibrators}\times C_{standard}/(W/V)$, ($A_{sample}$, sample OD value; $A_{calibration}$, calibrate the OD value; A_{blank}, blank OD value; $C_{calibrators}$, calibrator concentration, mmol/L; $C_{standard}$, protein concentration of the sample to be tested, gprot/L; W, sample quality, g; V, the total volume of ethanol added, L; prot, protein.). Wavelengths of 570 and 440 nm.

TABLE 2 Effect of dietary SBE on production performance of laying ducks[a].

Items[b]	SBE add levels, g/kg				SEM	*P*-value
	Control	0.5	1.0	1.5		
ADFI, g/d	131.850	131.857	131.478	131.991	0.085	0.927
AEW, g	61.891[b]	63.743[ab]	64.110[a]	61.391[b]	0.402	0.033
LR	0.7373	0.7504	0.7385	0.7349	0.008	0.927
FCR	2.718[a]	2.585[b]	2.501[bc]	2.710[ab]	0.017	0.044

[a]Different letters within a row denote significant differences ($P < 0.05$).
[b]Values represent the mean of six replicates ($n = 30$).
[c]ADFI, average daily feed intake; AEW, average egg weight; LR, laying rate; FCR, feed conversion ratio.

2.3.4. Detection of amino acids and fatty acids in eggs

On the 70th day of the experiment, three fresh duck eggs were randomly selected from each replicate (18 duck eggs in each group, fully mixed, and three samples taken for testing). At first, the fresh duck eggs were dried in a vacuum freeze-dryer (LYOQUEST-85PLUS, Telstar Electromechanical Equipment Shanghai Co., Ltd., China) until Constant weight was reached, secondly, the egg powder samples were obtained by grinding with high-speed multi-function grinder (model 800Y, Wuyi County Hainer Electric Co., Ltd., China).

Amino acids in whole eggs were analyzed according to the national standard for food safety GB/T5009.124-2016 (21). The brief steps are as follows: add 10–15 mL 6 mol/L hydrochloric acid solution and 4 drops of phenol into the hydrolysis tube, after freezing the hydrolysis tube for 5 min and then connected to the suction tube of the vacuum pump, evacuated (close to 0 Pa), filled with nitrogen gas. The vacuum-nitrogen filling step was repeated three times. The sealed tube was hydrolyzed in a 110°C hydrolysis furnace for 22 h, then cooled to room temperature and detected by an automatic amino acid analyzer (Biochrom 30, Biochrom Ltd., UK) at wavelengths of 570 and 440 nm. The 16 amino acids are as follows: aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), glycine (Gly), alanine (Ala), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), Lysine (Lys), arginine (Arg), and proline (Pro). All experimental steps were completed in strict accordance with the standard instructions, and the difference of the measurement results did not exceed 12% of the arithmetic mean.

The fatty acids in whole eggs were analyzed according to GB5009.168-2016 (22), the national standard for food safety. Brief steps are as follows: weigh 2 g of sample into a 50 mL test tube, add 20 mL of chloroform and 10 mL of methanol, sonicate for 10 min, and shake for 2 h. Add 6 mL of 0.9% sodium chloride aqueous solution, shake for 30 s, and let stand at 4°C for 22 h, then centrifuge at 3,500 r/min for 10 min, collect the lower chloroform layer solution, filter with filter paper. The filtrate was placed in a dry flask and dried in a vacuum drying oven. The single fatty acid methyl ester standard solution and the fatty acid methyl ester mixed standard solution were injected into the gas chromatograph separately to characterize the peaks. The individual fatty acids were analyzed by gas chromatograph (Agilent GC 6890N, Agilent, USA). The gas chromatographic conditions were as follows: the capillary column was a poly (dicyanopropyl siloxane) strongly polar stationary phase (100 m × 0.25 mm × 0.2 μm). The injector temperature was set at 270°C; the detector temperature was set at 280°C. The initial temperature program was 100°C for 13 min, 10°C/min to 180°C, duration 6 min, 1°C/min to 200°C, duration 6 min, and 4°C/min to 230°C, duration 6 min. The carrier gas is nitrogen, the split ratio was 100:1, and the injection volume was 1.0 μL. All experimental steps were performed in strict accordance with the standard requirements, and the difference of the measurement results did not exceed 10% of the arithmetic mean.

2.4. Statistical analysis

All data were analyzed by analysis of variance using the general linear model program of SPSS 25 (One-way ANOVA, LSD), and Duncan's multiple comparison test was used. A p-value of <0.05 was considered statistically significant. The experimental results of each group were expressed using the mean and standard error of the mean (SEM).

3. Results

3.1. Production performance

The effect of dietary SBE on production performance is listed in Table 2, the addition of 1.0 g/kg SBE significantly increased ($P < 0.05$) the average egg weight and feed conversion ratio of laying ducks. However, the addition of SBE to the diet had no significant ($P > 0.05$) effect on the average daily feed intake and laying rate of laying ducks. Although the difference in egg production rate was not significant, it can be found from Figure 1A that feeding 0.5 g/kg of SBE had a tendency to increase egg production rate in the middle part of the experiment, indicating that SBE has some effect in increasing egg production rate of ducks. Combined with the Figure 1B, the average daily feed intake was generally not very different between groups and the trend was not obvious.

3.2. Serum biochemical indices

Effect of dietary SBE on serum biochemical indexes is presented in Table 3, compared to the control group the addition of 1.0 and

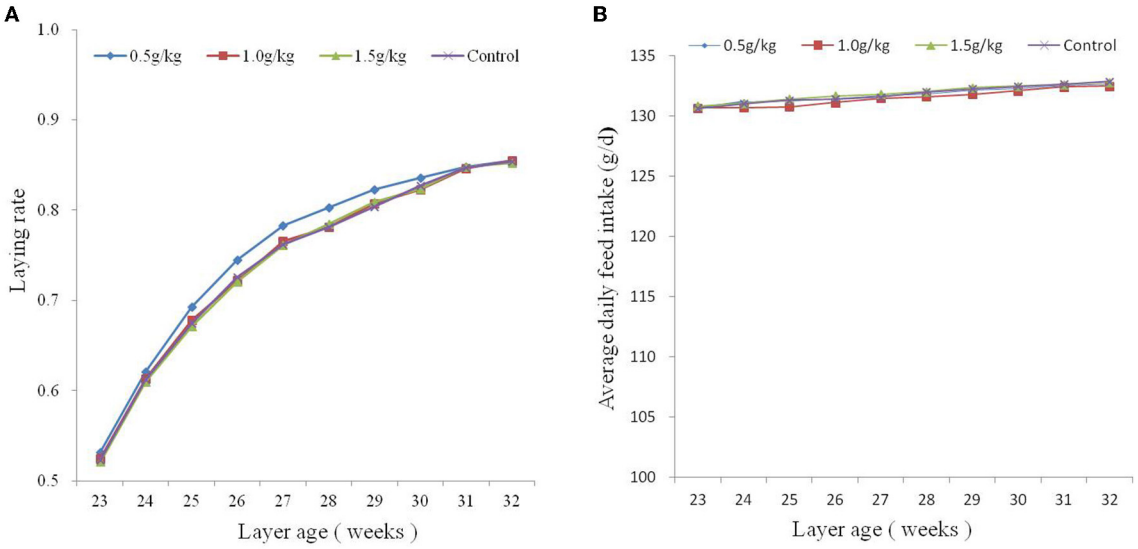

FIGURE 1
Plots of average daily feed intake **(A)** and laying rate **(B)** from 23 to 32 weeks of age.

TABLE 3 Effect of dietary SBE on serum biochemical indexes of laying ducks[a].

Items[b]	SBE add levels, g/kg				SEM	*P*-value
	Control	0.5	1.0	1.5		
Serum lipid index						
TC, mmol/L	4.074[a]	3.434[ab]	3.235[b]	3.301[b]	0.144	0.014
TG, mmol/L	4.040[a]	3.126[ab]	2.161[b]	2.762[b]	0.226	0.019
HDL-C, mmol/L	3.208	3.520	3.975	3.353	0.122	0.181
LDL-C, mmol/L	5.824[a]	5.313[ab]	4.893[ab]	3.787[b]	0.331	0.001
Serum antioxidant index						
SOD, U/mL	81.781[b]	89.478[a]	88.597[a]	85.619[ab]	0.936	0.046
T-AOC, U/mL	2.282[b]	2.544[b]	3.679[a]	3.402[a]	0.157	0.002
MDA, nmol/mL	8.824	10.441	9.471	8.721	0.518	0.546
GSH-Px, U/L	136.559[b]	155.544[a]	147.401[ab]	140.396[b]	2.502	0.043
Serum immunity index						
IgG, g/L	1.807[b]	2.342[a]	1.543[c]	1.618[bc]	0.065	<0.001
IgA, g/L	0.220[b]	0.251[a]	0.158[d]	0.185[c]	0.007	<0.001
IgM, g/L	0.384[b]	0.537[a]	0.283[c]	0.238[d]	0.019	<0.001

[a]Different letters within a row denote significant differences ($P < 0.05$).
[b]Values represent the mean of six replicates ($n = 12$).
[c]TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; MDA, malondialdehyde; GSH-Px, glutathione catalase; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M.

1.5 g/kg SBE significantly reduced ($P < 0.05$) the serum TC and TG levels, while serum GSH-Px and SOD levels were significantly higher ($P < 0.05$), and 1.5 g/kg SBE group significantly reduced ($P < 0.05$) levels of serum LDL-C. However, 0.5 g/kg SBE group significantly increased ($P < 0.05$) levels of serum T-AOC, IgG, IgA, and IgM than control group. No significant ($P > 0.05$) effect on the levels of HDL-C and MDA were detected among all groups.

3.3. Egg quality

Effect of dietary SBE on egg quality is given in Table 4, Compared to the control group, supplementation with 0.5 g/kg SBE to the diet significantly increased ($P < 0.05$) the egg shell strength of duck eggs, adding of 0.5 and 1.0 g/kg SBE significantly increased ($P < 0.05$) the yolk weight of duck eggs, and 1.5 g/kg SBE group significantly increased ($P < 0.05$) the egg white height

TABLE 4 Effect of dietary SBE on egg quality of laying ducks[a].

Items[b]	SBE add levels, g/kg				SEM	*P*-value
	Control	0.5	1.0	1.5		
Average egg weight, g	64.661[b]	65.154[b]	68.383[a]	64.476[b]	0.356	<0.001
Egg-shaped index	1.357	1.368	1.361	1.351	0.006	0.789
Eggshell strength, kgf/cm²	45.517[b]	53.419[a]	48.506[ab]	47.489[b]	0.988	0.037
Eggshell thickness, mm	0.231	0.234	0.239	0.231	0.002	0.515
Yolk weight g	19.001[c]	20.544[ab]	20.846[a]	19.714[bc]	0.182	0.001
Yolk specific gravity	0.300	0.309	0.310	0.303	0.002	0.090
Albumen height, mm	6.293[b]	6.444[ab]	6.473[ab]	6.867[a]	0.103	0.036
Yolk color	11.278	11.222	11.444	10.611	0.140	0.167
Haugh unit	75.993[b]	76.750[b]	77.479[ab]	80.571[a]	0.613	0.041
Yolk cholesterol, mmol/gprot	0.516[a]	0.459[ab]	0.416[b]	0.444[b]	0.012	0.026

[a]Different letters within a row denote significant differences ($P < 0.05$).
[b]Values represent the mean of six replicates (n = 18).

and haugh unit of duck eggs. In addition, feeding 1.0 and 1.5 g/kg SBE significantly reduced ($P < 0.05$) yolk cholesterol. However, SBE did not affect ($P > 0.05$) the egg shape index, shell thickness, yolk color and yolk specific gravity of duck eggs in comparison with the control group.

3.4. Amino acids in eggs

The effect of addition of SBE to the diet on amino acids in eggs is listed in Table 5. The inclusion of SBE significantly increased ($P < 0.05$) the content of essential amino acids and total amino acids compared with the control group, while the content of non-essential amino acids in eggs was significantly increased ($P < 0.05$) when adding of 0.5 and 1.0 g/kg SBE. In addition, SBE significantly increased ($P < 0.05$) the contents of threonine, serine, leucine, phenylalanine, histidine, lysine, aspartic acid, and methionine in eggs. Interestingly, the addition of 0.5 and 1.0 g/kg of SBE significantly increased ($P < 0.05$) the content of glutamic acid and tyrosine in the fresh tasting amino acids of eggs. SBE did not affect ($P > 0.05$) the content of arginine in eggs.

3.5. Fatty acid profile in eggs

The effect of addition of SBE to the diet on fatty acids in eggs is listed in Table 6. Compared to the control group, feeding of 1.0 g/kg SBE significantly reduced ($P < 0.05$) the content of saturated fatty acids (SFA) in eggs. In addition, duck eggs with SBE displayed significantly decrease ($P < 0.05$) in the contents of pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), heneicosan ic acid (C21:0), elaidic acid (C18:ln9t), heptadecenoic acid (C17:1), linoleic acid (C18:2n6c), linolelaidic acid (C18:2n6t), eicosadienoic acid (C20:2), α-linolenic acid (ALA: C18:3n3), docosapentaenoic acid (DPA: C20:5), and eicosatrienoic acid (C20:3n6). However, the contents of total MUFA of eggs in SBE groups were higher than ($P < 0.05$) that in the control group. Among them, the contents of oleic acid (C18:ln9c) and gondoic acid (C20:ln9) in MUFA were significantly increased ($P < 0.05$). Although SBE reduced the content of total polyunsaturated fatty acids (PUFA), but significantly increased ($P < 0.05$) the content of docosahexaenoic acid (DHA: C22:6n-3) when adding 1.0 and 1.5 g/kg SBE to the diets. In contrast, SBE had no obvious ($P > 0.05$) effect on the content of lauric (C12:0) and myristic (C14:0) acids in eggs.

4. Discussion

In laying duck farming production, economic efficiency is usually increased by increasing the average egg weight or improving the feed conversion ratio. Egg white is one of the main factors affecting the weight of eggs. Egg white are reported to consist of ovalbumin and oval mucin, and are secreted and synthesized in the enlarged portion of the oviduct (also known as the protein-secreting portion) (23). The synthesis and secretion of ovalbumin is regulated by estrogens (24). Among them, yolk protein is the main protein in egg yolk, and estrogen induces the synthesis of yolk protein (25). It has been reported that quercetin in flavonoids can increase the synthesis of estrogen (26), thus regulate yolk protein synthesis, increasing yolk and egg white weights, and it is noteworthy that the increased yolk weight in this study was an important cause of the increased egg weight. Chand et al. (27) confirmed that the addition of sea buckthorn seeds to the ration increased the weight of eggs. However, SBE does not consistently increase egg weight, and one study showed that estrogen promotes calcium absorption (28), but excessive flavonoids inhibit estrogen production (29), therefore, the lack of calcium leads to egg weight loss. There was no significant difference in average daily feed intake between the groups in this study, however, the increase in egg weight led to a decrease in feed conversion ratio, this was confirmed by the findings of BenMahmoud et al. (10).

TG in egg yolk is synthesized by the liver, transported to the ovary *via* the bloodstream, and absorbed into the developing follicle *via* receptor-mediated endocytosis (4). Studies have shown that

TABLE 5 Effect of dietary SBE on amino acids in eggs[a].

Items, %[b]	SBE add levels, g/kg				SEM	*P*-value
	Control	0.5	1.0	1.5		
Asparagine*	3.852[b]	4.088[a]	4.176[a]	4.040[a]	0.040	0.005
Threonine	2.450[b]	2.587[a]	2.629[a]	2.619[a]	0.026	0.019
Serine	3.328[b]	3.464[a]	3.494[a]	3.444[a]	0.024	0.032
Glutamic acid*	5.786[b]	6.197[a]	6.192[a]	5.803[b]	0.065	0.001
Glycine*	1.431[b]	1.477[ab]	1.525[a]	1.510[a]	0.013	0.010
Alanine*	2.117[b]	2.127[b]	2.222[a]	2.176[ab]	0.015	0.025
Valine	2.640[c]	2.734[bc]	2.795[ab]	2.826[a]	0.024	0.004
Methionine	1.308[c]	1.728[a]	1.386[b]	1.674[a]	0.055	<0.001
Isoleucine	1.982[c]	2.013[bc]	2.093[a]	2.067[ab]	0.015	0.012
Leucine	3.524[b]	3.665[a]	3.731[a]	3.695[a]	0.029	0.027
Tyrosine*	2.010[b]	2.190[a]	2.151[a]	2.075[b]	0.024	0.005
Phenylalanine*	3.182[b]	3.334[a]	3.390[a]	3.334[a]	0.028	0.015
Histidine	0.965[b]	1.009[a]	1.030[a]	1.013[a]	0.009	0.025
Lysine	3.078[b]	3.214[a]	3.286[a]	3.237[a]	0.027	0.011
Argnine	2.371	2.428	2.476	2.461	0.016	0.089
Proline	1.634[b]	1.670[b]	1.755[a]	1.718[ab]	0.009	0.009
TAA	41.660[b]	43.926[a]	44.331[a]	43.693[a]	0.363	0.011
EAA	19.130[b]	20.284[a]	20.341[a]	20.465[a]	0.185	0.007
NEAA	22.530[c]	23.642[ab]	23.990[a]	23.228[bc]	0.192	0.011
UAA	18.379[c]	19.414[ab]	19.656[a]	18.939[bc]	0.168	0.006
EAA/TAA	45.92	46.18	45.88	46.84		
EAA/NEAA	84.91	85.80	84.79	88.10		

[a]Different letters within a row denote significant differences ($P < 0.05$).
[b]Values represent the mean of six replicates ($n = 3$).
[c]TAA, total amino acids; EAA, essential amino acids; UAA, umami amino acids; EAA, threonine + valine + methionine + isoleucine + leucine + phenylalanine + lysine; UAA, asparagine + glutamic acid + glycine + alanine + tyrosine + phenylalanine.
*Belong to umami amino acids.

flavonoids can downregulate several adipogenic gene transcription factors, thereby reducing TG levels (30), and Yang et al. (31) showed that SBF can reduce serum TG levels. The source of TC is mainly through two routes: *in vivo* synthesis and dietary intake, with *in vivo* synthesis being mainly by the liver and, to a lesser extent, by the ovaries. Dietary intake is obtained through food. In addition to the TC required for the maintenance of the body, the remaining 2/3 of the TC in female birds is transported by the carrier LDL-C through the blood to the ovary, where it enters the follicle through receptor-mediated endocytosis, is deposited in the yolk, and is finally excreted by egg laying (30). During the synthesis of TC, HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase serves as a key rate-limiting enzyme in the TC synthesis pathway, and SBF inhibits the synthesis of HMG-CoA reductase, thereby inhibiting TC synthesis (32). Some studies have confirmed that consumption of sea buckthorn fruit flavonoids can reduce blood TG and TC levels (18). In the current study, addition of 1.5 g/kg SBE, reduced serum LDL-C levels, which is consistent with the results of Krejcarová et al. (33) and Ma et al. (34), confirming the ability of SBE to reduce lipids. This indicates that the addition of SBE to the diet can reduce cholesterol deposition in Egg.

Studies have demonstrated that quercetin can reduce oxidative stress in follicular granulosa cells and ensure normal ovarian development (35). Moreover, antioxidants can delay ovarian decline and increase the useful life of laying hens (36). The metabolism of the body is accompanied by an oxidative process that generates free radicals along with the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are harmful to the body, such as superoxide and hydrogen peroxide, and the body maintains the oxidative and antioxidant balance by scavenging free radicals (37). And the antioxidant effect is by enhancing the activity of antioxidant enzymes and inhibiting the activity of related oxidative enzymes, SOD and GSH-Px among antioxidant enzymes can induce the production of ROS scavenging enzymes. Studies have shown that SBF regulates Peroxidase through the Nrf_2-ARE (nuclear related factor 2-antioxidant response element) signaling pathway, and SOD is

TABLE 6 Effect of dietary SBE on fatty acids in eggs[a].

Items, %[b]	SBE add levels, g/kg				SEM	*P*-value
	Control	0.5	1.0	1.5		
Saturated fatty acid						
Lauric acid, C12:0	0.019	0.016	0.017	0.016	0.0006	0.115
Myristic acid, C14:0	0.420	0.456	0.432	0.468	0.009	0.159
Myristoleate, C15:0	0.028[a]	0.022[b]	0.021[c]	0.020[c]	0.0009	<0.001
Palmitate, C16:0	23.686[b]	23.577[b]	23.618[b]	24.203[a]	0.086	0.005
Margaric acid, C17:0	0.097[a]	0.069[b]	0.066[b]	0.057[c]	0.005	<0.001
Stearic acid, C18:0	5.909[a]	5.849[a]	5.320[b]	5.267[b]	0.094	<0.001
Arachidic acid, C20:0	0.036[a]	0.037[a]	0.032[b]	0.026[c]	0.001	<0.001
Heneicosanoic acid, C21:0	0.016[b]	0.019[a]	0.019[a]	0.019[a]	0.0005	0.041
Behenic acid, C22:0	0.279[b]	0.303[a]	0.281[b]	0.284[b]	0.003	0.001
lignoceric acid, C24:0	0.041[a]	0.040[a]	0.033[b]	0.034[b]	0.001	<0.001
Monounsaturated fatty acid						
Myristoleate, C14:1	0.034[b]	0.042[b]	0.041[b]	0.059[a]	0.003	0.001
Palmitoleate, C16:1	2.675[bc]	2.474[c]	2.704[b]	2.997[a]	0.067	0.001
Heptadecenoic acid, C17:1	0.078[a]	0.067[bc]	0.070[b]	0.065[c]	0.002	0.001
Elaidic acid, C18:ln9t	0.208[a]	0.075[c]	0.163[b]	0.137[b]	0.020	<0.001
Oleic acid, C18:ln9c	55.165[c]	56.338[b]	57.082[a]	56.357[b]	0.225	0.001
Eicosenoic acid, C20:ln9	0.020[c]	0.235[a]	0.125[b]	0.105[b]	0.044	<0.001
Polyunsaturated fatty acid						
Linolelaidic acid, C18:2n6t	0.083[a]	0.006[c]	0.056[b]	0.064[b]	0.011	<0.001
Linoleic acid, C18:2n6c	9.162[a]	8.322[b]	7.834[bc]	7.585[c]	0.197	0.001
Linolenic acid methyl ester, C18:3n3 (ALA)	0.370[a]	0.105[c]	0.216[b]	0.193[b]	0.044	<0.001
Methyl linolenate, C18:3n6	0.147[b]	0.145[b]	0.165[a]	0.164[a]	0.003	<0.001
Eicosadienoic acid, C20:2	0.190[a]	0.184[b]	0.174[c]	0.142[d]	0.006	<0.001
Eicosatrienoic acid, C20:3n6	0.211[a]	0.136[bc]	0.118[c]	0.164[b]	0.021	<0.001
Arachidonic acid, C20:4n6	1.357[a]	1.261[b]	1.152[c]	1.307[ab]	0.025	0.001
Diphenylamine, C22:5n3 (DPA)	0.150[a]	0.103[b]	0.104[b]	0.097[b]	0.006	<0.001
Docosahexaenoic Acid, C22:6n3 (DHA)	0.037[b]	0.036[b]	0.089[a]	0.090[a]	0.012	<0.001
Total SFAs	32.941[b]	32.773[b]	32.458[c]	33.319[a]	0.102	0.001
Total MUFAs	63.729[b]	65.005[a]	65.343[a]	64.851[a]	0.105	0.003
Total PUFAs	9.435[a]	8.513[b]	8.064[bc]	7.790[c]	0.036	0.001
Total n-3 PUFAs	0.557[a]	0.245[c]	0.410[b]	0.379[b]	0.053	<0.001
Total n-6 PUFAs	10.961[a]	9.871[b]	9.325[b]	9.283[b]	0.020	0.001

[a]Different letters within a row denote significant differences ($P < 0.05$).
[b]Values represent the mean of six replicates ($n = 3$).
[c]Among them, n-3 PUFA includes C18:3n3, C22:6n3 and DPA; n-6 PUFA includes C18:2n6c C18:2n6t C18:3n6, C20:3n6 and C20:4n6.

responsible for the breakdown of superoxide anions into H_2O_2 and O_2. GSH-Px further reduces the active peroxide to harmless alcohol and water (38). T-AOC reflects the body's ability to resist oxidation. In the present study, SBE could increase the content of SOD, GSH-Px, and T-AOC in serum, which indicated that SBE may improve the anti-oxidation ability of laying ducks and slow down the damage of oxidative stress. However, the content of SOD and GSH-Px in serum were decreased with further addition of SBE, indicating that excessive SBE could not further improve the antioxidant capacity of laying ducks.

The immunity of the organism is related to immune factors. Sea buckthorn fruit flavonoids improve the immunity of the organism

by modulating immune-related regulatory factors *in vitro* and stimulating pro-inflammatory factors (IL-6, interleukin-6) and tumor necrosis factor (TNF-α, tumor necrosis factor-α) (39). The intestine is the largest immune organ of the animal organism (40). Attri et al. (41) found that sea buckthorn juice increased the diversity of Lactobacillus and Bacteroides in the colonic site, and a substantial increase in probiotics such as Bifidobacterium was found in the descending colonic site, and Bifidobacterium inhibited harmful bacteria, improve gastrointestinal barrier function, maintain intestinal microecological stability, and regulate intestinal immune homeostasis (42). Bifidobacteria can also promote the growth of B lymphocytes and regulate immune function (43). Organismal immunoglobulins are mainly composed of IgG, IgA, and IgM and are synthesized and secreted by B lymphocytes (44). It can be speculated that SBE may promote the growth of B lymphocytes by increasing the number of intestinal bifidobacteria, thereby increasing the content of IgG, IgA, and IgM in serum immunoglobulins. In this study, the serum IgG, IgA, and IgM contents of ducks were significant at 0.5 g/kg SBE addition, but with the increase of SBE addition, the IgG, IgA, and IgM contents decreased instead, probably because the high SBE addition changed the intestinal microbial structure of ducks in a direction unfavorable to the improvement of immunity, and even decreased immunity. In addition, methionine and cysteine have the effect of enhancing immune function (45), and the addition of SBE in this study increased the content of methionine and cysteine in eggs, which in turn improved the immunity of laying ducks.

Eggshell strength, egg white height, and haugh units are important indicators for evaluating egg quality. Calcium has a great influence on eggshell indicators, and increasing calcium absorption can improve eggshell strength and eggshell thickness (46). Estrogen has been shown to promote calcium absorption (28), the intestine and kidney contain a large number of estrogen receptors (47). When flavonoids bind to estrogen receptors on the small intestine and kidney, they promote calcium absorption in the small intestine and calcium reabsorption in the kidney. Flavonoids can improve eggshell strength by modulating estrogen and thus calcium metabolism (48). In the current study, supplementation with SBF to the ration could improve the eggshell strength of duck eggs, excess flavonoids inhibited estrogen synthesis (29), were responsible for the decrease in eggshell strength. A previous study showed that antioxidant properties are critical to maintaining the antioxidant protection of the oviduct during eggshell formation (49), so improving the antioxidant capacity is also an important reason for the improvement of the eggshell strength. Therefore, the amount of SBE added to the ration should not be too high in production. In addition SBF can also increase the synthesis and secretion of egg mucin by regulating estrogen (50), and β-ovalmucin in eggmucin combines with O-glycoside carbohydrates to form a gel structure that makes egg white sticky and directly increases egg white height (51). Egg white height was positively correlated with haugh unit, and increased egg white height in this study led to increased haugh units.

In addition to the apparent egg quality, the content and type of amino acids and fatty acids in duck eggs are also important indicators of egg quality. It has been shown that genistein flavonoids activate MAPK signaling pathway (activating Ser/Thr-protein kinase) and up-regulate insulin signaling pathway and glycolysis process in chicken liver to promote the conversion of glucose to amino acids (52), thus it was hypothesized that the addition of SBF to the diet could increase the TAA content in eggs. The higher the UAA content in the egg, the better the egg taste. When the expression of umami substance regulatory genes was high, the content of umami substance in meat also increased (53). In addition, antioxidants also reduced the loss of UAA, and it was speculated that SBE could regulate the expression of umami substance genes, which needed further verification. In the present study, the total UAA content in the eggs increased, especially the content of Asp, Glu, and Tyr increased significantly. The highest UAA content was found when 1.0 g/kg SBE was added, and the UAA content decreased when 1.5 g/kg SBE was added. Therefore, the addition of SBE could increase the content of EAA in duck eggs, and the moderate amount of SBE may increase the content of TAA, NEAA, and UAA in duck eggs, while the excessive amount of SBE may result in the decrease of content of TAA, NEAA, and UAA in duck eggs.

Most fatty acids in birds are synthesized and metabolized by the liver (54) and deposited in eggs *via* blood transport. In this study, the levels of SFA, PUFA, n-3 PUFA, and n-6 PUFA were reduced in eggs. On the one hand, peroxisome proliferator-activated receptor-alpha (PPARα) are important transcription factors for hepatic fatty acid metabolism (55), SBF can up-regulate the expression of PPARα (56), promote fatty acid oxidation and inhibit fatty acid synthesis (57), thus reducing fatty acid content (58). Additionally, flavonoids inhibit fatty acid synthase (FAS) activity to reduce fatty acid synthesis (59), thus suggesting that SBE reduces fatty acid content in eggs. Furthermore, fatty acids are the main component of TG (60), and in this study, SBF reduced serum TG levels, which resulted in lower fatty acid levels in eggs. In the present study SBE reduced the content of ALA and DPA in eggs, but interestingly inclusion of 1.0 and 1.5 g/kg SBE increased the content of DHA in eggs, which was 2.4 times higher than the control group. It has been shown that the human body can convert ALA to DHA by prolonging enzymes and desaturases (3), and DPA in laying hens can be efficiently converted to DHA (61). It is speculated that SBE may promote the conversion of ALA and DPA to DHA in laying ducks.

5. Conclusion

The addition of 1.0 g/kg SBE to the diet may increase the egg weight of laying ducks, improve the utilization of feed nutrients. Appropriate addition of SBE to the diet can improve the quality of eggs and the content of amino acids in eggs. SBE could also reduce blood lipids and yolk cholesterol, improve the fatty acid profile of eggs, and increase the antioxidant capacity and immunity of laying ducks. Therefore, these results suggest that SBE can be used as an effective feed additive in laying duck production.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Guizhou University Sub-committee of Experimental Animal Ethics (Guiyang, China; No. EAE-GZU-2021-E012).

Author contributions

B-nY: data collation and draft writing. S-lY: revise the first draft and supervise the completion of the test. F-yL, J-yY, AL, B-gZ, and GF: assist with feeding trials and writing. All authors have read and approved the final manuscript.

Funding

This work was supported by National Nature Science Foundation of China (No. 31960682), the Chinese Government Grant for Supporting the Development of Local Science and Technology (No. [2019]4021), Agricultural Major Special Project of Guizhou Province (No. [2019]5203), the Special Research Projects of Guizhou Province (No. [2020]1Y041), and the Guizhou University College Students SRT Plan Project (No. GD SRT 20), respectively.

Acknowledgments

We thank Guizhou University for providing the places and instruments needed for the experiment, S-lY Teacher for guiding the paper, F-yL for processing the data for the experiment, Editage for providing the article polishing service and all the students who have worked hard for this article.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 27 March 2023
DOI 10.3389/fvets.2023.1147020

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

RECEIVED 18 January 2023
ACCEPTED 23 February 2023
PUBLISHED 27 March 2023

CITATION
Suliman GM, Hussein EOS, Al-Owaimer AN, Alhotan RA, Al-Garadi MA, Mahdi JMH, Ba-Awadh HA, Qaid MM and Swelum AA-A (2023) Betaine and nano-emulsified vegetable oil supplementation for improving carcass and meat quality characteristics of broiler chickens under heat stress conditions. *Front. Vet. Sci.* 10:1147020. doi: 10.3389/fvets.2023.1147020

Betaine and nano-emulsified vegetable oil supplementation for improving carcass and meat quality characteristics of broiler chickens under heat stress conditions

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Introduction: This research aimed to examine the effects of water-added betaine (BET) and/or nano-emulsified vegetable oil (MAGO) on carcass and meat quality characteristics of broilers raised under thermoneutral (TN) and heat stress (HS) conditions.

Methods: On day 21, 640 birds (Ross 308) were randomly assigned to one of two thermal conditions (thermoneutral 22 ± 1°C and heat stress 32 ± 1°C) each containing four treatment groups: Control, BET, MAGO, and a mixture of both (BETMAGO) in a 2 × 4 factorial arrangement (eight groups). Each group has eight replicates, with ten birds each. The birds' carcass and meat quality characteristics were evaluated at 35 days.

Results and discussion: The dressing percentage, breast, leg, wing, heart, initial pH, color change, cooking loss (CL), water-holding capacity (WHC), shear force (SF), and texture profile with exception of springiness significantly affected by the treatments. The results showed that HS had negative effects on carcass weight and relative weights of the breast, spleen, and heart. Moreover, HS increased dressing percentage, wing, initial pH, final core temperature, initial lightness, WHC, and hardness. Significant differences in interactions between treatments and temperature were observed in the spleen, WHC, and SF.

Conclusion: Water supplemented with BET effectively improved carcass dressing percentage, breast weight, and meat quality in terms of water-holding capacity and tenderness under HS conditions. More studies on the use of BET and/or MAGO at different levels were recommended.

KEYWORDS

betaine, magic oil, carcass traits, meat quality, heat stress, broiler chickens

Introduction

Poultry meat is generally considered a rich source of nutrients, including high-quality proteins, vitamins, and minerals. Additionally, it has a low unsaturated fatty acid profile, making it an optimal dietary choice for all age groups (1). Poultry provides humans with nutrition and fiber through meat, eggs, and feathers.

The poultry industry has expanded quickly in Saudi Arabia during the past few decades. It has the potential to attract a considerable number of investments within a short period of time, garner government support to achieve self-sufficiency, compete with imported products, and address the considerable increase in the rate of consumption of poultry meat in the Kingdom of Saudi Arabia over the past few years. However, heat stress is a major concern in the poultry industry, affecting broiler chickens' mass gain and immunity (2). In addition, chronic heat stress has a negative impact on the quality of poultry meat (3). Supplementing Magic oil and probiotics is recommended for the optimal growth of broilers in poultry programs, particularly for male birds, during the period of 0 to 30 days of age (4). It has been found that supplementing male broilers with Magic oil and probiotics, especially during the first 30 days of their lives, can result in good meat chewiness due to higher springiness and lower hardness and cohesiveness. Furthermore, these supplements were found to be associated with the most convenient cooking loss value. Natural herbs or their derivatives, such as *Rumex nervosus* leaves, used as phytogenic feed additives improved meat quality and some carcass traits in broilers and are believed to be a promising non-traditional feed source in the future (5).

Betaine, or tri-methyl glycine, is a naturally occurring chemical compound that serves as an organic osmolyte in microbes and flora exposed to osmotic stress and dehydration (6). Under severe osmotic stress, water migrates from the cell toward higher concentrations of solutes outside the cell (7–10). The constant loss of water causes cells to shrink and eventually die. As a result, osmolytes must regulate osmotic pressure to conserve water and maintain cell integrity.

Several studies have shown that natural antioxidants supplied before and after slaughter improve carcass traits, shelf life, and meat quality. For example, natural antioxidants can be added to animal feed or drinking water, applied to the meat surface, or used in active packaging (11). However, supplementation of these additives in feed or drinking water has been proven to be a better method than other strategies (12, 13), as it allows animals to spread the substance more effectively in the body and absorb tolerable and safe levels of the compound (14). Nanoemulsions are used in food manufacturing to encapsulate, protect, deliver, and transport hydrophobic (poorly water-soluble) bioactive components such as nutrients, nutraceuticals, antimicrobials, and antioxidants (15, 16). They consist of tiny oil droplets suspended in water that serve as vehicles for essential oil bioavailability (17). The pH of meat is an important factor in determining meat quality because it influences the color, texture, and WHC of the breast meat when the muscles are stiff. Within the first 15 m following death, pH plays a significant role in determining the shelf life of meat products (18).

Abudabos et al. (19) and Ghanima et al. (20) have investigated the use of nano-emulsified vegetable oil as a Magic oil in the diets of broiler chickens and rabbits (20). However, few studies have explored the potential benefits of supplementing drinking water with this oil. As a result, there is a need to investigate the effects of adding these supplements to drinking water and assess their effectiveness. Thus, this experiment aimed to investigate the effects of water-added betaine and/or Magic oil on the carcass traits and meat quality of broiler chickens subjected to thermal stress conditions.

Materials and methods

Ethical approval

The experiment was conducted in accordance with the code of ethics and guidelines for the use of animals in research, and the protocol was approved by the King Saud University Ethics Committee (Approval No. KSU-SE-21-02).

Chemical specifications of Magic oil™ and Betafin® S6

Magic oil™ (Atcopharma El-Menofia, Egypt) contains 98.5% nano-emulsified crude oil, including 26% monounsaturated fat, 59% polyunsaturated fat (50% linoleic acid; n-6 & 7% linolenic acid; n-3), 14% saturated fat, and vitamin E.

Betafin® S6 (Danisco Animal Nutrition, Marlborough, Wiltshire SN81XN, United Kingdom) is a natural betaine ideal for drinking water applications. It contains ≥ 91% anhydrous betaine [1-Carboxy-N, N, N-trimethylolethane-aminium hydroxide inner salt (trimethylglycine)] with ≥ 5% anti-caking agent (calcium stearate), moisture (halogen drying) ≤ 2 %, chloride (IC) ≤ 1,000 ppm, sulfate (IC) ≤ 1,000 ppm, and heavy metals ≤ 10 ppm.

Bird's management and treatment groups

The experiment was conducted in the Animal Production Department, College of Food and Agricultural Sciences, KSU, Saudi Arabia. The broiler chicks (Ross 308) were procured from the Alkhumasia commercial hatchery in <city>Riyadh</city>, Saudi Arabia, and individually weighed to ensure they were assigned to similar groups (46.3 g ± 0.13). The broiler chicks were raised under the recommended temperatures, with the initial brooding temperature set at 33°C on day 0. This was gradually decreased by 1°C per week until it reached 24°C on day 20. On day 21, a total of 640 straight-run broiler chicks with comparable body weights (1,010 g ± 11.56) were selected and randomly assigned to 64-floor cage pens (100 cm width, 100 cm length) located in two environmentally controlled rooms. Each cage with 10 birds was considered the experimental unit. Therefore, a 2 × 4 factorial arrangement was applied. Eight experimental groups (eight replicates per group) were formed by using two conditions with rising temperatures [thermoneutral (TN) and constant heat stress (HS)] and four water-added supplementation treatments (control, BET, MAGO, and BETMAGO). The birds in one of the two experimental rooms were continuously kept under the TN condition (22 ± 1°C), while the birds in another room were raised

under constant heat stress conditions (HS; 32 ± 1°C). The birds were given one of four water additive treatments: control (no additives), Magic oil (MAGO) at a rate of 1 mL/L drinking water, betaine (BET) at a rate of 1 g/L drinking water, or a mixture of Magic oil and betaine (MAGOBET: a mixture of 1 g betaine and 1 mL Magic oil/L drinking water). The eight treatment groups were as follows: group 1, TN + CON; group 2, TN + 1 g/L drinking water MAGO; group 3, TN + 1 g/L drinking water BET; group 4, TN + 1 g/L drinking water MAGOBET combination; group 5, HS + CON; group 6, HS + 1 g/L drinking water MAGO; group 7, HS + 1 g/L drinking water BET; and group 8, HS + 1 g/L drinking water MAGOBET combination. The presence of HS has a negative impact on the slaughter weight, carcass weight, internal organ weights, and quality of poultry meat (3). Therefore, in the current study, half of the broilers were exposed to the HS challenge during the finisher period, while the other half were not (20–35 days). Therefore, during the same period of the HS challenge, from 20 to 35 days, all birds under TN and HS conditions received water supplements with Magic oil and betaine Magic oil and betaine combination to mitigate the adverse effects of chronic heat stress in stressed broilers and compare their effects with those of the birds reared under TN conditions. The lighting protocol was set at 22 h of light and 2 h of darkness per day.

To meet the nutritional recommendations for the Ross 305 strain, standard experimental diets were formulated based on commercial practice recommendations in Saudi Arabia (Table 1). These diets were divided into three feeding periods: starter (0–10 d), grower (11–20 d), and finisher (21–35 d), which corresponded to changes in the birds' protein and energy requirements as they aged. The diets were given in pellet form and were made using a corn-soybean meal (CSBM) base.

Carcass measurements

On day 35, 16 birds were chosen at random from each group. Feathers, heads, and shanks were removed after slaughter, and the residual carcasses were sectioned to split the breast and thigh. Fat, liver, heart, gizzard, wings, and drumstick were all separated and weighed in the same way. The percentage of yield for each part was calculated using dressing weight (21).

Meat characteristics

PH and temperature

pH and temperature were measured directly in the breast sample after 1 h postmortem using a microprocessor *pH*-meter (Model PH 211, Hanna Instruments). Two readings were conducted for each carcass, and the mean value was computed.

Meat color

The CIELAB Color System is a color space used to describe color. L* represents lightness, a* represents the degree of redness, and b* represents the degree of yellowness. Color measurements were taken on the breast meat 15 min after slaughter using a chroma meter (Konica Minolta, CR-400-Japan). Additional color parameters were calculated, including color change (ΔE), b/a ratio, chroma (C), and hue angle (A°) (22).

Cooking loss

The frozen breast muscle (∼100 g) was thawed overnight at 4°C. Once the internal temperature reached 70°C, the meat was cooked on a commercial tabletop grill. A thermocouple thermometer (Ecoscan Temp JKT, Eutech Instruments) was inserted into the center of the muscle to measure the cooking temperature. Cooking loss was calculated as the difference between the initial and final weight of the muscles by weighing them before and after cooking.

Shear force

The shear force was measured using a cooked meat sample that had been subjected to an earlier handling process to determine the amount of cooking loss, as outlined by Wheeler et al. (23). Five round cores measuring 1.27 cm in diameter were then removed from each muscle sample that was parallel to the direction of the muscle fibers after they had been cooled to room temperature (21°C). With the aid of a handheld coring tool, cores were obtained. Using a texture analyzer with a Warner-Bratzler attachment, the Texture Analyzer (TA-HD-Stable MicroSystems, England) was used to calculate the SF, which was defined as the maximum force (N) vertical to the fibers. The crosshead speed that was chosen was 200 mm/min.

Water-holding capacity (WHC)

The Wilhelm et al. (24) method was used to calculate WHC. For each sample, the pectoral muscle was divided into two replicates weighing ∼2 g each. The samples were then sandwiched between two Plexiglas disks and two filter papers and placed under a 10 kg weight for 5 m. The sample was then weighed, and the difference between the initial and final weights was used to calculate the WHC.

Statistical analysis

All analysis was carried out using the Statistical Analysis System (25) in a two-way ANOVA. The experimental design was a randomized complete block design (RCBD) with room "raising conditions" ($n = 2$) as a blocking factor. Means for variables showing significant differences were tested using the PDIFF option. The probability value was set at $P < 0.05$. Means ± standard errors of the mean (SEM) were used to express all values.

10.3389/fvets.2023.1147020

TABLE 1 Composition of experimental diets and nutrients analysis.

Ingredients (%)	Starter (0–10 d)	Grower (11–20 d)	Finisher (21–35 d)
Corn grain	57.65	61.32	60.64
Soybean meal (48% CP)	35.43	31.79	30.48
Palm oil	2.06	2.61	4.63
Dical phos	1.92	1.73	1.42
Limestone	1.06	0.96	1.42
Common salt	0.36	0.36	0.35
L-Threonine	0.30	0.12	0.07
Methionine	0.38	0.33	0.28
LLysine HCl	0.28	0.22	0.15
Choline Cl70	0.06	0.06	0.06
Minvit Arasco 0.5%	0.50	0.50	0.50
Nutrients		Analysis	
Dry Matter %	89.614	89.576	85.664
ME kcal/kg	3000	3070	3200
CP %	22.4	20.7	19.5
Arginine %	1.52	1.399	1.273
Lysine %	1.435	1.29	1.131
Methionine %	0.711	0.641	0.566
Methionine + Cysteine %	1.08	0.99	0.882
Threonine %	1.143	0.9	0.803
Tryptophan %	0.277	0.254	0.228
Valine %	1.017	0.951	0.901
Ether extract	4.601	5.256	7.236
Linoleic acid %	1.597	1.714	4.496
Crude Fiber %	2.65	2.589	2.523
Calcium %	0.96	0.87	0.937
Total phosphorus %	0.713	0.663	0.625
Available phosphorus %	0.48	0.44	0.4
Sodium %	0.16	0.16	0.16

Results

Carcass traits

The HS effects and experimental treatments and their interactions on carcass characteristics and the relative weights of body components of broiler chickens at day 35 are presented in Table 2. Treatments, HS, or their interaction had no effect ($P > 0.05$) on the relative weights of carcass characteristics and the relative weights of the body components of the broiler chickens.

The treatments had significant effects on the dressing percentage ($P = 0.008$) relative to the weights of the breast ($P = 0.032$), leg ($P = 0.011$), wing, and heart ($P < 0.0001$). BET supplementation increased the percentage of hot carcass dressing compared to the control. Compared to the control, the treatments reduced the relative weight of the heart. Except for the spleen, the treatment*temperature interaction did not affect the body components' relative weight.

HS reduced the broiler chickens' final live weight, hot and cold carcass weight, and relative weights of the breast, spleen, and heart ($P < 0.05$). In contrast, HS increased the percentage of hot carcass dressing and the relative weight of the broiler chickens' wings ($P < 0.05$).

Meat quality

The initial and final pH and temperature of the breast samples from 35-day-old broiler chickens are shown in Table 3. Treatments and rising temperatures had significant interactional effects on

TABLE 2 Effect of the treatments on carcass characteristics and body components of broiler chickens at d 35 under thermoneutral (TN) and heat stress (HS) raising conditions.

Treatments (TRT)	Raising Condition (RC)	Final LW (kg)	HCW (kg)	CCW (kg)	Dressing (%)	[1]Body components were computed as a ratio to the carcass weight							
						Breast %	Leg %	Wing %	Gizzard (%)	Liver %	Spleen %	Heart %	Abdominal fat %
Effect of interactions between treatments and raising condition (TRT∗RC)													
Control[1]	TN	2.43	1.83	1.85	75.23	48.96	37.60	9.03	1.83	2.68	0.14	0.67	1.73
MAGO[2]	TN	2.38	1.80	1.79	75.62	47.57	37.89	9.37	1.73	2.65	0.18	0.63	1.61
BET[3]	TN	2.53	1.95	1.93	76.99	49.50	36.51	8.65	1.73	2.67	0.17	0.62	1.70
BETMAGO[4]	TN	2.43	1.85	1.85	76.19	48.70	38.44	9.10	1.81	2.57	0.14	0.65	1.68
Control	HS	2.05	1.57	1.56	76.77	47.54	37.45	9.41	1.89	3.31	0.12	0.61	1.74
MAGO	HS	2.06	1.59	1.58	77.30	46.59	38.53	9.66	1.90	2.39	0.10	0.53	1.68
BET	HS	2.11	1.65	1.64	78.08	48.38	37.01	9.00	1.71	2.25	0.10	0.53	1.82
MAGOBET	HS	2.16	1.67	1.66	77.41	46.93	37.78	9.45	1.73	2.13	0.11	0.52	1.64
SEM		62.60	50.63	48.54	0.45	0.632	0.485	0.148	0.06	0.30	0.01	0.03	0.12
p-values		0.668	0.678	0.600	0.912	0.929	0.514	0.983	0.196	0.184	0.002	0.523	0.946
Effect of treatments (TRT)													
Control		2.24	1.70	1.71	76.00^{b}	48.25^{ab}	37.52^{ab}	9.22^{ab}	1.86	3.00	0.13	0.64^{a}	1.73
MAGO		2.22	1.70	1.68	76.46^{b}	47.08^{b}	38.21^{a}	9.51^{a}	1.82	2.52	0.14	0.58^{b}	1.65
BET		2.32	1.80	1.78	77.54^{a}	48.94^{a}	36.76^{b}	8.82^{b}	1.72	2.46	0.13	0.58^{b}	1.76
BETMAGO		2.30	1.76	1.75	76.80^{ab}	47.82^{ab}	38.11^{a}	9.27^{a}	1.77	2.35	0.12	0.59^{b}	1.66
SEM		44.26	35.80	34.32	0.32	0.45	0.45	0.34	0.11	0.04	0.21	0.01	0.02
p-values		0.346	0.141	0.153	0.008	0.032	0.011	<0.0001	0.130	0.131	0.415	0.043	0.735
Effect of raising condition (RC)													
TN[5]		2.444^{a}	1.858^{a}	1.854^{a}	76.01^{b}	48.68^{a}	37.61	9.03^{b}	1.78	2.65	0.16^{a}	0.64^{a}	1.68
HS[6]		2.093^{b}	1.621^{b}	1.607^{b}	77.39^{a}	47.36^{b}	37.69	9.38^{a}	1.81	2.52	0.11^{b}	0.55^{b}	1.72
SEM		31.30	25.32	24.27	0.23	0.32	0.24	0.07	0.03	0.15	0.01	0.01	0.06
p-values		<0.0001	<0.0001	<0.0001	<0.0001	0.004	0.790	0.001	0.504	0.542	<0.0001	<0.0001	0.772

FLW, Final live weight; HCW, Hot carcass weight; CCW, Cold carcass weight; $^{a-c}$Mean values of different superscripts on the same column are significantly different at ($P < 0.05$). [1]Control, no additive; [2]MAGO, Magic oil (1 mL/L drinking water); [3]BET, betaine (1 g/L drinking water); [4]BETMAGO, a mixture of the 1 g betaine and 1 mL Magic oil/L drinking water; [5]TN, thermoneutral environment (22°C); [6]HS, heat stress environment 33°C, SEM, Standard Error of Mean.

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10.3389/fvets.2023.1147020

TABLE 3 Effect of the treatments on the pH and core temperature at 1 and 24 h postmortem of breast muscle of broiler chickens at d 35 under thermoneutral (TN) and heat stress (HS) raising conditions.

Treatments (TRT)	1 h P.M			24 h P.M	
	Raising condition (RC)	pH	Temp °C	pH	Temp °C
Effect of interactions between treatments and raising condition (TRT*RC)					
Control[1]	TN	6.07	30.01	5.89	16.90
MAGO[2]	TN	6.16	29.55	5.78	17.26
BET[3]	TN	6.06	28.80	5.80	16.84
BETMAGO[4]	TN	6.13	28.43	5.88	16.86
Control	HS	6.26	22.36	5.85	17.78
MAGO	HS	6.34	22.00	5.87	18.21
BET	HS	6.23	20.76	5.88	17.91
MAGOBET	HS	6.13	21.64	5.83	17.90
SEM		0.03	0.72	0.02	0.17
p-values		0.011	0.851	0.001	0.934
Effect of treatments (TRT)					
Control		6.17^{b}	26.18	5.87	17.34
MAGO		6.26^{a}	25.78	5.82	17.73
BET		6.14^{b}	24.78	5.84	17.37
BETMAGO		6.13^{b}	25.03	5.85	17.38
SEM		0.02	0.51	0.02	0.12
p-values		0.001	0.184	0.171	0.060
Effect of raising condition (RC)					
TN[5]		6.10^{b}	29.20^{a}	5.84	16.96^{b}
HS[6]		6.24^{a}	21.69^{b}	5.86	17.95^{a}
SEM		0.02	0.36	0.01	0.08
p-values		<0.0001	<0.0001	0.199	<0.0001

$^{a-c}$Mean values of different superscripts on the same column are significantly different at ($P < 0.05$). [1]Control, no additive; [2]MAGO, Magic oil (1 mL/L drinking water); [3]BET, betaine (1 g/L drinking water); [4]BETMAGO, a mixture of the 1 g betaine and 1 mL Magic oil/L drinking water; [5]TN, thermoneutral environment (22°C); [6]HS, heat stress environment 33°C, SEM, Standard Error of Mean.

the initial pH of breast meat ($P < 0.05$). The treatments and rising temperatures had no impact on the ultimate pH ($P > 0.05$). However, the ultimate pH significantly differed due to their interaction ($P = 0.001$). Stressed birds had a higher initial pH (6.24 *vs.* 6.10, $P < 0.0001$), lower initial core temperature (21.69 *vs.* 29.20°C, $P < 0.0001$), and a higher final core temperature (17.95 *vs.* 16.96°C, $P < 0.0001$) of the breast muscle compared to the unstressed birds.

The initial and final meat color derivatives of breast samples from 35-day-old broiler chickens are shown in Table 4. Although the color change was noticeably amplified in the birds that were given BET or MAGO alone, water supplementation treatments had no significant main or additive effects on breast color. The L* value increased over time (1 and 24 h postmortem) in the breasts of the chickens that were given experimental water treatment.

When compared to the unstressed birds, the heat-stressed birds had higher initial lightness (51.66 *vs.* 50.52, $P = 0.005$) and lower final lightness (53.74 *vs.* 51.05, $P < 0.0001$). Furthermore, the heat-stressed birds had lower initial yellowness (8.11 *vs.* 8.56, $P = 0.081$) and final yellowness (10.62 *vs.* 11.47, $P = 0.012$) of the breast muscle than the unstressed birds. Color change (4.34 *vs.* 5.45, $P = 0.004$) and chroma saturation (10.75 *vs.* 11.58, $P = 0.012$) were lower in the HS-exposed birds than in the HS-unexposed birds.

The WHC, CL, SF, and texture profile analysis (TPA) of breast samples from 35-day-old broiler chickens are presented in Table 5. The WHC, CL, SF, and texture profile analysis (TPA) of breast samples are significantly ($P<0.05$) influenced by treatments and/or rising temperatures. However, the values for springiness were unaffected ($P > 0.05$) by the water treatments. Furthermore, the thermal condition had no effect on CL or chewiness ($P > 0.05$). The interactions between water treatments and thermal conditions had no effect ($P > 0.05$) on all meat parameters, except for WHC and SF.

WHC values of breast meat were higher in the HS-exposed chickens (33.62 *vs.* 31.91%, $P = 0.002$) and those who were given BET (36.31). Nevertheless, there was evidence of an interaction between the temperature regime and BET, as seen in the breasts of the birds that were fed BET (36.31 *vs.*

TABLE 4 Effect of the treatments on meat color derivatives of breast muscle of broiler chickens at d 35 under thermoneutral (TN) and heat stress (HS) raising conditions.

Treatments (TRT)	Raising condition (RC)	Li∗1	ai∗1	bi∗1	Lu∗2	au∗2	bu∗2	Color change	b:a ratio	Chroma	Hue angle
Effect of interactions between treatments and raising condition (TRT∗RC)											
Control[1]	TN	49.72	−1.08	8.58	53.33	−1.06	11.01	5.05	−4.62	11.12	−50.45
MAGO[2]	TN	51.24	−1.29	8.12	54.70	−1.00	11.72	6.34	−8.23	11.84	−62.54
BET[3]	TN	50.74	−1.18	8.47	53.62	−1.14	11.59	5.74	−3.36	11.71	−50.19
BETMAGO[4]	TN	50.37	−1.39	9.08	53.30	−0.77	11.54	4.66	−5.56	11.62	−41.07
Control	HS	52.04	−0.94	8.15	53.05	−0.47	10.99	4.13	−6.05	11.07	−43.03
MAGO	HS	50.70	−1.34	8.67	50.16	−0.38	11.18	4.73	−8.27	11.37	−43.13
BET	HS	52.49	−1.33	7.56	50.37	−0.63	10.06	4.88	−7.19	10.16	−52.70
MAGOBET	HS	51.40	−1.54	8.07	50.64	−1.10	10.27	3.62	−6.58	10.38	−61.46
SEM		0.56	0.22	0.36	0.94	0.34	0.47	0.53	3.96	0.46	16.86
p-values		0.07	0.90	0.13	0.15	0.45	0.36	0.89	0.97	0.34	0.68
Effect of treatments (TRT)											
Control		50.88	−1.01	8.37	53.19	−0.77	11.00	4.59^{ab}	−5.33	11.10	−46.74
MAGO		50.97	−1.31	8.39	52.43	−0.69	11.45	5.53^{a}	−8.25	11.61	−52.84
BET		51.61	−1.26	8.02	51.99	−0.88	10.83	5.31^{a}	−5.28	10.94	−51.45
BETMAGO		50.88	−1.47	8.57	51.97	−0.93	10.90	4.14^{b}	−6.07	11.00	−51.26
SEM		0.40	0.16	0.25	0.67	0.24	0.33	0.38	2.80	0.33	11.92
p-values		0.50	0.23	0.47	0.53	0.89	0.55	0.04	0.86	0.46	0.99
Effect of raising condition (RC)											
TN[5]		50.52^{b}	−1.23	8.56^{a}	53.74^{a}	−0.99	11.47^{a}	5.45^{a}	−5.44	11.58^{a}	−51.06
HS[6]		51.66^{a}	−1.29	8.11^{b}	51.05^{b}	−0.65	10.62^{b}	4.34^{b}	−7.02	10.75^{b}	−50.08
SEM		0.28	0.11	0.18	0.47	0.17	0.23	0.27	1.98	0.23	8.43
p-values		0.01	0.74	0.08	<0.0001	0.16	0.01	0.01	0.57	0.01	0.93

$^{a-c}$Mean values of different superscripts on the same column are significantly different at ($P < 0.05$). [1]Control, no additive; [2]MAGO, Magic oil (1 mL/L drinking water); [3]BET, betaine (1 g/L drinking water); [4]BETMAGO, a mixture of the 1 g betaine and 1 mL Magic oil/L drinking water; [5]TN, thermoneutral environment (22°C); [6]HS, heat stress environment 33°C, SEM, Standard Error of Mean.

10.3389/fvets.2023.1147020

TABLE 5 Effect of the treatments on chicken breast objective eating quality measures at d 35 under thermoneutral (TN) and heat stress (HS) raising conditions.

Treatments (TRT)	Raising condition (RC)	WHC (%)	CL (%)	SF (N)	Texture profile analysis (TPA)			
					Hardness (N)	Springiness	Cohesiveness	Chewiness
Effect of interactions between treatments and raising condition (TRT*RC)								
Control[1]	TN	29.97	16.28	7.18	7.43	0.85	0.51	3.42
MAGO[2]	TN	32.58	21.95	6.50	7.34	0.83	0.51	3.30
BET[3]	TN	33.94	19.47	5.32	7.80	0.86	0.55	3.79
BETMAGO[4]	TN	31.15	21.55	9.26	7.91	0.83	0.55	3.68
Control	HS	35.17	16.28	4.81	7.75	0.77	0.47	2.81
MAGO	HS	31.20	24.15	5.07	9.23	0.75	0.51	3.79
BET	HS	36.31	19.47	4.94	10.07	0.75	0.52	4.13
MAGOBET	HS	31.83	21.55	5.15	9.45	0.76	0.52	3.89
SEM		0.77	1.82	0.36	0.45	0.02	0.02	0.27
p-values		<0.0001	0.91	<0.0001	0.16	0.72	0.72	0.14
Effect of treatments (TRT)								
Control		32.57^{b}	16.28^{b}	5.99^{b}	7.59^{b}	0.81	0.49^{b}	3.12^{b}
MAGO		31.89^{b}	23.05^{a}	5.79^{bc}	8.28^{ab}	0.79	0.51^{ab}	3.55^{ab}
BET		35.13^{a}	19.47^{ab}	5.13^{c}	8.93^{a}	0.80	0.54^{a}	3.96^{a}
BETMAGO		31.49^{b}	21.55^{a}	7.21^{a}	8.68^{a}	0.79	0.54^{a}	3.78^{a}
SEM		0.54	1.28	0.26	0.32	0.01	0.01	0.18
p-values		<0.0001	0.002	<0.0001	0.02	0.70	0.04	0.01
Effect of raising condition (RC)								
TN[5]		31.91^{b}	19.81	7.07^{a}	7.62^{b}	0.84^{a}	0.53^{a}	3.55
HS[6]		33.62^{a}	20.36	4.99^{b}	9.13^{a}	0.76^{b}	0.51^{b}	3.65
SEM		0.39	0.91	0.18	0.23	0.01	0.01	0.13
p-values		0.002	0.67	<0.0001	<0.0001	<0.0001	0.05	0.56

$^{a-c}$Mean values of different superscripts on the same column are significantly different at ($P < 0.05$). WHC, water-holding capacity; CL, cooking loss; [1]Control, no additive; [2]MAGO, Magic oil (1 mL/L drinking water); [3]BET, betaine (1 g/L drinking water); [4]BETMAGO, a mixture of the 1 g betaine and 1 mL Magic oil/L drinking water; [5]TN, thermoneutral environment (22°C); [6]HS, heat stress environment 33°C, SEM, Standard Error of Mean.

33.94%) rather than those who were in the control group (35.17 *vs.* 29.97%). Cooking loss was significantly ($P = 0.002$) higher in experimentally treated birds' breast meat than in the control group.

The lesser shear force value was reported by the BET treatment group (5.13 N), resulting in the lowest shear force value of chickens that drank BET and were housed under HS conditions (4.94 N), indicating a significant level of tenderness., SF and shear energy are the maximum force and energy needed to cut the sample; thus, lower values indicate that the meat was more tender. The highest value of shear force was higher in the TN-exposed chickens (7.07 *vs.* 4.99 N, $P < 0.0001$) and was higher in the birds that received BETMAGO treatment (7.21 N, $P < 0.0001$), indicating tough meat, resulting in treatment*thermal interaction, as shown by the BETMAGO treatment group under TN (9.26 N). In the texture profile analysis, the BET and then BETMAGO treatments had the highest values for hardness, cohesiveness, and chewiness.

Discussion

This study compared the efficacy of Magic oil, a natural nano-emulsified vegetable oil, with betaine on carcass characteristics and breast quality in broiler chickens. In this case, BET increased the dressing percentage and breast percentage significantly. This suggests that supplementing BET with water improved breast weight, which increased the dressing percentage by alleviating the negative effects of HS. The addition of 0.05% methionine increased meat yield by ~1.5 percentage points, whereas 0.04% betaine only increased breast meat yield by 0.3 to 0.6 percentage points (26). They concluded that there was no evidence to suggest that betaine served as a replacement for DL-methionine as an essential amino acid supplement in the broiler chickens' diets. However, BET supplementation improved carcass values slightly. Additionally, the study found that BET supplementation improved the yield of breast muscle compared to the control group. The findings of

Waldroup et al. (27) and McDevitt et al. (28) are also consistent with our findings. BET supplementation improved carcass dressing by increasing slaughter and raising carcass weights more than BETMAG, indicating that BET alone is a preferable supplement without the need for MAG. Waldroup and Fritts (29) found a rise in dressing percentage in the broilers that were fed 0.1% BET at 42 days. Furthermore, when compared to TN, HS reduced breast yield. Temperature also had an effect on the relative weight of the spleen, which was significantly lower in the broilers raised under HS than in those raised under TN. According to recent studies, lymphoid organ growth in heat-stressed broilers has slowed (19, 30, 31). In addition, the weight loss could be due to a decrease in feed intake, which results in fewer nutrients for the appropriate growth of lymphoid organs under HS conditions (32).

Meat pH is an important parameter in determining meat quality that occurs during muscle stiffness; it can affect the texture, color, and WHC of meat. pH is an important factor in determining the shelf life of meat products within the first 15 m after death (18). Usually, a rapid drop in meat pH can cause protein denaturation, resulting in pale meat with low WHC. Several studies have suggested that nano-emulsion-based products can positively influence the physicochemical and sensory properties of the breast muscles (33). To the best of our knowledge, to date, there have not been many studies that examine how nanoemulsified vegetable oil affects meat characteristics. In a current study, BET seemed to have a greater impact on the physicochemical properties of filets than MAGO. According to Smith et al. (34), breast meat color was unaffected by BET, but HS increased L* and decreased b*. According to Akşit et al. (35), the broilers raised under cyclic HS had pectoral meat that was lighter but not yellower. Since there is a link between muscle pH at 24 h and color, pH is one of the factors influencing meat color (36). Although pH values at 1-h postmortem were higher in the MAGO group, resulting in significant differences with the other water supplementation groups, for all groups, the pH values at 24-h postmortem were within the range of raw meat (5.82–5.87). These values were also similar to those reported in other studies (37, 38), which reported similar values for breast muscle. This indicates that there were no quality problems with the breast meat in any of the water additive treatment groups.

The current study found that HS increased initial pH or lightness, which contradicts previous research that found that both consistent and cyclic HS can lower pH at1 h and pH at 24 h postmortem (39, 40). These experiments may show that the duration of HS is an essential factor that can affect pH. In this study, the treatments, temperature regime, and interactions between them had an effect on the initial pH. Even if breast muscle under TN conditions at 24-h postmortem showed a higher lightness, the pH values in both raising temperatures were within the range for normal meat. The pH at 1 h postmortem was higher in the breast meat of the birds supplemented with MAGO and reared under HS due to an increased pH in the main effect of MAGO treatment and the main effect of HS. As expected, muscle pH decreased over the first 24 h postmortem. Therefore, the declining pH between the 1 and 24 h was the highest in the breast muscle of the chickens supplemented with MAGO and reared under HS due to an increased pH decline in the main effect of MAGO treatment and the main effect of HS.

The addition of betaine led to an improvement in the breast muscle, which correlated with better postmortem WHC, tenderness, and texture profile of the meat. HS reduced hot and cold carcass weight, the relative weights of breast, spleen, and heart, and meat quality. Combining MAGO with BET did not improve BET's beneficial effects on reducing the adverse effects of HS. However, when MAGO and BET were used together, the relative weights of the leg and wing showed a positive impact compared to BET alone, but the results were unsatisfactory compared to the control group.

Antioxidant activities of BET improve meat quality in birds under HS conditions (41). When subjected to HS, BET alters osmotic pressure, retains water within cells (42), and minimizes energy spent in the Na+ K+ pump, which minimizes thermogenesis from metabolic activities (43). Because a bird's body is unable to produce additional heat when heat production is reduced, deep body temperature and respiration rate decrease. We found that HS decreased initial core temperature, initial yellowness, final lightness, final yellowness, and color change—chroma, SF, springiness, and cohesiveness. HS increased dressing percentage, wing percentage, initial pH, final core temperature, initial lightness, WHC, and hardness. Because of their thermoregulatory system (covered feathers and lack of sweat glands), broilers are more susceptible to unwanted high temperatures, and global warming makes broilers especially vulnerable to HS (44). Several studies have demonstrated that chronic HS increased L* values, cooking loss, drip loss, shear force, and hardness values, decreasing the springiness and pH24h and a* values of the PM muscle (3, 41). The presence of water within muscle cells is crucial, as water loss can reduce the weight and thus the cost-effective value of the final meat product. It can also affect the tenderness and texture profile of the meat. The current study's findings support the findings of Fouad et al. (45) and Wang et al. (46), indicating that BET can increase breast yield, while HS decreases it because HS reduces total water content by increasing water loss, whereas BET can improve it. Heat stress likely causes membrane leakage, allowing water to escape from muscular cells. This mechanism should account for the lower total muscle water content and breast yield. Betaine, an osmolyte, prevents dehydration by preserving water in the cells and maintaining osmotic pressure (42). The present results disagree with those of Zhu et al. (47) and Abudabos et al. (19), which showed that HS increased shear force.

Texture measurements, such as SF, are made up of skeletal muscle fibers and connective tissue, and they are a method of measuring the force needed to shear connective tissue and myofibrils in a typical frame of meat. The tenderization method of meat is started by calpain-mediated fiber proteolysis of skeletal muscle, which results in smaller fragments and an increase in MFI (48). In this case, the HT group had a lower SF. However, Olson and Stromer (49) found that SF is related to other muscle indices, i.e., MFI, that were higher in the HT group. In TPA parameters, the meat is exposed to conditions similar to those in the mouth, i.e., sensory properties are measured. The HT factor worsened all parameters except chewiness. Notably, the joint use of BET and MAGO may have a variety of benefits for product quality and yield.

More research is needed to determine betaine's ability to reduce methionine addition and make betaine supplementation cost-effective, particularly when combined with different levels of

methionine and choline. More research is needed to determine BETMAGO's ability to reduce BET or MAGO supplementation alone and make BETMAGO supplementation cost-effective, particularly when combined with different levels of BET and MAGO.

Conclusion

Based on the findings, it was concluded that using betaine as an aqueous additive in broilers conferred overall benefits in terms of improvement in the carcass dressing percentage, breast weight, and meat quality in terms of water-holding capacity and tenderness without using nano-emulsified vegetable oil. However, more research is needed to explore the extended effects of nano-emulsified vegetable oil and/or betaine combinations, particularly with respect to shelf life, antioxidant activity, and nutritional value, including meat analysis, fatty acid profiles, meat composition, and microbiological analysis.

Data availability statement

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

Ethics statement

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

Author contributions

GS and EH: conceptualization. AA-O, GS, MA-G, and EH: methodology. JM: software. RA, GS, and EH: validation. RA: formal analysis. AA-O, GS, and EH: investigation. AA-O: resources and funding acquisition. HB-A: data curation. MQ, GS, and EH: writing the original draft preparation. AS: writing, reviewing, and editing and supervision. GS: visualization. GS and AA-O: project administration. All authors have read and agreed to the published version of the manuscript.

Funding

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia, for funding this research work through Project No. (IFKSURG-2-477).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 23 August 2023
DOI 10.3389/fvets.2023.1238070

OPEN ACCESS

EDITED BY
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RECEIVED 10 June 2023
ACCEPTED 10 August 2023
PUBLISHED 23 August 2023

CITATION
Azzam MM, Chen W, Xia W, Wang S, Zhang Y, El-Senousey HK and Zheng C (2023) The impact of *Bacillus subtilis* DSM32315 and L-Threonine supplementation on the amino acid composition of eggs and early post-hatch performance of ducklings.
Front. Vet. Sci. 10:1238070.
doi: 10.3389/fvets.2023.1238070

The impact of *Bacillus subtilis* DSM32315 and L-Threonine supplementation on the amino acid composition of eggs and early post-hatch performance of ducklings

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Poultry requires Threonine, an essential amino acid, and its metabolites for proper metabolic function. Threonine is crucial in the biosynthesis of mucin, which is essential for intestinal health and nutrient absorption. *Bacillus subtilis* (*B. subtilis*) is a potential substitute for antibiotic growth promoters in the poultry industry. The current study was designed to evaluate the simultaneous effect of L-Threonine (Thr) and *B. subtilis* DSM32315 supplementation on laying duck breeders in order to maximize performance. A total number of 648 female 23-week-old Longyan duck breeders were assigned to a 3×2 factorial design with six replicates of 18 birds per replicate. L-Thr was added to the control diet at concentrations of 0, 0.7, and 1.4g/kg, equating to 3.9, 4.6, and 5.3g Thr/kg, with or without *B. subtilis* strain DSM 32315 (0.0 and 0.5g/kg). Increasing Thr concentrations improved egg production and ducklings' hatchling weight ($p<0.05$). In addition, L-Thr supplementation resulted in a tendency for decreased feed conversion ratio without affecting egg quality. There was no significant effect ($p>0.05$) of the dietary Thr levels on egg yolk and albumen amino acid concentrations. In contrast, the addition of *B. subtilis* decreased the concentrations of amino acids, excluding proline, in the egg white (albumen) and the egg yolk ($p<0.05$). Furthermore, the supplementation of *B. subtilis* decreased ($p<0$ 0.001) the hatching weight of ducklings. The addition of *B. subtilis* without L-Thr decreased ($p<0.05$) the hatchability of fertile eggs and the hatching weight of ducklings compared to those of ducks fed dietary L-Thr along with *B. subtilis* ($p<0.001$). The combining L-Thr at 0.7g/kg with *B. subtilis* DSM 32315 at 0.5g/kg could increase eggshell quality, hatchability, and hatching weight. The current study revealed that the combination supplemented of L-Thr and *B. subtilis* DSM 32315 is recommended due to its positive effects on the eggshell percentage, hatchability and the body weights of newly hatched ducklings when dietary Thr was added at a rate of 0.7 g/kg and *B. subtilis* DSM 32315 at 0.5 g/kg. In addition, adding L-Thr separately at 0.7 g/kg could improve the egg production of duck breeders. Further studies are required to find the proper dosages of *B. subtilis* DSM 32315 with co-dietary inclusion of limiting amino acids in the diets of duck breeders. The findings of these trials will support feed additive interventions to transition into antibiotic-free diets.

KEYWORDS

ducks, Threonine, *Bacillus subtilis*, amino acid composition of eggs, hatch weight of ducklings

1. Introduction

The use of antibiotic growth promoters (AGP) has been banned in poultry farms worldwide. Therefore, global efforts in the poultry industry are focused on the development of effective feeding strategies that provide comparable benefits. *Bacillus subtilis* DSM 32315 has been effectively utilized as a substitute for AGP in broiler feeding (1–3). *Bacillus subtilis* DSM 32315 has unique beneficial effects on broilers; dietary supplementation of *B. subtilis* DSM 32315 improved the growth performance of birds and the intestinal microflora balance (4). The addition of *B. subtilis* to laying hens' diets increased egg production, health, and egg quality' (5, 6). Furthermore, the application of dietary *B. subtilis* (C-3102) improved the egg weight, fertility and hatchability, and eggshell thickness in laying hen breeders (7). *Bacillus subtilis* supplementation has a significant impact on the levels of a wide range of chemical metabolites in the chicken intestine, particularly those related to amino acids (8). Moreover, adding a probiotic to an amino acid supplement has been demonstrated to have a synergistic or additive effect on productivity, egg quality, and immunity (9). Therefore, the combination of Thr (an essential amino acid) and probiotic *Bacillus* may increase the absorptive area and functional ability of the intestine, thereby increasing production and egg quality.

Threonine is an essential amino acid for poultry because it is the third most limiting amino acid, and its metabolites are necessary for the metabolic process. Thr is essential for mucin biosynthesis, which is crucial for intestinal health and the absorption of nutrients. Previous research has demonstrated that Thr supplementation significantly increases egg production in laying ducks and breeder hens (10–12). However, previous studies mainly focused on the effects of supplementing *B. subtilis* DSM 32315 as a potential probiotic in diets as individual supplements. To our knowledge, no prior study has evaluated the combined effects of dietary Thr and *B. subtilis* on laying duck breeders, as far as we are aware. Consequently, this study aimed to investigate the interactive effects of dietary Thr and *B. subtilis* on laying duck breeders' reproductive performance, the eggs' amino acid composition, and early post-hatch duckling performance'.

2. Materials and methods

2.1. Experimental design and dietary treatments

In a randomized block design, six hundred and forty-eight of female Longyan duck breeders, with comparable body weight (1.10 ± 0.09 kg), 23 weeks old, were assigned to a 3 × 2 factorial design with 6 replicates pens containing 18 ducks and each duck was housed in one cage [18 ducks (cages)/ replicate]. Ducks were raised at 24°C and 74% RH during the experimental period. In addition, a 17-h photoperiod per d was adopted. The basal control diet met the requirements of the breed (Table 1). L-Threonine (L-Thr, 98.5%, ThreAMINO, Evonik Industries AG, Essen, Germany) was supplemented to the control diet at 0, 0.7, and 1.4 g /kg, corresponding 3.9, 4.6, and 5.3 g total Thr /kg with or without *B. subtilis* strain DSM 32315 0.0 and 0.5 g/kg, *B. subtilis* strain DSM32315 according to the manufacturer's recommendation [min. 2×10^9 CFU/g (Gut Care® PY1; Evonik Industries AG, Essen, Germany)]. The dietary amino acids were analyzed using HITACHIL-8900 (Hitachi, Ltd., Tokyo, Japan) (Table 2) (13). The Animal Care Committee of Guangdong Academy of Agriculture Sciences (Guangdong, PRC) approved the study.

2.2. Laying performance, productive performance, and egg quality

The number of eggs and feed intake for each replicate were recorded in order to calculate egg production and the feed conversion

TABLE 1 Composition and nutrient levels of the control basal diet.

Ingredients	Kg
Yellow Corn	325
Wheat	300
Peanut meal	130
Wheat bran	121
Limestone	84
Di-calcium phosphate	12
DL-Met	2.5
L-Lys	5
L-Trp	0.5
L-Ile	1.5
L-Val	1.5
L-Thr	0.0
Zeolite carrier	3.5
Salt	3.5
Premix[1]	10
Total	1,000
Nutrient levels, %	
ME (MJ/kg)	10.46
Crude protein	15.9
Calcium	3.5
Methionine	0.42
Lysine	0.89

[1]Provided per kilogram of diet: Vitamins: A, 12500 IU; D3, 4,125 IU; E, 15 IU; K, 2 mg; B1 1 mg; B2 8.5 mg; calcium pantothenate 50 mg; niacin 32.5 mg; pyridoxine 8 mg; biotin 2 mg; folic acid 5 mg; VB12 5 mg. Minerals: Zn 90 mg; I 0.5 mg; Fe 60 mg; Cu 8 mg; Se 0.2 mg; Co 0.26 mg; choline chloride 500 mg.

10.3389/fvets.2023.1238070

TABLE 2 Analyzed levels of amino acid in the experimental diets (3 × 2).[1]

Items, %	Treatments					
	- Bacillus subtilis (0.0 g/kg)			*+ Bacillus subtilis (0.5 g/kg)*		
	L-Thr supplementation (g/kg)			L-Thr supplementation (g/kg)		
L-Thr supplementation, g/kg	0.0	0.7	1.4	0.0	0.7	1.4
Essential amino acid						
Arginine	1.39	1.5	1.33	1.44	1.45	1.44
Histidine	0.35	0.33	0.33	0.34	0.35	0.35
Isoleucine	0.64	0.60	0.62	0.64	0.70	0.64
Leucine	1.06	1.02	1.01	1.06	1.08	1.10
Lysine	1.11	0.99	1.00	1.04	0.99	1.00
Methionine	0.32	0.30	0.34	0.35	0.34	0.43
Phenylalanine	0.72	0.70	0.70	0.71	0.73	0.74
Threonine	0.41	0.45	0.53	0.42	0.46	0.54
Valine	0.81	0.76	0.76	0.80	0.79	0.83
Tyrosine	0.52	0.50	0.50	0.55	0.55	0.56
Nonessential amino acid						
Alanine	0.69	0.62	0.64	0.66	0.67	0.69
Aspartic acid	1.43	1.35	1.30	1.39	1.37	1.39
Glutamic acid	3.32	3.30	3.31	3.344	3.53	3.55
Glycine	0.78	0.74	0.75	0.79	0.79	0.80
Proline	0.90	0.90	0.90	0.91	0.94	0.95
Serine	0.67	0.63	0.63	0.67	0.66	0.67

[1]Values are the results of a chemical analysis conducted in duplicate. Thr, Threonine.

ratio (g feed intake/g egg produced). Each female breeder was inseminated (artificially) twice weekly with 100 L of pooled sperm at the age of 40 weeks. Fifty eggs from each replicate ($n = 300$ eggs per treatment) were incubated using an egg incubator under (Dezhou Jingxiang Technology Co, Dezhou, China) at 37.2°C to 38.0°C and 60 to 75% RH for 28 day. The fertility, hatchability, and ducklings' weights were determined. Three eggs per replicate ($n = 18$ eggs per treatment) were collected at the end of the experiment to determine egg quality, as described by Ruan et al. (14). In addition, both albumen and yolk were extracted and kept at −20°C to determine amino acid content.

2.3. Amino acid analysis of egg albumen and yolk

The albumen and yolk samples were dried to constant weight and then defatted and hydrolyzed. An amino acid analyzer (HITACHIL-8900, Hitachi, Ltd., Tokyo, Japan) was used according to the AOAC method 994.12 (15). Briefly, a hundred milligrams of each sample were mixed with 800 μLof 0.1 MHCl and the oxygen was expelled bypassing nitrogen into the ampoule. Then, 800 μL of hexane was added and mixed by vertexing for1 min, and centrifuged at 22,000 × g for 5 min, and the lower layer was collected. After acid hydrolysis with 6 NHCl at 110°C for 20 h, the amino acid contents were measured, except for cysteine and methionine. Methionine and cystine were measured by acid oxidation before hydrolysis with 6 NHCl (16). The absolute concentration was determined from the peaks of the sample and standard by comparing the peak area obtained for each amino acid with the peak area of the amino acid standards.

2.4. Early post-hatch performance of ducklings

At hatch, the total number of ducklings ($n = 468$) were assigned to the same dietary treatment groups (3 × 2 factorial design) for early post-hatch performance of ducklings. Each treatment group included six replicates of 13 ducklings. Ducklings were provided the same starter diets (Supplementary Table S1). Pens were supplied with water and feeders to provide *ad libitum* access to feed. On day 7 of age, body weight and feed consumption were determined, and mortality rates were recorded daily.

2.5. Statistical analysis

The data were analyzed with SPSS16.0 using a 3 × 2 factorial experimental design that included 2 factors: L-Thr (0, 0.7, and 1.4 g/kg) and *B. subtilis* strain DSM 32315 (0.0 and 0.5 g/kg) in order to determine the effects of dietary Thr and probiotic *B. subtilis* and their interaction on the reproductive performance of laying duck breeders, the amino acid composition of eggs, and early post-hatch duckling performance. A two-way ANOVA (SPSS 16; SPSS Inc., Chicago, IL) and Tukey's multiple comparisons were performed to compare means. The a and b superscripts refer to a significant difference ($p < 0.05$), and * indicates a tendential difference between treatments at a specific time point ($p = 0.051 \sim 0.06$).

3. Results

3.1. Productive performance

The productive performance data are shown in Table 3. Increasing the L-Thr levels increased egg production rate compared with those of birds fed the control diet (66.22 ± 0.02 (0.7 g Thr /kg) and 66.14 ± 0.02% (1.4 g Thr /kg) *VS* 54.78 ± 0.02% (0.0 g Thr /kg); $p < 0.05$). The addition of *B. subtilis* did not affect the laying performance. Thr and probiotic (*B. subtilis*) co-supplementation had no effect on laying performance ($p > 0.05$).

3.2. Egg quality

The results of egg quality are depicted in Table 4. Both Thr and *B. subtilis* demonstrated no significant effect on ($p > 0.05$) albumen height, Haugh unit, eggshell thickness, eggshell strength, or percentage of albumen, yolk, and eggshell.

10.3389/fvets.2023.1238070

TABLE 3 Effect of dietary L-Thr levels and *Bacillus subtilis* addition on laying performance of laying duck breeders from week 23 to 48 of age[1].

Probiotic (*Bacillus subtilis*) g/kg	L- Thr (g/kg)	Egg production (%)	ADFI (g/d/bird)	Egg weight (g)	Egg mass (g/d)	FCR (g:g)
0.0	0.0	55.45	181	68.93	38.27	4.79
	0.7	65.83	177	73.17	48.65	3.84
	1.4	61.17	183	65.75	40.47	4.64
0.5	0.0	54.11	179	65.71	36.32	5.46
	0.7	66.61	184	73.21	49.67	4.04
	1.4	71.11	177	72.28	51.78	3.53
SEM		4.31	3.89	3.44	4.91	0.52
p-value						
Thr		0.02	0.97	0.23	0.06*	0.06*
Bacillus subtilis		0.38	0.86	0.69	0.39	0.85
Bacillus subtilis X Thr		0.39	0.26	0.36	0.38	0.22

[1]$n = 6$ replicates/treatment of 18 duck breeders per replicate. SEM, Standard error of mean. *indicates a tendential difference between treatments at a specific time point ($p = 0.051 \sim 0.06$). Thr, Threonine; ADFI, average daily feed intake; FCR, feed conversion ratio (feed intake/weight gain).

TABLE 4 Effect of dietary L-Thr levels and *Bacillus subtilis* addition on egg quality at 48-week-old age.[1]

Probiotic (*Bacillus subtilis*) g/kg	L- Thr (g/kg)	Albumen Height (mm)	Haugh unit	Eggshell thickness (mm)	Eggshell strength (kgf)	Albumen (%)	Yolk (%)	Eggshell (%)
0.0	0.0	6.06	75.04	0.32	3.41	58.552	32.534	8.91
	0.7	5.84	75.51	0.332	3.76	59.482	31.2	9.31
	1.4	5.89	76.64	0.328	3.67	59.01	31.749	9.24
0.5	0.0	5.71	75.05	0.318	3.88	59.26	31.25	9.49
	0.7	5.40	71.28	0.321	3.72	58.428	32.556	9.01
	1.4	5.72	71.69	0.33	3.67	58.195	32.68	9.12
SEM		0.29	2.67	0.007	0.213	0.646	0.628	0.187
p value								
Thr		0.65	0.83	0.34	0.88	0.84	0.83	0.98
Bacillus subtilis		0.18	0.17	0.48	0.41	0.45	0.51	0.73
Bacillus subtilis X Thr		0.89	0.62	0.61	0.40	0.34	0.08	0.051*

[1]$n = 3$ eggs per replicate ($n = 18$ eggs per treatment). SEM, Standard error of mean. *indicates a tendential difference between treatments at a specific time point ($p = 0.051 \sim 0.06$).

3.3. Amino acid composition of egg

The amino acid composition results of egg albumen and yolk are displayed in Tables 5, 6. Except for proline, the addition of *B. subtilis* decreased ($p < 0.05$) the concentrations of amino acids in the egg white (albumen). *B. subtilis* supplementation decreased the concentrations of methionine, isoleucine, valine, and glycine while increasing the concentrations of cysteine, tyrosine, and serine ($p < 0.05$) in the egg yolk. In contrast, there was no significant influence ($p > 0.05$) of the dietary L-Thr on the amino acid concentrations in egg albumen and egg yolk.

3.4. Reproductive performance

The results of fertility and hatchability are presented in Table 7. Both Thr and *B. subtilis* demonstrated no significant effect on the reproductive performance ($p > 0.05$). The addition of *B. subtilis* without L-Thr decreased ($p < 0.05$) the percentage of egg hatchability compared with those of ducks fed diets of Thr at 0.7 and 1.4 g /kg along with dietary *B. subtilis* ($p < 0.001$).

3.5. Early post-hatch performance of ducklings

The findings of the early growth performance of ducklings are presented in Table 8. Increasing the L-Thr levels increased the hatching weight of ducklings compared with those of birds fed the control diet [33.98 ± 0.054 (0.7 g Thr /kg) and 34.17 ± 0.054 (1.4 g Thr /kg) *VS* 32.86 ± 0.054 g (0.0 g Thr /kg); $p < 0.001$]. In addition, the hatching weight of ducklings was higher in birds fed L-Thr supplementation at 1.4 g /kg compared to those of birds fed L-Thr

10.3389/fvets.2023.1238070

TABLE 5 Effect of dietary L-Thr levels and *Bacillus subtilis* addition on amino acid composition (g/100 CP) of albumen in laying duck breeders[1].

Amino acid	Treatments						SEM	*p*-Value		
	- *Bacillus subtilis* (0.0 g/kg)			+ *Bacillus subtilis* (0.5 g/kg)				Thr	*Bacillus*	*Bacillus subtilis* × Thr
	L-Thr supplementation (g/kg)			L-Thr supplementation (g/kg)						
	0	0.7	1.4	0	0.7	1.4				
Essential amino acid										
Arginine	3.62	3.59	3.57	3.35	3.33	3.33	0.043	0.70	<0.001	0.93
Isoleucine	2.52	2.43	2.40	2.29	2.27	2.29	0.041	0.30	<0.001	0.36
Leucine	5.85	5.83	5.78	5.43	5.38	5.43	0.068	0.84	<0.001	0.74
Lysine	5.31	5.31	5.30	4.99	4.94	4.97	0.062	0.94	<0.001	0.89
Methionine	4.09	4.11	4.29	3.92	3.87	3.72	0.096	0.99	<0.001	0.09
Phenylalanine	5.87	5.87	5.80	5.44	5.46	5.54	0.070	0.98	<0.001	0.38
Threonine	5.14	5.12	5.11	4.79	4.76	4.82	0.058	0.89	<0.001	0.80
Valine	4.27	4.13	4.12	3.91	3.88	3.93	0.058	0.31	<0.001	0.38
Histidine	1.59	1.58	1.57	1.44	1.43	1.45	0.018	0.91	<0.001	0.83
Semi-essential amino acid										
Cysteine	2.02	1.97	2.00	1.84	1.74	1.71	0.048	0.21	<0.001	0.581
Tyrosine	3.23	3.22	3.25	3.02	2.97	3.00	0.046	0.75	<0.001	0.803
Non- essential AAs										
Alanine	3.94	3.89	3.89	3.64	3.62	3.62	0.041	0.65	<0.001	0.91
Aspartic acid	7.84	7.88	7.87	7.40	7.34	7.40	0.091	0.95	<0.001	0.83
Glutamic acid	11.83	11.82	11.76	11.02	10.97	11.10	0.125	0.95	<0.001	0.71
Glycine	2.98	2.98	2.96	2.75	2.70	2.71	0.032	0.59	<0.001	0.69
Serine	7.04	7.03	7.01	6.58	6.58	6.62	0.077	0.99	<0.001	0.89
Proline	3.10	3.13	3.20	3.06	3.09	3.13	0.042	0.17	0.13	0.95

$n = 18$ egg albumen per replicate. SEM, Standard error of mean; Thr, Threonine.

supplementation at 0.7 g /kg (34.17 ± 0.054 vs. 33.98 ± 0.054 g, $p < 0.001$). In contrast, supplementation of *B. subtilis* decreased duckling hatching weight ($p < 0$ 0.001). The addition of *B. subtilis* without L-Thr decreased hatching weight compared with those of ducks fed dietary Thr, along with dietary *B. subtilis* [31.95 ± 0.072 (0.0 g Thr /kg + 0.5 g *B. subtilis /kg*) vs. 34.08 ± 0.072 (0.7 g Thr / kg + 0.5 g *B. subtilis /kg*) and 33.98 ± 0.072 g (1.4 g Thr /kg + 0.5 g *B. subtilis /kg*); $p < 0.001$].

4. Discussion

According to our knowledge, this is the first study to examine the simultaneous effects of Thr and *B. subtilis* supplementation on laying duck breeders. Following the restriction of antibiotics as growth promoters in many countries, the use of probiotics or synthetic amino acids in poultry diets has significantly increased (17–20). Previous studies found that a combination of amino acids and probiotics could synergistically or additively compensate to improve production performance, egg quality, and immunity (9). Therefore, we hypothesized that the combination of Thr and *B. subtilis* DSM32315 may improve the amino acid profile of eggs and the early post-hatch performance of ducklings fed antibiotic-free diets. In the present study, increasing dietary Thr intake improved egg production. It is well known that Thr is an indispensable amino acid because it cannot be synthesized in the body. In laying breeds, it has been reported that increasing dietary Thr level enhanced egg production, egg weight, egg mass, feed conversion ratio, albumen weight, and Haugh units (10, 21). Threonine has been shown to have a beneficial impact on nutrient absorption by augmenting the surface area of the villi (10). This may help to elucidate why the supplementation of Thr has been observed to enhance productive performance. Also, previous studies have reported an improvement in egg production in birds fed probiotics *B. subtilis* (5, 22, 23). However, in the present study, the addition of *B. subtilis* did not affect the laying performance. There is a scarcity of experiments on the effect of *B. subtilis* on laying ducks. Consistent with the findings of the present studies, the prior study revealed that probiotic supplementation (*Clostridium butyricum* and a combination of *Saccharomyces boulardii* and *Pediococcus acidilactici*) (24) or *B. subtilis* RX7 and B2A supplementation did not impact the laying rate under no stress conditions. Other studies have reported an improvement in egg production in birds fed probiotics *B. subtilis* (5, 22) under no stress conditions. These contradictory results may be attributed to differences in the addition level, diet

TABLE 6 Effect of dietary L-Thr levels and *Bacillus subtilis* addition on amino acid composition (g/100 CP) of yolk in laying duck breeders[1].

Amino acid	Treatments						SEM	*P*-Value		
	- *Bacillus subtilis* (0.0 g/kg)			+ *Bacillus subtilis* (0.5 g/kg)				Thr	*Bacillus*	*Bacillus subtilis* × Thr
	L-Thr supplementation (g/kg)			L-Thr supplementation (g/kg)						
	0.0	0.7	1.4	0.0	0.7	1.4				
Essential amino acid										
Arginine	1.898	1.881	1.828	1.820	1.817	1.829	0.086	0.93	0.51	0.88
Isoleucine	1.283	1.556	1.421	1.044	1.071	1.096	0.118	0.44	0.001	0.57
Leucine	2.274	2.540	2.417	2.279	2.279	2.293	0.150	0.67	0.30	0.67
Lysine	2.149	2.381	2.272	2.161	2.162	2.183	0.138	0.69	0.38	0.70
Methionine	0.580	0.548	0.521	0.485	0.314	0.328	0.057	0.12	0.001	0.46
Phenylalanine	1.248	1.374	1.322	1.244	1.214	1.232	0.072	0.79	0.16	0.56
Threonine	1.316	1.504	1.415	1.507	1.500	1.514	0.084	0.56	0.17	0.52
Valine	1.448	1.688	1.550	1.163	1.202	1.246	0.117	0.48	0.001	0.64
Histidine	0.790	0.835	0.809	0.749	0.746	0.759	0.045	0.89	0.11	0.85
Semi-essential amino acid										
Cysteine	0.256	0.233	0.213	0.366	0.402	0.455	0.029	0.71	<0.001	0.09
Tyrosine	0.882	0.862	0.861	1.136	1.148	1.139	0.064	0.99	<0.001	0.96
Non- essential AAs										
Alanine	1.520	1.450	1.372	1.408	1.428	1.445	0.060	0.65	0.68	0.31
Aspartic acid	2.493	2.640	2.525	2.560	2.545	2.549	0.128	0.85	0.98	0.80
Glutamic acid	3.652	3.623	3.436	3.436	3.417	3.450	0.157	0.80	0.29	0.71
Glycine	1.219	0.895	0.855	0.859	0.845	0.851	0.081	0.054*	0.045	0.07
Serine	1.695	2.001	1.892	2.372	2.366	2.372	0.123	0.47	<0.001	0.44
Proline	1.125	1.083	1.076	1.112	1.156	1.102	0.055	0.82	0.53	0.73

$n = 18$ egg yolk per replicate. SEM, Standard error of mean; Thr, Threonine. *indicates a tendential difference between treatments at a specific time point ($p = 0.051 \sim 0.06$).

TABLE 7 Effect of dietary L-Thr levels and *Bacillus subtilis* addition on fertility and hatchability rate in laying duck breeders.[1]

Probiotic (*Bacillus subtilis*) g/Kg	L-Thr (g/kg)	Fertility (%)	Hatchability of fertile eggs(%)	Mortality, %
0.0	0.0	80.97	67.60^{ab}	0.048
	0.7	86.64	64.25^{ab}	0.035
	1.4	84.61	69.72^{ab}	0.037
0.5	0.0	83.99	54.16^{b}	0.062
	0.7	83.31	71.76^{a}	0.052
	1.4	86.02	64.62^{ab}	0.042
SEM		3.74	3.72	
P-value				
Thr		0.71	0.13	0.61
Bacillus subtilis		0.90	0.23	0.39
Bacillus subtilis X Thr		0.68	0.02	0.93

$n = 50$ eggs per replicate. SEM, Standard error of mean; Thr, Threonine. Means sharing different letters (superscripts a, b) differ significantly.

composition, species and concentrations of *B. subtilis*, age of birds, different microorganisms used, and the experiential conditions (25). Since the study was conducted under normal conditions, *B. subtilis* DSM32315 has no effect on laying performance. Further studies are needed to evaluate the effects of *B. subtilis* DSM32315 on breeder-laying ducks under different stress conditions.

The current study showed that Thr supplementation had no effects on the egg quality parameters. However, eggshell percentage increased after supplementing a combination of *B. subtilis* DSM32315 and Thr. Studies have shown that supplementing laying hens with probiotics improves eggshell quality and decreases the number of damaged eggs (26, 27). Inclusion of *B. subtilis* in hen laying hen diets improved eggshell quality as a result of enhancing calcium absorption (28). Ovalbumin is the predominant protein in albumen; it provides essential amino acids for chicken embryo development and is a valuable source of amino acids required for embryonic development (29). Herein, the addition of *B. subtilis* decreased methionine, isoleucine, and valine, in the egg albumen and yolk. This finding indicates that *B. subtilis* may affect essential amino acids and limit their retention and decreasing embryonic growth rates.

In breeding programs and production, the hatchability rate is an essential factor. Intriguingly, the percentage of ducklings that successfully hatched and their hatching weights increased when birds were fed *B. subtilis* with 0.7 g/kg Thr. Daris et al. (26) suggested that dietary *B. subtilis* improve eggshell quality, which led to enhance hatchability in broiler breeder hens.

TABLE 8 Effect of dietary L-Thr levels and *Bacillus subtilis* addition on early post-hatch performance of ducklings.

Probiotic (*Bacillus subtilis*) g/Kg	L- Thr (g/kg)	Progeny early growth performance (ducklings)					
		Hatching weight, g (d 1)	BW, g (d7)	BWG, g	ADFI (g/d/bird)	FCR (g: g)	Mortality, %
0.0	0.0	33.78^{b}	135	101	169	1.67	0
	0.7	33.89^{b}	128	94.125	153	1.68	0
	1.4	34.36^{a}	128	93.256	139	1.49	0
0.5	0.0	31.95^{c}	127	94.733	125	1.31	0
	0.7	34.08^{ab}	134	99.872	138	1.38	0
	1.4	33.98^{b}	132	97.619	149	1.53	0
SEM		0.072	3.431	3.43	13.47	0.147	-
Thr		<0.001	0.90	0.76	0.96	0.97	-
Bacillus subtilis		<0.001	0.85	0.67	0.13	0.09	-
Bacillus subtilis X Thr		<0.001	0.09	0.16	0.15	0.35	-

n = 6 replicates/ treatment of 13 ducklings per replicate. SEM, Standard error of mean; Thr, Threonine; BW, body weight; BWG, body weight gain; ADFI, average daily feed intake; FCR, feed conversion ratio (feed intake/weight gain). Means sharing different letters (superscripts a-c) differ significantly.

5. Conclusion

The current study revealed that the combination supplemented of L-Thr and *B. subtilis* DSM 32315 is recommended due to its positive effects on the eggshell percentage, hatchability and the body weights of newly hatched ducklings when dietary Thr was added at a rate of 0.7 g/kg and *B. subtilis* DSM 32315 at 0.5 g/kg. In addition, adding L-Thr separately at 0.7 g/kg could improve the egg production of duck breeders. Further studies are required to find the proper dosages of *B. subtilis* DSM 32315 with co-dietary inclusion of limiting amino acids in the diets of duck breeders. The findings of these trials will support feed additive interventions to transition into antibiotic-free diets.

Data availability statement

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

Ethics statement

The Animal Care and Use Committee of Guangdong Academy of Agriculture Sciences approved all experimental procedures (GAASIAS-2019-020).

Author contributions

MA and CZ: conceptualization. MA, HE-S and CZ: methodology. MA: investigation and writing–original draft preparation. WX and SW: data curation. YZ, WC, and WX: formal analysis. MA and HE-S: writing–review and editing. CZ: supervision. CZ and MA: funding acquisition. All of the authors contributed to the article and approved the final version.

Funding

This study was supported by the China Agriculture Research System of MOF and MARA (Grant No. CARS-42-13), the Modern Agricultural Industry Technology System Innovation Team of Guangdong Province(Grant No. 2019KJ137), the Special Fund for Scientific Innovation Strategy-Construction of High Level Academy of Agricultural Sciences (R2020PY-JX008, 202106TD), and the Science and Technology Program of Guangdong Province (Grant No. 2019A050505007, 2021A0505050003), Foreign Expert Project (QNL20200130001) and Researchers Supporting Project Number (RSPD2023R731), King Saud University (Riyadh, Saudi Arabia).

Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1238070/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 28 November 2023
DOI 10.3389/fvets.2023.1298587

OPEN ACCESS

EDITED BY
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RECEIVED 21 September 2023
ACCEPTED 15 November 2023
PUBLISHED 28 November 2023

CITATION
Paneru D, Tellez-Isaias G, Arreguin-Nava MA, Romano N, Bottje WG, Asiamah E, Abdel-Wareth AAA and Lohakare J (2023) Effect of fenugreek seeds and *Bacillus*-based direct-fed microbials on the growth performance, blood biochemicals, and intestinal histomorphology of broiler chickens. *Front. Vet. Sci.* 10:1298587. doi: 10.3389/fvets.2023.1298587

Effect of fenugreek seeds and *Bacillus*-based direct-fed microbials on the growth performance, blood biochemicals, and intestinal histomorphology of broiler chickens

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Background: The objective of the present study was to evaluate the potential synergistic impact of the combination of fenugreek seeds (FS) and *Bacillus*-based direct-fed microbials (DFM) on growth performance, intestinal health, and hematological parameters of broiler chickens.

Methods: A total of 160 one-day-old (Ross 308) broiler chicks were randomly assigned to a 2 × 2 factorial arrangement, with two levels of FS (0 and 5 g/kg) and two levels of *Bacillus*-DFM (0 and 0.1 g/kg), with five replicates of 8 birds each.

Results: The result showed that dietary supplementation of FS at 5 g/kg did not improve the growth performance of broilers but impaired the early growth performance by reducing body weight gain and increasing feed conversion ratio, which was recovered during finisher phase. Dietary supplementation of *Bacillus*-based DFM at 0.1 g/kg did not affect the performance variables but increased the feed conversion ratio. The interaction of fenugreek seeds and *Bacillus*-based DFM showed synergistic effects on growth performance during the later stages of production. However, antagonistic effects were observed on the blood parameters and the gut morphology.

Conclusion: This study demonstrated that FS and DFM had different effects on the broiler health and production depending on the phase of production. The interaction between FS and DFM revealed synergistic effects on growth performance during the finisher phase, but antagonistic effects on blood parameters and gut morphology. Further studies are needed to elucidate the underlying mechanisms and optimize the dosage and combination of FS and DFM for broiler health and production.

KEYWORDS

broilers, nutrition, intestinal morphology, fenugreek, *Bacillus*-DFM

Introduction

In recent years, there has been growing concern about the impact of antibiotics used in poultry feed on public health and the environment, prompting a need to reassess this controversial practice (1–4). The addition of subtherapeutic doses of antibiotics to poultry feed has resulted in the emergence of antibiotic-resistant pathogenic bacteria (5) and the accumulation of antibiotic residues in poultry meat and eggs (6). Consequently, the European Commission has imposed restrictions on the use of antibiotic growth promoters AGPs (7), and the United States Food and Drug Administration has requested that pharmaceutical companies cease labeling antimicrobials as growth promoters in agriculture animals under Guidance-213 and that antibiotic can only be administered for therapeutic purposes under veterinarian supervision (8). This has intensified the search for alternative, non-antibiotic solutions to maintain or improve poultry health and performance while addressing health and safety concerns for consumers, which drives the growing preference for natural and safe alternatives such as phytogenic and direct-fed microbials (DFM) (9, 10).

Fenugreek (*Trigonella foenum-graecum*) is a potential alternative to antibiotics in poultry due to its nutritional and bioactive properties. The high soluble fiber content of fenugreek seeds (FS) is thought to improve gut health by promoting the growth of beneficial bacteria and reducing harmful bacteria (11). This, in turn, may lead to improved feed efficiency and digestive health, as well as increased growth and disease resistance in poultry. Additionally, FS contains various bioactive compounds, such as saponins, alkaloids, and flavonoids, which possess health-promoting effects (12). These compounds may modulate the immune system, provide antioxidant activity, and regulate hormones, potentially contributing to improved health and performance in poultry (13–15). FS has anti-inflammatory and antimicrobial properties, which may help prevent and control infections in poultry (16).

Bacillus-based DFMs, another promising alternative to AGPs, are probiotics intended to improve gut health and enhance nutrient utilization in broiler chickens (17, 18). The mode of action of DFMs includes (a) augmentation of beneficial bacteria populations, such as *Lactobacillus* and *Bifidobacterium* spp., (b) reduction of pathogenic bacteria through competitive exclusion or by producing bacteriocins, (c) stimulation of metabolism by increasing both endogenous and exogenous digestive enzymes, (d) amelioration of ammonia production, (e) neutralization of enterotoxins (f) enhancement of the immune system, and (g) generation of beneficial metabolic byproducts, such as volatile fatty acids (19, 20).

Furthermore, as effective natural probiotics and phytogenic become more widely available, they are becoming a popular alternative to antibiotics in chickens to boost productivity and reproduction (16). Fenugreek seeds, which are high in dietary fiber, soluble fiber, and biologically active phytochemicals, act as a prebiotic (16) and may have synergistic effects, offering health-promoting benefits or stimulating broiler growth performance. However, few research have focused on the utilization of fenugreek seeds and *Bacillus*-DFMs to improve broiler chicken growth performance, gut integrity, and health. Furthermore, the synergistic benefits of probiotics and fenugreek seeds in broilers have not been consistently established in previous studies. It is hypothesized that fenugreek seeds and DFM will influence intestinal health and improve intestinal absorption, which altogether improves performance.

For this reason, the proposed studies aimed to assess the potential synergistic impact of the combination of FS and *Bacillus*-based DFM on growth performance, intestinal health, blood biochemical, and hematological parameters of broiler chickens.

Materials and methods

All the experimental procedures were conducted in accordance with the ethical guidelines set by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas at Pine Bluff and were approved under protocol number #UAPB2020-04. The protocols adhered to the principles outlined in the National Institutes of Health's (NIH) Guide for the Care and Use of Laboratory Animals to ensure the humane treatment of all animals involved in the experiments.

Preparation of fenugreek seeds and *Bacillus*-based direct-fed microbials

Fenugreek seeds (Deep Foods Inc., Union, NJ) were procured from a local supplier in Little Rock, AR. The seeds were ground using a Grinding Grain Mill (Thomas Scientific, Swedesboro, NJ) utilizing a 1 mm sieve to achieve a medium consistency. The ground seeds were then added on the top of starter and finisher diets at the rate of 5 g/kg of the diets in the experimental groups requiring FS. The GC–MS analysis of the fenugreek seed extract was done previously using gas chromatography–mass spectrometry (21). The extract of fenugreek seeds powder contained several active compounds, with the most abundant ones being 2-phenyl-4-(trimethylsilyl) furan at 25.45%, followed by 9,17-octadecadienal at 11.26%, curlone at 10.92%, α-curcumene at 10.31%, and cis,cis,cis-7,10,13-hexadecatrienal at 8.1%. Additionally, other compounds were present in smaller percentages, including tetradecanal, β-sesquiphellandrene, zingiberene, β-bisabolene, 7-methoxymethyl-2,7-dimethylcyclohepta-1,3,5-triene, caryophyllene oxide, 5-fluoro-1,1,3,3-tetramethyl-1,3-dihydroisobenzofuran, thymol, linoleic acid, p-cymene, α–tumerone, trans-caryophyllene, palmitic acid, and benzaldehyde, each in varying proportions.

Norum™, a patented *Bacillus*-direct fed microbial (DFM) from the University of Arkansas, consisting of three *Bacillus* strains selected for their enzyme activity. Norum™ was sourced from Eco-Bio/Euxxis Bioscience LLC and added to feed at 0.1 g/kg for experimental groups. The *Bacillus* strains within Norum™ were identified through whole-genome sequencing as *Bacillus subtilis* (AM1002), *Bacillus amyloliquefaciens* (AM0938), and *Bacillus licheniformis* (JD17) (22).

Birds and experimental design

One-day-old male broiler chicks (Ross 308; $n = 160$) were obtained from a commercial hatchery (Keith Smith, Hot Springs, AR). Upon arrival, the chicks were weighed and randomly assigned to four treatment groups in a 2×2 factorial design, with five pens per treatment and 8 birds per pen. Each floor pen ($180 \times 90 \times 48\ cm^3$) was covered with pine wood shavings and equipped with separate feeders and drinkers (Harris Farms, Tractor Supply Co., Pine Bluff, AR). The

housing temperature was carefully controlled throughout the growth cycle of the birds, starting at 33.5°C at the time of placement and gradually decreasing to 23.6°C by day 42 following the breeder recommendation. The birds were monitored twice daily to ensure their well-being, and factors such as room temperature, bird condition, mortality, and the availability of feed and water were checked during each inspection to ensure optimal conditions for growth and health. The treatments consisted of a corn-soybean meal basal diet (Table 1) supplemented with two levels of FS (F0 = 0 g/kg FS and F1 = 5 g/kg FS) and two levels of DFM (D0 = 0 g/kg DFM and D1 = 0.1 g/kg DFM). The diets were formulated to meet or exceed the nutrient requirements of Ross 308, with two-phase feeding system (starter, 0 to 21 days) and (finisher, 22 to 42 days) (23). Feed was provided in mash form. All birds were offered *ad libitum* access to feed and water throughout the study period.

TABLE 1 Ingredient composition of the basal diet for starter and finisher growth periods, as-fed basis.

Ingredient (%)	Starter (0 to 21 days)	Finisher (22 to 42 days)
Corn	59.4	66.5
Soybean meal	32.9	25.3
Pro plus[1]	2.5	0
Meat and bone meal, 50%	0	2.5
Poultry oil	2.01	3.14
Sodium chloride	0.38	0.31
Sodium bicarbonate	0	0.05
Limestone	0.8	0.7
Dicalcium phosphate	1.13	0.85
Vitamin Premix[2]	0.1	0.1
Mineral premix[3]	0.1	0.1
Choline Chloride	0.1	0.1
Selenium PMX 0.06%	0.02	0.02
Santoquin	0.02	0.02
L-Lysine HCL	0.17	0.10
DL-Methionine	0.3	0.21
L-Threonine	0.11	0.05
Copper chloride	0.02	0
Xylanase	0	0.01
Phytase	0.02	0.02
Total	100	100
Calculated analysis		
ME (kcal/kg)	3015.00	3090.00
Crude protein (%)	22.30	20.00
Lysine (%)	1.18	1.05
Methionine (%)	0.59	0.53
Total calcium (%)	0.90	0.84
Available phosphorus (%)	0.45	0.42

[1]Pro-Plus is an animal by-product blend with a crude protein content of 60% (H. J. Baker & Bros. Inc., Little Rock, AR).
[2]Vitamin premix (provided the following per kilogram of diet): vitamin A (trans retinyl acetate), 3,600 IU; vitamin D_3 (cholecalciferol), 800 IU; vitamin E (DL-α-tocopheryl acetate), 7.2 mg; vitamin K3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxin, 1.2 mg; cobalamine, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.
[3]Mineral premix (provided the following per kilogram of diet): Mn (from $MnSO_4H_2O$), 40 mg; Zn (from ZnO), 40 mg; Fe (from $FeSO_47H_2O$), 20 mg; Cu (from $CuSO_45H2$ O), 4 mg; I [from $Ca(IO_3)_2H_2O$], 0.64 mg; Se (from sodium selenite), 0.08 mg.

Proximate and mineral nutrient analysis

Feed samples were collected during bagging and analyzed for proximate and mineral nutrients at University of Arkansas (Fayetteville, AR). Table 2 shows the analyzed composition of all experimental diets. Dry matter was determined by drying the samples at 55°C overnight. Crude protein was determined by the Kjeldahl method as described by (24). Ether extract was determined by the Soxhlet method following the procedure of AOSC (25). Neutral detergent fiber and acid detergent fiber were determined by the methods of (26) and (27), respectively. Mineral nutrients were determined by digesting the samples with HNO_3 and H_2O_2 and measuring them by atomic absorption spectrophotometry as described by (28).

Data collection

Data on live body weight (BW) and feed intake (FI) per pen were recorded at the end of both the starter period (d 21) and the finisher period (d 42) and used to calculate the mean values for body weight gain (BWG), FI, and feed conversion ratio (FCR). Mortality was recorded as it occurred.

At the end of the experimental period (d 42), two birds from each pen with a mean body weight were selected and humanely euthanized via rapid decapitation technique. Blood was collected from the jugular vein into 3 ml BD Vacutainer EDTA Blood Collection Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) for whole blood analysis. The whole blood samples were analyzed for white blood cell (WBC) count, hemoglobin, total protein, heterophil, lymphocyte, monocyte, and basophil counts. Another 3 ml of blood was collected in BD Vacutainer Serum Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) to obtain blood serum. The collected blood was allowed to clot for approximately 2 h at room temperature and then centrifuged at 2000 x g for 15 min using a Centrifuge 5430R (Eppendorf SE, Enfield, CT) to separate the serum. The serum samples were analyzed for cholesterol, albumin (AL), globulin (GL), albumin/globulin ratio (A: G), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT). The whole blood and serum samples were analyzed by an external laboratory, the Arkansas State Veterinary Laboratory in Little Rock, AR.

The birds utilized for blood collection were then subjected to evisceration to obtain segments of the small intestine for detailed morphological examination. Samples of the mid-region of the jejunum and ileum were collected, washed with 10X phosphate-buffered saline, and then fixed in Bouin's solution for 24 h. The samples were then preserved in 70% ethanol for 24 h and underwent a progressive dehydration process using increasing ethanol concentrations, followed by clearing in xylene. The samples were embedded in paraffin wax and sectioned at 5 µm thickness using a manual rotary microtome (Leica Biosystems, Buffalo Grove, IL). Two

10.3389/fvets.2023.1298587

TABLE 2 Analyzed composition of the experimental diets for starter and finisher periods, as DM basis.[1]

Item, %	Starter				Finisher			
	F0		F1		F0		F1	
	D0	D1	D0	D1	D0	D1	D0	D1
Dry matter	91.3	90.8	90.9	91	91.3	91	91	91.1
Crude protein	24.4	23.5	24.5	25.7	21.4	22.1	22.1	21.8
Ether extract	5.3	5.82	5.63	5.36	7.06	7.09	7.85	6.61
NDF	14.5	13.2	12.4	11.7	11.2	13.5	11.4	13.7
ADF	6	5.7	5.7	5.7	5.8	6.3	5.4	5.5

[1]F0 = first level of fenugreek seeds, 0 g/kg of diet; F1 = second level of fenugreek seeds, 5 g/kg of diet; D0 = first level of *Bacillus*-based direct-fed microbial, 0 g/kg of diet; D1 = Second level of *Bacillus*-based direct-fed microbial, 0.1 g/kg of diet.

TABLE 3 Growth performance of birds during the starter phase (0–21 days).[1]

FS	DFM	Starter Phase (0 to 21 days)		
		BWG (g)	FI (g)	FCR (g)
Main effects				
F0		934[a]	1,210	1.30[b]
F1		863[b]	1,181	1.37[a]
	D0	918	1,214	1.32
	D1	879	1,176	1.34
SEM		14	18	0.01
p-values				
FS		0.0027	0.2689	<0.0001
DFM		0.0708	0.1627	0.1908
Interaction effects				
F0	D0	960	1,244	1.30
F0	D1	907	1,176	1.30
F1	D0	875	1,184	1.35
F1	D1	851	1,177	1.38
SEM		20	26	0.01
p-value				
FS × DFM		0.4707	0.2440	0.2347

[a,b]Means within a column not sharing a common superscript differ significantly ($p < 0.05$).
[1]FS = fenugreek seeds; DFM = direct-fed microbials; F0 = 0 g/kg FS; F1 = 5 g/kg FS; D0 = 0 g/kg DFM; and D1 = 0.1 g/kg DFM.

sections, taken at different depths, were stained with hematoxylin and eosin, and images were taken at 200x magnification. Five villi and crypts that were perpendicular to the muscularis mucosae and had a clear boundary with the adjacent structure were selected for further analysis. The height and depth of five randomly selected villi and crypts from each replicate were measured using Leica software (Leica DM3000, Leica Biosystems, Buffalo Grove, IL).

Statistical analysis

Replicate pens were considered as the experimental unit for all analyses. The data were analyzed using a 2 × 2 factorial design with the Fit Model platform of JMP Pro 16.1 (SAS Institute Inc., Cary, NC) to examine the main effects and interactions between FS and Bacillus-DFM. Interaction effects that revealed significant differences were separated using the Tukey HSD, while main effects that displayed significant differences were separated using Student's *t*-test. Statistical significance was set at a *p*-value of less than 0.05. The results were presented as least square means and the pooled standard error of the mean.

Results

Growth performance

The effects of fenugreek seeds (FS) and direct-fed microbials (DFM) on the growth performance of broiler chickens during the starter phase (0 to 21 days) are shown in Table 3. The main effect of FS was significant for body weight gain (BWG) and feed conversion ratio (FCR), but not for feed intake (FI). Chickens fed with F0 had higher BWG than those fed with F1 ($p = 0.0027$). Chickens fed with F0 also had lower FCR than those fed with F1 ($p < 0.0001$). There was no significant difference in FI between F0 and F1 ($p = 0.2689$). The main effect of DFM was not significant for any of the performance parameters. Chickens fed with D0 had similar BWG, FI and FCR as those fed with D1 ($p > 0.05$). There was no significant interaction between FS and DFM for any of the performance parameters ($p > 0.05$).

The effects of fenugreek seeds (FS) and direct fed microbials (DFM) on the performance of broiler chickens during the finisher phase (22 to 42 days) are shown in Table 4. The main effects of FS and DFM were not significant for any of the performance parameters. Chickens fed with F0 had similar BWG, FI and FCR as those fed with F1 ($p > 0.05$). Chickens fed with D0 had similar BWG, FI and FCR as those fed with D1 ($p > 0.05$). There was a significant interaction between FS and DFM for BWG ($p = 0.0353$), but not for FI and FCR ($p > 0.05$). Chickens fed with F0D0 had the highest BWG among all the treatments, while chickens fed with F0D1 had the lowest BWG.

The effects of FS and DFM on the performance of broiler chickens during the overall phase (0 to 42 days) are shown in Table 5. The main effect of FS was not significant for BWG, FI or FCR. Chickens fed with F0 had similar BWG, FI and FCR as those fed with F1 ($p > 0.05$). The main effect of DFM was significant for FCR, but not for BWG and FI. Chickens fed with D0 had similar BWG and FI as those fed with D1 ($p > 0.05$). Chickens fed with D0 had lower FCR than those fed with D1 ($p = 0.0495$). There was a significant interaction between FS and DFM for BWG ($p = 0.0328$), but not for FI and FCR ($p > 0.05$).

10.3389/fvets.2023.1298587

TABLE 4 Growth performance of birds during the finisher phase (22–42 days).[1]

FS	DFM	Finisher phase (22 to 42 days)		
		BWG (g)	FI (g)	FCR (g)
Main effects				
F0		1738	2,883	1.67
F1		1708	2,718	1.60
	D0	1762	2,747	1.57
	D1	1,683	2,854	1.71
SEM		49	66	0.05
p-values				
FS		0.6642	0.0972	0.3305
DFM		0.2683	0.2715	0.0706
Interaction effects				
F0	D0	1857[a]	2,852	1.55
F0	D1	1620[b]	2,914	1.80
F1	D0	1668[ab]	2,642	1.58
F1	D1	1747[ab]	2,794	1.62
SEM		69	94	0.07
p-value				
FS × DFM		0.0353	0.6428	0.1487

[a,b]Means within a column not sharing a common superscript differ significantly ($p < 0.05$).
[1]FS = fenugreek seeds; DFM = direct-fed microbials; F0 = 0 g/kg FS; F1 = 5 g/kg FS; D0 = 0 g/kg DFM; and D1 = 0.1 g/kg DFM.

TABLE 5 Growth performance of birds during the overall phase (0–42 days).[1]

FS	DFM	Overall Phase (0–42 days)		
		BWG (g)	FI (g)	FCR (g)
Main effects				
F0		2,672	4,093	1.54
F1		2,570	3,898	1.52
	D0	2,680	3,961	1.48[b]
	D1	2,562	4,030	1.58[a]
SEM		52	73	0.03
p-values				
FS		0.1898	0.0786	0.6928
DFM		0.1317	0.5123	0.0495
Interaction effects				
F0	D0	2817[a]	4,096	1.46
F0	D1	2527[b]	4,090	1.62
F1	D0	2543[ab]	3,826	1.50
F1	D1	2598[ab]	3,971	1.54
SEM		74	104	0.05
p-value				
FS × DFM		0.0328	0.4777	0.1689

[a,b]Means within a column not sharing a common superscript differ significantly ($p < 0.05$).
[1]FS = fenugreek seeds; DFM = direct-fed microbials; F0 = 0 g/kg FS; F1 = 5 g/kg FS; D0 = 0 g/kg DFM; and D1 = 0.1 g/kg DFM.

Chickens fed with F0D0 had the highest BWG among all the treatments, while chickens fed with F0D1 had the lowest BWG.

Hematological parameters

The effects of fenugreek seeds (FS) and direct fed microbials (DFM) on the hematological parameters of broiler chickens on d 42 are shown in Table 6. The main effect DFM was significant for white blood cells (WBC), total protein (TP), lymphocyte count and heterophil: lymphocyte ratio (H: L), but not for heterophil count and basophil count. Chickens fed with F0 had similar WBC, TP, heterophil count, lymphocyte count, basophil count and H: L as those fed with F1 ($p > 0.05$). Chickens fed with F0 had lower Hb than those fed with F1 ($p = 0.0268$). Chickens fed with D0 had higher WBC and TP than those fed with D1 ($p < 0.05$). Chickens fed with D0 had similar Hb, heterophil count and basophil count as those fed with D1 ($p > 0.05$). Chickens fed with D0 had higher lymphocyte count and lower H: L than those fed with D1 ($p < 0.05$). There was a significant interaction between FS and DFM for WBC, TP, heterophil count and lymphocyte count ($p < 0.05$), but not for Hb, basophil count and H: L ($p > 0.05$). Chickens fed with F0D0 had the highest WBC, heterophil count and lymphocyte count among all the treatments, while chickens fed with F0D1 had the lowest WBC and lymphocyte count.

Blood biochemical parameters

The effects of FS and DFM on the serum biochemical parameters of broiler chickens on d 42 are shown in Table 7. The main effects of FS and DFM were not significant for any of the serum biochemical parameters. Chickens fed with F0 had similar albumin, globulin, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and cholesterol as those fed with F1. Chickens fed with had similar albumin, globulin, AST, GGT and cholesterol as those fed with D1. There was no significant interaction between FS and DFM for any of the serum biochemical parameters ($p > 0.05$).

Intestinal histomorphology

The effects of FS and DFM on the villus height (Vh), crypt depth (Cd) and villus height: crypt depth ratio (Vh:Cd) of the jejunum of broiler chickens at d 42 are shown in Table 8. The main effects of FS and DFM were significant for Cd and Vh:Cd, but not for Vh. Chickens fed with F0 had similar Vh as those fed with F1 ($p > 0.05$). Chickens fed with F0 had higher Cd and lower Vh:Cd than those fed with F1 ($p < 0.05$). Chickens fed with D0 had similar Vh as those fed with ($p > 0.05$). Chickens fed with D0 had lower Cd ($p < 0.05$) and numerically higher Vh:Cd ($p = 0.0702$) than those fed with D1. There was a significant interaction between FS and DFM for Vh and Cd ($p < 0.05$), but not for Vh:Cd ($p > 0.05$). Chickens fed with F1D0 had the highest Vh among all the treatments, while chickens fed with F0D1 had the highest Cd. However, FS also induced inflammation and monocyte infiltration in the jejunum epithelium and lamina propria (Figure 1), which may impair the gut barrier function and increase the susceptibility to pathogens.

10.3389/fvets.2023.1298587

TABLE 6 Hematological parameters of birds fed fenugreek seeds (FS) and Direct fed microbials (DFM) on d 42.[1]

FS	DFM	WBC (10^3/μl)	Hb (g/dl)	TP (g/dl)	HC (n/μl)	LC (*n*/μl)	BC (*n*/μl)	HLR (*n*/*n*)
Main effects								
F0		20.59	10.54[b]	2.82	8,879	5,935	5,696	1.76
F1		19.14	11.08[a]	2.84	8,601	5,526	4,957	1.80
	D0	21.34[a]	10.77	3.00[a]	8,720	6767[a]	5,815	1.34[b]
	D1	18.39[b]	10.86	2.67[b]	8,761	4694[b]	4,838	2.22[a]
SEM		0.96	0.27	0.08	424	551	427	0.17
p-values								
FS		0.2905	0.0268	0.8387	0.6469	0.6033	0.2300	0.8745
DFM		0.0368	0.7174	0.0053	0.9457	0.0120	0.1155	0.0009
Interaction effects								
F0	D0	24.00[a]	10.51	3.11	9839[a]	7803[a]	6,334	1.30
F0	D1	17.18[b]	10.57	2.53	7919[b]	4067[b]	5,057	2.23
F1	D0	18.67[ab]	11.03	2.89	7600[b]	5731[ab]	5,295	1.38
F1	D1	19.60[ab]	11.14	2.80	9603[a]	5321[ab]	4,619	2.23
SEM		1.50	0.27	0.13	571	885	686	0.23
p-values								
FS × DFM		0.0073	0.9134	0.0335	0.0025	0.0405	0.6221	0.8763

[a,b]Means within a column not sharing a common superscript differ significantly ($p < 0.05$).

[1]FS = fenugreek seeds; DFM = direct-fed microbials; F0 = 0 g/kg FS; F1 = 5 g/kg FS; D0 = 0 g/kg DFM; and D1 = 0.1 g/kg DFM; WBC = white blood cell count; Hb = hemoglobin content; TP = total protein level; HC = heterophil count; LC = lymphocyte count; BC = basophil count; HLR = heterophil to lymphocyte ratio.

TABLE 7 Blood serum parameters of birds fed fenugreek seeds (FS) and Direct fed microbials (DFM) on d 42.[1]

FS	DFM	Albumin (g/dl)	Globulin (g/dl)	AST (IU/L)	GGT (IU/L)	Cholesterol (mg/dl)
Main effects						
F0		0.69	1.35	245	21.34	86.6
F1		0.69	1.33	252	22.40	89.6
	D0	0.70	1.34	243	21.39	88.0
	D1	0.68	1.34	253	22.35	88.2
SEM		0.03	0.07	15	0.88	4.9
p-values						
FS		0.9596	0.8114	0.7672	0.3991	0.6639
DFM		0.6132	0.9909	0.6354	0.4445	0.986
Interaction effects						
F0	D0	0.74	1.42	242	21.78	88.6
F0	D1	0.64	1.28	248	20.90	84.6
F1	D0	0.66	1.26	245	21.00	87.5
F1	D1	0.72	1.40	259	23.80	91.7
SEM		0.04	0.10	21	1.30	7.2
p-value						
FS × DFM		0.0674	0.1489	0.8678	0.1479	0.5581

[1]FS = fenugreek seeds; DFM = direct-fed microbials; F0 = 0 g/kg FS; F1 = 5 g/kg FS; D0 = 0 g/kg DFM; and D1 = 0.1 g/kg DFM; AST = Aspartate aminotransferase; GGT = Gamma-glutamyl transferase.

The effects of FS and DFM on the Vh, Cd, and Vh:Cd of the ileum of broiler chickens at d 42 are shown in Table 9 and presented in Figure 2. The main effects of FS and DFM were significant for all the ileal parameters. Chickens fed with F0 had higher Vh, lower Cd and higher Vh:Cd than those fed with F1 ($p < 0.05$). Chickens fed with D0 had higher Vh, lower Cd and higher Vh:Cd than those fed with D1 ($p < 0.05$). There was a significant interaction between FS and DFM for Vh and Vh:Cd ($p < 0.05$), but not for Cd ($p > 0.05$). Chickens fed with

F0D0 had the highest Vh and Vh:Cd among all the treatments, while chickens fed with F1D1 had the lowest Vh and Vh:Cd.

TABLE 8 Jejunal morphology of birds fed fenugreek seeds (FS) and direct fed microbials (DFM) on d 42.[1]

FS	DFM	Vh (μm)	Cd (μm)	Vh:Cd
Main effects				
F0		1750	169[a]	10.51[b]
F1		1837	147[b]	12.55[a]
	D0	1764	149[b]	11.97
	D1	1823	168[a]	11.09
SEM		31	3	0.33
p-values				
FS		0.0557	<0.0001	0.0001
DFM		0.1878	<0.0001	0.0702
Interaction effects				
F0	D0	1674[b]	149[b]	11.33
F0	D1	1826[ab]	189[a]	9.70
F1	D0	1854[a]	148[b]	12.61
F1	D1	1820[ab]	146[b]	12.49
SEM		44	4	0.47
p-value				
FS × DFM		0.0415	<0.0001	0.1151

[a,b]Means within a column not sharing a common superscript differ significantly ($p < 0.05$).
[1]FS = fenugreek seeds; DFM = direct-fed microbials; F0 = 0 g/kg FS; F1 = 5 g/kg FS; D0 = 0 g/kg DFM; and D1 = 0.1 g/kg DFM; Vh = villus height; Cd = crypt depth; Vh: Cd = villus height to crypt depth ratio.

Discussion

The present study investigated the effects of fenugreek seeds (FS) and direct-fed microbials (DFM) on the growth performance, blood parameters and gut health of broiler chickens. FS are seeds of the plant *Trigonella foenum-graecum* L., which are widely used as a spice and flavor enhancer in human food (29). The Food and Drug Administration has classified them as generally recognized as safe (GRAS), meaning that they have a history of safe use in food and are unlikely to cause adverse effects when consumed in normal amounts (30). However, some studies in animals have raised concerns about the potential toxicity of high doses of FS or their extracts, which contain high levels of flavonoid glycosides (31). Flavonoid glycosides are natural compounds that have antioxidant and anti-inflammatory properties, but they can also induce oxidative stress and organ damage when ingested in excess. The *Bacillus*-based DFM used in this study, Norum™ (Eco Bio/Euxxis Bioscience LLC, Fayetteville, AR), is a spore-based DFM culture consisting of three *Bacillus* strains: *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* with a stable *Bacillus* spore content of ~3×10^{11} spores/g (22). These *Bacillus* strains can produce various enzymes (xylanase, cellulase, phytase, lipase, protease, and β-glucanase) that can improve the digestibility and nutrient availability of the feed, as well as modulate the intestinal microbiota and enhance the gut health and immunity of poultry (31, 32).

In the present study, the results showed that FS had a negative effect on the growth performance of broilers during the starter phase, but not during the finisher phase. DFM had a non-significant effect on the growth performance of broilers during either phase. There was an interaction between FS and DFM for BWG during the finisher and overall phases, indicating that the combination of FS and DFM may

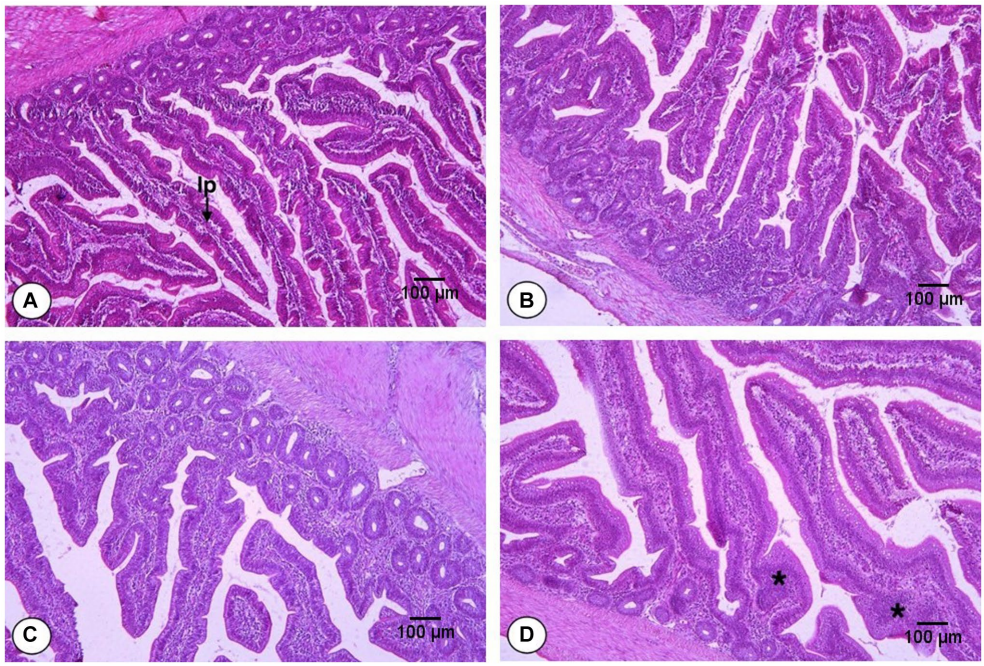

FIGURE 1
Histomorphology from the jejunum of broilers fed fenugreek seeds (FS) and Direct fed microbials (DFM) on d 42; F0D0 group. **(A)** F0D1 group, **(B)** F1D0 group, **(C)** F1D1 group (**D**; x200 magnification; H&E Staining). The "lp" represents the lamina propria, which is the smooth muscle fiber inside the intestinal villi. The asterisks (*) indicate the inflammation sites in the villi.

10.3389/fvets.2023.1298587

have some synergistic effects on broiler growth. The negative effect of FS on the growth performance of broilers during the starter phase may be attributed to the high saponin content in FS, which are known to have anti-nutritional properties such as reducing nutrient digestibility, interfering with bile acid absorption, and inhibiting intestinal enzymes (33). Earlier studies have demonstrated that broilers have little endogenous enzyme production during starter phase which might be negatively affected by the presence of antinutritional factors in the diet thus negatively affecting the growth performance during starter phase (34). FS may also have altered the intestinal microbiota of broilers, which play an important role in nutrient metabolism, immune modulation, and pathogen exclusion (35). These factors may have impaired the growth potential of broilers during the starter phase, when they are more sensitive to dietary changes and environmental stressors. The lack of effect of FS on the growth performance of broilers during the finisher phase may be due to their adaptation to the presence of FS in their diet over time and developed mechanisms to overcome its anti-nutritional effects. Broilers may also have benefited from some positive effects of FS on their health and immunity, which may have compensated for its negative effects on their growth. For instance, FS has been shown to have antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory properties in various animal models (36, 37). It is also possible that broilers may have received adequate levels of nutrients from other sources in their diet, which may have mitigated the impact of FS on their nutrient utilization. It is worth noting that we observed similar results in our previous experiment with the inclusion of 2.5 to 10 g/kg of FS in the broiler diet (38).

The lack of effect of DFM on the growth performance of broilers during either phase may be due to several factors. DFM may not have been able to colonize or persist in the gastrointestinal tract of broilers, due to competition from native microorganisms or unfavorable environmental conditions. It is also possible that DFM may not have

TABLE 9 Ileal morphology of birds fed fenugreek seeds (FS) and direct fed microbials (DFM) on d 42.[1]

FS	DFM	Vh (μm)	Cd (μm)	Vh: Cd
Main effects				
F0		742[a]	105[b]	7.24[a]
F1		704[b]	120[a]	5.94[b]
	D0	751[a]	107[b]	7.20[a]
	D1	695[b]	117[a]	5.98[b]
SEM		6	3	0.17
p-values				
FS		<0.0001	0.0003	<0.0001
DFM		<0.0001	0.0051	<0.0001
Interaction effects				
F0	D0	801[a]	98[b]	8.24[a]
F0	D1	683[b]	111[ab]	6.24[b]
F1	D0	701[b]	115[a]	6.15[b]
F1	D1	706[b]	124[a]	5.72[b]
SEM		8	4	0.24
p-value				
FS × DFM		<0.0001	0.6946	0.0021

[a,b]Means within a column not sharing a common superscript differ significantly ($p < 0.05$).
[1]FS = fenugreek seeds; DFM = direct-fed microbials; F0 = 0 g/kg FS; F1 = 5 g/kg FS; D0 = 0 g/kg DFM; and D1 = 0.1 g/kg DFM; Vh = villus height; Cd = crypt depth; Vh: Cd = villus height to crypt depth ratio.

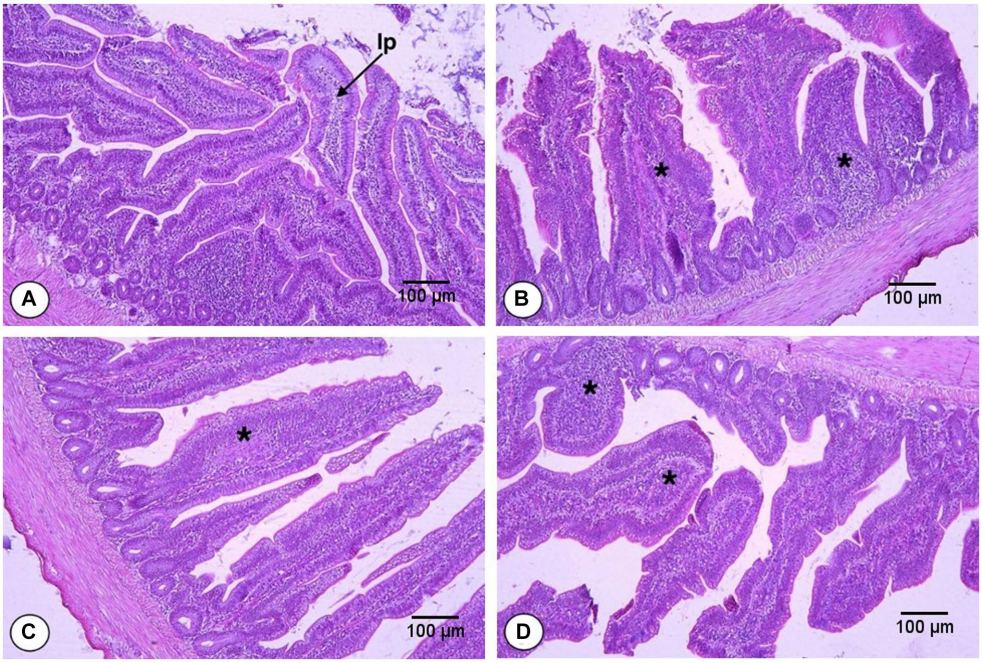

FIGURE 2
Histomorphology from the ileum of broilers fed fenugreek seeds (FS) and Direct fed microbials (DFM) on d 42; F0D0 group. **(A)** F0D1 group, **(B)** F1D0 group, **(C)** F1D1 group (**D**; x200 magnification; H&E Staining). The "lp" represents the lamina propria, which is the smooth muscle fiber inside the intestinal villi. The asterisks (*) indicate the inflammation sites in the villi.

produced sufficient amounts or types of metabolites that could enhance the growth performance of broilers, such as short-chain fatty acids, vitamins, enzymes, or antimicrobial substances (39). DFM may not have interacted with the host immune system or intestinal epithelium in a way that could improve the growth performance of broilers, such as modulating inflammatory responses, enhancing barrier function, or stimulating mucosal immunity (38). The interaction between FS and DFM for BWG during the finisher and overall phases may be due to some synergistic or antagonistic effects between these two feed additives on broiler growth. For instance, FS and DFM may have synergized to improve the intestinal health and immunity of broilers by reducing oxidative stress, enhancing intestinal morphology, increasing beneficial bacteria, and suppressing pathogenic bacteria (40). Alternatively, FS and DFM may have antagonized each other by interfering with their respective modes of action or bioavailability. For example, FS may have reduced the efficacy or survival of DFM by inhibiting their growth or activity with its saponins or other phytochemicals. Conversely, DFM may have reduced the bioavailability or absorption of FS by degrading its active components or binding to them with their cell wall components.

The results showed that DFM had a significant effect on some hematological parameters, such as white blood cells (WBC), total protein (TP), lymphocyte count and heterophil: lymphocyte ratio (H:L), but not on others, such as hemoglobin (Hb), heterophil count and basophil count. FS had a significant effect only on Hb, but not on any other hematological parameters. There was an interaction between FS and DFM for WBC, TP, heterophil count and lymphocyte count, indicating that the combination of FS and DFM may have some synergistic or antagonistic effects on the blood profile of broilers. The reduction in WBC, TP, and lymphocyte count in broilers fed a DFM diet may be due to an alteration of the gut microbiome, leading to a reduction in systemic inflammation and a decrease in the production of cytokines, which are signaling molecules involved in the regulation of the immune system. An increase in the H:L ratio can indicate an activation of the immune system, as heterophils play a role in the body's initial response to infection and inflammation (41). In such cases, the increase in heterophils can reflect an increase in the body's ability to respond to and combat pathogens. It is not entirely clear why Bacillus-DFM specifically would increase the HLR, but some studies suggest that certain Bacillus strains have immune-stimulatory effects that can enhance the body's ability to fight off infections and improve overall immunity (42). However, more research is needed to fully understand the mechanisms by which *Bacillus*-DFM might influence the H:L ratio and the immune system. The interaction of FS and DFM decreased the TP levels, WBC count, heterophil count, and lymphocyte count. This suggests that FS and DFM may have antagonistic effects on the blood parameters of broiler chickens. FS may contain some anti-nutritional factors, such as saponins or flavonoids, that may bind to or degrade some components of DFM, such as bacterial cell wall or enzymes, reducing their bioavailability or activity in the gut (43, 44). Alternatively, DFM may produce some metabolites or enzymes that may alter the pH or enzymatic activity in the gut, affecting the digestion or absorption of some components of FS, such as iron or flavonoids (45). These interactions may result in reduced efficacy or adverse effects of FS or DFM on the observed hematological parameters. The lack of effect of FS and DFM on the serum biochemical parameters may be attributed to several factors. FS and DFM may not have reached sufficient levels or durations in the gastrointestinal tract or bloodstream of broilers to exert their effects on the serum biochemical parameters. FS and DFM may not have altered the metabolic pathways or functions of the liver, kidney, or other organs that are involved in the synthesis or degradation of the serum biochemical parameters. FS and DFM may not have affected the homeostatic mechanisms that regulate the serum biochemical parameters within a narrow range. The serum biochemical parameters are important indicators of the health and physiological status of broilers, as they reflect the functions of various organs and systems (46). The normal ranges of these parameters may vary depending on the age, sex, breed, diet, environment, and health condition of broilers (47). The values of these parameters in this study were within the normal ranges reported for broiler chickens. This suggests that FS and DFM did not cause any adverse effects on the health and physiology of broilers.

The effect of FS on the intestinal morphology may be attributed to several factors. FS may contain some bioactive compounds, such as flavonoids or oligosaccharides, that may stimulate the growth and differentiation of intestinal cells, increasing the intestinal surface area and nutrient absorption capacity. Some studies have reported that FS supplementation can increase Vh, Cd, and Vh: Cd ratio of jejunum in broilers (48). However, another possible reason for the negative effect of FS on inflammation and monocyte infiltration in jejunum may be due to anti-nutritional factors, such as saponins, that may have cytotoxic and immunomodulatory effects on intestinal cells, inducing oxidative stress and inflammatory response (49). Some studies have reported that FS supplementation can cause inflammation and monocyte infiltration in jejunum in rats (50). Therefore, FS supplementation may have both positive and negative impacts on the jejunum morphology and gut health of broilers, which might depend on the dosage and growth stage of the birds.

One possible reason for the antagonistic effect of FS and DFM on Vh and Cd of jejunum is that FS and DFM may interfere with each other's absorption or metabolism in the gut. FS may contain anti-nutritional factors, such as saponins or flavonoids, that may bind to or degrade the components of DFM, such as bacterial cell wall or enzymes, reducing their bioavailability or activity in the gut. Alternatively, DFM may produce some metabolites or enzymes that may alter the pH or enzymatic activity in the gut, affecting the digestion or absorption of some components of FS, such as iron or flavonoids. These interactions may result in reduced efficacy or adverse effects of FS or DFM on jejunum morphology and gut health. These results are consistent with our previous study that reported similar effects of FS on ileum morphology in broilers (38). Moreover, FS also induced inflammation and monocyte infiltration in the ileum epithelium and lamina propria (Figure 2), which may impair the gut barrier function and increase the susceptibility to pathogens. FS contains saponins, alkaloids, and flavonoids that can induce inflammation, which can negatively impact gut health and result in decreased ileum villus height to crypt depth ratio (50, 51). DFM supplementation decreased the Vh, increased the Cd and decreased the Vh:Cd of ileum in broilers, which is contrary to some previous studies that reported that DFM increased or did not affect the Vh and decreased the Cd of ileum in broilers (52, 53). However, the effects of DFM on intestinal morphology may depend on the strain, dose, form, duration, and combination of DFM used. The combination of FS and DFM decreased the Vh and Vh:Cd of ileum in broilers, suggesting the possible antagonistic effects on the ileum morphology of broilers. It is

possible that FS may interfere with the colonization or activity of DFM in the intestine and reduce their beneficial effects on intestinal morphology. The interaction between FS and DFM for some intestinal parameters may be due to some synergistic or antagonistic effects between these two feed additives on the intestinal morphology and absorptive surface area of broilers. For instance, FS and DFM may have synergized to increase the Vh and Vh:Cd of broilers by enhancing their nutrient absorption and digestion with their enzymatic or prebiotic properties. Alternatively, FS and DFM may have antagonized each other by interfering with their respective modes of action or bioavailability. For example, FS may have reduced the efficacy or survival of DFM by inhibiting their growth or activity with its saponins or other phytochemicals. Conversely, DFM may have reduced the bioavailability or absorption of FS by degrading its active components or binding to them with their cell wall components.

Conclusion

This study demonstrated that FS and DFM had different effects on the broiler health and production depending on the phase of production. The interaction between FS and DFM revealed synergistic effects on growth performance during the finisher phase, but antagonistic effects on blood parameters and gut morphology. Further studies are needed to elucidate the underlying mechanisms and optimize the dosage and combination of FS and DFM for broiler health and production. Future studies should also evaluate the effects of FS and DFM on other aspects such as immunity, microbiota, and carcass quality.

Data availability statement

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

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas at Pine Bluff and were approved under protocol number #UAPB2020-04. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DP: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. GT-I: Conceptualization, Validation, Writing – review & editing. MA-N: Conceptualization, Resources, Validation, Writing – review & editing. NR: Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. WB: Conceptualization, Validation, Writing – review & editing. EA: Data curation, Formal Analysis, Methodology, Writing – review & editing. AA-W: Validation, Writing – review & editing. JL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by USDA – NIFA – Sustainable Agricultural Systems (grant numbers 2019-69012-29905) and the USDA – NIFA – 1890 Institution Teaching, Research and Extension Capacity Building Grants Program (grant number 2017-38821-26403).

Conflict of interest

MA-N was employed by the company Eco-Bio LLC.

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 07 December 2023
DOI 10.3389/fvets.2023.1302801

OPEN ACCESS

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RECEIVED 27 September 2023
ACCEPTED 24 November 2023
PUBLISHED 07 December 2023

CITATION
Liu S, Xiao G, Wang Q, Tian J, Feng X, Zhang Q and Gong L (2023) Effects of dietary *Astragalus membranaceus* and *Codonopsis pilosula* extracts on growth performance, antioxidant capacity, immune status, and intestinal health in broilers. *Front. Vet. Sci.* 10:1302801. doi: 10.3389/fvets.2023.1302801

Effects of dietary *Astragalus membranaceus* and *Codonopsis pilosula* extracts on growth performance, antioxidant capacity, immune status, and intestinal health in broilers

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The objective of this study was to examine the effects of dietary Chinese herbal medicine (CHM) consisting of *Astragalus membranaceus (Fisch.) Bunge* (AMT) and *Codonopsis pilosula (Franch.) Nannf* (CPO) extracts on growth performance, antioxidant capacity, immune status, and intestinal health of broiler chickens. Two groups were formed, each consisting of six replicates of 12 one-day-old healthy male 817 white feather broilers. Broilers were fed either a basal diet (CON group) or a basal diet supplemented with 500 mg/kg CHM. The trial lasted 50 days. The results showed that CHM supplementation resulted in enhanced feed efficiency and antioxidant capacity in both the serum and liver, while it reduced uric acid and endotoxin levels, as well as diamine oxidase activity ($p < 0.05$). Additionally, CHM treatment increased the height of jejunum villi and upregulated *Claudin-1* expression in the jejunal mucosa accompanied by an increase in the mRNA levels of interleukin-6 (IL-6), interferon-γ (IFN-γ), interferon-β (IFN-β), tumor necrosis factor-α (TNF-α), and anti-inflammatory cytokine interleukin-10 (IL-10) ($p < 0.05$). The presence of dietary CHM caused an increase in the proportions of *Bacteroidetes* and unclassified *Bacteroidales* but led to a decrease in those of *Firmicutes* and *Alistipes* ($p < 0.05$). The composition of the jejunal mucosa microbiota was correlated with the feed conversion ratio, serum metabolites, and gene expression based on Spearman correlation analysis. The findings indicated that the consumption of dietary CHM improved the utilization of feed, increased the mRNA expression of pro-inflammatory cytokines in the jejunal mucosa, and decreased the endotoxin level and activities of diamine oxidase and lactate dehydrogenase in the serum, which could potentially be linked to changes in the gut microbiota of broiler chickens.

KEYWORDS

Chinese herbal medicine extract, *Astragalus membranaceus*, *Codonopsis pilosula*, growth performance, antioxidation, immune response, intestinal barrier function, gut microbiota

1 Introduction

In recent years, chicken meat has been in high demand as a source of protein. Consequently, commercial chicken farms prioritize faster growth and higher feed efficiency as their primary objectives. However, attaining these objectives requires ensuring the health of the chickens, good digestion and absorption, and protection against pathogens. Owing to the exhaustive ban on antibiotics in feed, broiler chickens have become increasingly vulnerable to illnesses, resulting in diminished performance as they face the threat of contagious agents, stressors, and nutritional insufficiencies (1, 2). Hence, many researchers are focusing on finding effective solutions to address or mitigate this issue.

Chinese herbal medicines (CHM) and their extracts have been utilized as adjuvants to mitigate the risk of disease and enhance the growth performance of livestock (3–5). The profusion of herbal remedies, *Astragalus membranaceus (Fisch.) Bunge* (AMT) and *Codonopsis pilosula (Franch.) Nannf* (CPO) has been used in traditional oriental medicine. AMT, one of the most widely used tonic herbs in many Asian regions, has been reported to possess components that demonstrate various biological effects, including antioxidative, anti-inflammatory, and antiviral properties (6–8). AMT has been reported to scavenge oxygen free radicals, making it an important plant in preventing mucosal injury in the intestine, liver, and plasma (9). CPO is known for its ability to enhance spleen function, promote liver health, and exhibit anti-tumor, antioxidant, and antibacterial effects (10–12). Studies have also shown that AMT and CPO have immunomodulatory effect, improve immune defense function of broiler chickens and mice, and have nourishing effects and can be used as immune adjuvants (13, 14). These two plants are often used in tandem to combat multiple of diseases because their synergistic effects are believed to promote lipid oxidation, protect against illnesses, and ameliorate inflammation (15, 16).

Recently, researchers found that the combination of CPO and AMT could re-establish the immune balance of the intestinal flora and alleviate colonic mucosal injury in mice with colitis (16). Synergistic extracts of AMT and CPO showed antioxidant activity in weaned piglets and influenced the relative proportion of Firmicutes and Bacteroidetes in the gastrointestinal tract (17). However, to the best of our knowledge, the combined effects of AMT and CPO extracts in broiler chickens have not yet been thoroughly studied. Therefore, the aim of this study was to explore the effects of adding CHM, by considering the example of AMT and CPO extracts, to the diet on the growth performance, immune response, antioxidant capacity, and intestinal health of broiler chickens.

2 Materials and methods

2.1 Experimental design, birds, diets, and management

A total of 144 one-day-old male 817 white feather broilers (Nanhai little white chicken) with similar initial weights were purchased from Muyuan Foods Co., Ltd., (Guangzhou, China). The broilers were allocated to two groups, with six replicates per group and 12 chicks per replicate. Birds were fed with either a basal diet or a basal diet supplemented with 500 mg/kg CHM. The determination of the CHM dosage was based on a preliminary experiment conducted by the research group members, taking into account the dosage of extracts mentioned in our previous study (18). The CHM product was purchased from Guangdong Huakang Biopharmaceutical Co., LTD (Guangzhou, China), which mainly consists of a mixture of extracts of AMT and CPO. The main component of this product is polysaccharide. The diet was developed based on the nutrient needs of broiler chickens (Table 1), ensuring that all essential nutrients were provided in amounts that met or surpassed the requirements set for chickens (NRC, 1994). In this study, all the chicks were kept in a controlled environment with specific dark-and-light cycles. All chicks

TABLE 1 Composition and nutrient content of experimental diets.

Diets	Starter (1–20 days)	Finisher (21–50 days)
Ingredients (g/kg)		
Corn	58.75	57.66
Soybean meal (43% CP)	27.70	27.21
Corn gluten meal (60% CP)	5.00	5.00
Limestone	1.43	1.22
Calcium hydrogen phosphate (16.5%)	1.10	1.04
L-lysine Sulfate (70%)	0.66	0.39
DL-methionine (98.5%)	0.33	0.21
NaCl	0.28	0.32
L-Threonine	0.16	0.06
Peanut meal	3.00	0
Choline chloride (50%)	0.08	0.08
Premix [1]	0.35	0.35
Phytase (20,000 IU)	0.01	0.01
Sodium humate	0	0.15
Lard	1.15	6.30
Total (%)	100	100
Nutrient content [2]		
ME (Kcal/kg)	2941.61	3231.28
CP (g/kg)	21.92	19.61
CEE (g/kg)	3.65	8.87
CF (g/kg)	2.51	2.33
Ca (g/kg)	0.90	0.80
Total P (%)	0.57	0.54
Lys (%)	1.48	1.21
Met (%)	0.64	0.52
Cys (%)	0.30	0.27
Met+Cys (%)	0.94	0.78
L-Threonine	0.92	0.79

[1] Premix is provided for each kilogram of diet: vitamin A, 12,000 IU; vitamin D3, 3,000 IU; vitamin E, 10 IU; vitamin K3, 2 mg; vitamin B1, 1 mg; vitamin B2, 3 mg; vitamin B6, 2 mg; vitamin B12, 0.01 mg; niacin, 20 mg; calcium pantothenate, 4 mg; biotin, 0.05 mg; folic acid, 0.5 mg; Mn, 100 mg; Fe, 100 mg; Zn, 80 mg; Cu, 20 mg; I, 3 mg; and Se, 0.5 mg.
[2] Nutrient levels were calculated values.
ME, metabolizable energy; CP, crude protein; CEE, crude ether extract; *CF*, crude fiber.

were subjected to the same light cycle arrangement (24 L:0 D) 24 h before the experiment. From the second day of age, the brightness gradually decreased and reached 18 L:6 D on the seventh day. From the eighth day onwards, the light cycle was maintained at 16 L:8 D until the end of the experiment. Furthermore, the chicks were provided with unrestricted access to both water and feed throughout the entire duration of the trial.

2.2 Sample collection

On the 50th day, after an eight-hour fasting period, measurements were made of the body weight (BW) and the amount of feed remaining in each replicate. Afterwards, the growth performance parameters for each replicate were computed, which included the mean average daily feed intake (ADFI), mean average daily gain (ADG), and the feed conversion ratio (FCR). FCR = feed intake (g) / weight gain (g). Additionally, any instances of mortality throughout the entire 50-day duration of the experiment were taken into consideration. One chicken was selected from each replicate after weighting at d 50 for blood and tissue samples collection. Following the extraction of around 4 mL of blood from the brachial vein, it was subsequently centrifuged at a temperature of 4°C for 15 min with a force of 3,000 g. After centrifugation, serum was stored at −80°C for further analysis. Following blood collection, chickens were sacrificed through exsanguination. Liver tissue samples were collected uniformly from the left side of each chicken, immediately placed in liquid nitrogen, and subsequently stored at −80°C to facilitate determination of antioxidant capacity. Furthermore, the jejunum, jejunal mucosa, and the contents of the cecum were systematically collected and preserved using different techniques. Both the jejunal mucosa and jejunal tissue samples were obtained from the middle section of the intestinal segment. The jejunal mucosa was collected by scraping the jejunum using a scalpel. Additionally, the scraped jejunal tissue, measuring approximately 3–4 cm in length, was preserved for further analysis. Histomorphological analyses were conducted on sections of scraped jejunal tissue that were preserved in formaldehyde.

2.3 Serum biomarkers measurement

Serum biomarkers were tested using detection kits from the Institute of Bioengineering, Nanjing Jiancheng (Nanjing, China). These markers include alanine aminotransferase, alkaline phosphatase, total cholesterol, albumin, low-density lipoprotein cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), lactate dehydrogenase (LDH), and uric acid (UA). Additionally, diamine oxidase (DAO) and endotoxin concentrations were measured a DAO assay kit and an endotoxin detection kit, respectively. Interleukin 2 (IL-2), interleukin-10 (IL-10), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), secretory immunoglobulin A (sIgA) and transforming growth factor-β (TGF-β) were measured in each group with chicken specific enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Biomedicine, China). All determination procedures were conducted following the instructions given by the manufacturer (Nanjing Jiancheng Biomedicine, China).

2.4 Serum and liver antioxidant capacity

The antioxidant levels in serum and livers were assessed with kits to measure various indicators, including total superoxide dismutase (T-SOD), malondialdehyde (MDA), catalase (CAT), total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-Px). The kits were purchased from Nanjing Jiancheng BioEngineering, and the determination procedure was conducted following the kits' instructions. Before homogenizing with nine times normal saline, the liver samples were thawed on ice, maintaining a ratio of 1 gram of tissue to 9 milli-liters of saline. The website of Nanjing Jiancheng company has a technical support section that offers details about technical approaches.[1] After centrifugation, the supernatant from liver homogenate was obtained for determining and analyzing the index.

2.5 Intestinal tissue morphology

Morphology is an accepted method for studying the change of tissue structure, such as livers (19), hearts (20), and jejunum (21). In this study, tissue specimens from the jejunum were collected and preserved in a formaldehyde solution for further analysis. Afterwards, the samples were immersed in paraffin and sliced into segments. The serial sections that were affixed onto a slide, which was then treated with hematoxylin and eosin staining. Six complete crypto-villi units were selected from each sample for analysis. Measurements of the small intestine villi and crypts were performed using the high-definition LEICA imaging system (version DFC290, Heilbrugger, Switzerland). To assess the morphology of the tissue, measurements of villus height and crypt depth were taken. The measurement of villi height starts from the villi tip and ends at the junction of the villi recess, while crypt depth refers to the depth of the invagination between neighboring villi. Calculate the villus/crypt ratio by dividing the villi height by the crypt depth. This ratio provides insight into the structural integrity of the intestinal tissue.

2.6 Quantitative PCR

We used TransZol reagent (TransGen Biotech, Beijing, China) to extract RNA from jejunal mucosa and with the NanoDrop 2000C Ultramicrospectrophotometer (Thermo, Shanghai, China) to measure the quality of the RNA (OD 260/280). RNA was reverse-transcribed by RT EasyTMII Kit, and cDNA was synthesized. A specific primer sequence was combined with cDNA, and real-time quantitative PCR was conducted to analyze cDNA gene expression. The primers were created with the assistance of the NCBI primer tool and their specificities were confirmed. Qingke Biological Co., Ltd. synthesized the primers utilized in the research, and all pairs of primers exhibited an amplification efficiency of approximately 100% (Table 2). The PCR amplification system contained a total volume of 20 μL, and the cycling conditions consisted of 30 s at 95°C, followed by 10 s at 95°C and 30 s at 60°C for 40 cycles. To determine the relative gene

1 http://www.njjcbio.com/

TABLE 2 The primer sequence of the gene.

Gene	GenBank	Sequence (5′–3′)	TM °C
β-actin	NM_205518.2	F: CATTGTCCACCGCAAATGCT R: AAGCCATGCCAATCTCGTCT	57.2
IL-6	NM_204628.2	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	56.6
IL-10	NM_001004414.4	F: CGCTGTCACCGCTTCTTCA R: CTTTGTTCTCATCCATCTTCTC	57.0
IL-1β	XM_046931582.1	F: CGACATCAACCAGAAGTGCTT R: GTCCAGGCGGTAGAAGATGA	56.8
IL-22	NM_001199614.1	F: GCCCTACATCAGGAATCGCA R: TCTGAGAGCCTGGCCATTTC	57.8
IL-17A	NM_204460.2	F: GAAGGTGATACGGCCAGGAC R: TGGGTTAGGCATCCAGCATC	56.8
IFN-β	NM_001024836.2	F: TGCAACCATCTTCGTCACCA R: GGAGGTGGAGCCGTATTCT	56.68
IFN-γ	NM_205149.2	F: ACACTGACAAGTCAAAGCCGC R: AGTCGTTCATCGGGAGCTTG	58.6
TNF-α	XM_046927265.1	F: TGTGTATGTGCAGCAACCCGTAGT R: GGCATTGCAATTTGGACAGAAGT	57.8
MUC-2	XM_040673077.2	F: TTCATGATGCCTGCTCTTGTG R: CCTGAGCCTTGGTACATTCTTGT	58.0
ZO-1	XM_046925214.1	F: CTTCAGGTGTTTCTCTTCCTCCTC R: CTGTGGTTTCATGGCTGGATC	56.6
Claudin-1	NM_001013611.2	F: GGTATGGCAACAGAGTGGCT R: CAGCCAATGAAGAGGGCTGA	57.0
Occludin	XM_046904540.1	F: GATGGACAGCATCAACGACC R: CATGCGCTTGATGTGGAAGA	58.0

IL-6, interleukin-6; IL-10, interleukin-10; IL-1β, interleukin-1β; IL-22, interleukin-22; IL-17A, interleukin-17A; IFN-γ, interferon-γ; IFN-β, interferon-β; TNF-α, tumor necrosis factor-α; MUC-2, Mucin-2; ZO-1, Zonula occludens-1.

expression, real-time quantitative PCR was used using the $2^{-\Delta\Delta Ct}$ method (20, 22).

2.7 16S sequencing and cecal microbiota analysis

The 16s rRNA sequencing of feces was entrusted to Lianchuan Biotechnology Co., LTD (Hangzhou, China). The hexadecyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from the contents of the cecum. The concentration of DNA was determined and the quality of the DNA extraction was evaluated using agarose gel electrophoresis, employing an ultraviolet spectrophotometer. DNA obtained from the samples was used to amplify the 16S rRNA V3-V4 region. PCR products were purified using AM-Pure XT beads from Beckman Coulter Genomics in Danvers, MA, USA. Subsequently, the quantification was performed using Qubit from Invitrogen in the United States. The refined PCR samples were evaluated using an Agilent 2,100 Bioanalyzer (Agilent, USA) and the Illumina Library Quantification Kit from Kapa Biosciences (Woburn, MA, USA). The samples obtained through sequencing were divided based on barcode information for the double-ended data. Data was spliced and filtered after the joint and barcode sequences were removed. Afterwards, dada 2 was used in conjunction with qiime DADA 2 denoise-paired to carry out length filtering and denoising, which led to the detection of ASV (feature) sequences and the generation of an ASV (feature) abundance chart. Singletons ASV were then removed. The acquired ASV (characteristic) sequences and ASV (characteristic) abundance table were utilized for the analysis of alpha diversity and beta diversity. The alpha diversity analysis assesses domestic diversity using six indexes: observed_species, shannon, simpson, chao1, goods_coverage, and pielou_e. Additionally, four types of distances (unweighted_unifrac, weighted_unifrac, jaccard, bray_curtis) were calculated to evaluate the diversity between habitats (samples/groups). The characterization of microorganismal features differentiating the fecal microbiota was performed using the linear discriminant analysis (LDA) effect size (LEfSe) method.[2] We analyzed the Spearman's correlations using the heatmap function from the R package (version 3.6.3). Spearman correlation methodology can be the website,[3] and use OmicStudio tools on[4] perform clustering correlation heat map with symbols (23).

2 https://www.omicstudio.cn/tool/60
3 https://www.omicstudio.cn
4 https://www.omicstudio.cn/tool

TABLE 3 Effects of CHM on growth performance of broilers.

Items	CON	CHM	*p*-value
1 d BW, g	36.20 ± 0.01	36.2 ± 0.01	1.00
50 d BW, g	2015.00 ± 62.65	2036.67 ± 120.97	0.80
1–50 d			
ADG, g/d	40.38 ± 1.28	40.83 ± 2.47	0.80
ADFI, g/d	78.34 ± 3.52	74.31 ± 2.64	0.08
FCR	1.94 ± 0.06	1.82 ± 0.02	0.02

BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio; CON, control; CHM, Chinese herbal medicine.

TABLE 4 Effects of CHM on serum physiological and biochemical indexes of broilers.

Items	CON	CHM	*p*-value
Triglycerides, mmol/L	0.75 ± 0.19	0.87 ± 0.13	0.20
Total cholesterol, mmol/L	4.26 ± 0.67	5.30 ± 0.75	0.03
HDL-C, mmol/L	3.62 ± 1.51	5.76 ± 0.87	0.01
Low-density lipoprotein cholesterol, mmol/L	3.43 ± 0.86	3.02 ± 0.57	0.36
Albumin, g/L	20.87 ± 4.03	22.17 ± 2.56	0.52
UA, μmol/L	353.52 ± 72.75	250.44 ± 26.51	0.01
LDH, U/L	6072.58 ± 257.85	5529.26 ± 437.67	0.03
Alkaline phosphatase, mg/L	155.56 ± 29.88	154.94 ± 53.75	0.98
Aspartate aminotransferase, U/L	15.36 ± 3.88	24.68 ± 9.77	0.05
Alanine aminotransferase, U/L	21.12 ± 12.44	14.30 ± 6.60	0.26
DAO, U/L	18.13 ± 5.28	7.88 ± 1.74	0.01
Endotoxin, U/L	1.43 ± 0.50	0.83 ± 0.42	0.04

HDL-C, high-density lipoprotein cholesterol; UA, uric acid; LDH, lactate dehydrogenase; DAO, diamine oxidase; CON, control; CHM, Chinese herbal medicine.

2.8 Statistical analysis

All data was analyzed using independent samples *t*-test in SPSS version 23.0 software (SPSS, Inc., Chicago, IL, USA). The data were expressed as mean ± standard deviation (SD). Values at $p < 0.05$ were statistically significant and values at $0.05 < p < 0.10$ are trending.

3 Results

3.1 Growth performance

Data on growth performance are shown in Table 3. Compared with those in the CON group, the BW and ADG were higher in the CHM group; however, there were no significant differences observed among BW, ADG, and ADFI between the two groups from day 1 to day 50. However, FCR of the CHM group was lower than that of the CON group ($p = 0.02$).

3.2 Serum physiological and biochemical indexes

The effects of CHM on the physiological serum parameters of broilers are presented in Table 4. Compared with the CON group, the CHM group showed a significant increase in total cholesterol ($p = 0.03$) and HDL-C ($p = 0.01$) serum levels, while exhibiting a decrease in UA level ($p = 0.01$) and LDH activity ($p = 0.03$). Aspartate aminotransferase level ($p = 0.05$) was higher in the CHM group than in the CON group. In addition, the CHM group exhibited a significant decrease in DAO activity ($p = 0.01$) and endotoxin concentration ($p = 0.04$). Serum levels of triglycerides, low-density lipoprotein cholesterol, albumin, alkaline phosphatase, and alanine aminotransferase were not significantly affected by the experimental treatments.

3.3 Immune response

As shown in Table 5, serum levels of immune factors were determined using enzyme-linked immunosorbent assay (ELISA) kits, whereas mRNA levels of immune factors in the jejunal mucosa of broilers were quantified using quantitative polymerase chain reaction (qPCR). Compared with the CON group, the CHM group exhibited decreased levels of IL-10 ($p = 0.08$) in the serum. Moreover, birds in CHM group had increased mRNA expression levels of pro-inflammatory cytokines IL-6 ($p = 0.02$), interferon-γ (IFN-γ) ($p = 0.01$), interferon-β (IFN-β) ($p = 0.01$) and TNF-α ($p = 0.01$), as well as anti-inflammatory cytokine IL-10 ($p = 0.01$) in the jejunal mucosal tissue compared to the birds in the CON group.

TABLE 5 Effects of CHM on the expression levels of immune factors in the serum and jejunal mucosa of broilers.

Items	CON	CHM	*p*-value
Serum			
IL-2, ng/L	22.712 ± 5.30	27.47 ± 6.03	0.18
IL-6, ng/L	94.03 ± 15.99	92.18 ± 20.90	0.87
TNF-α, ng/L	193.65 ± 14.71	183.97 ± 13.68	0.25
TGF-β, ng/L	2652.14 ± 242.39	2354.87 ± 340.84	0.11
IL-10, ng/L	4.80 ± 1.66	3.35 ± 0.71	0.08
sIgA, ng/L	170.80 ± 16.67	188.50 ± 36.93	0.30
Jejunal mucosa mRNA abundance			
IL-6	1.00 ± 0.02	2.38 ± 0.83	0.02
IL-1β	1.00 ± 0.03	1.42 ± 0.69	0.29
IL-22	1.00 ± 0.01	1.12 ± 0.65	0.72
IL-17A	1.00 ± 0.01	1.66 ± 1.06	0.26
IFN-γ	1.00 ± 0.01	2.16 ± 0.31	0.01
TNF-α	1.03 ± 0.04	2.57 ± 0.62	0.01
IL-10	1.00 ± 0.01	3.56 ± 0.90	0.01
IFN-β	1.00 ± 0.02	1.83 ± 0.26	0.01

IL-2, interleukin-2; IL-6, interleukin-6; IL-10, interleukin-10; IL-22, interleukin-22; IL-17A, interleukin-17A; IFN-γ, interferon-γ; IFN-β, interferon-β; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; sIgA, secretory immunoglobulin A; CON, control; CHM, Chinese herbal medicine.

TABLE 6 Effects of CHM on serum and hepatic antioxidant capacity of broilers.

Items	CON	CHM	*p*-value
Serum			
MDA, nmol/mL	3.52 ± 1.09	2.98 ± 9.62	0.26
CAT, U/mL	4.03 ± 0.88	5.02 ± 1.60	0.21
T-AOC, U/mL	3.86 ± 1.08	7.57 ± 2.13	0.01
T-SOD, U/mL	45.03 ± 6.8	54.47 ± 4.15	0.02
GSH-Px, μmol/L	1249.27 ± 273.88	1639.38 ± 175.37	0.02
Liver			
MDA, nmol/mgprot	5.13 ± 0.80	4.40 ± 0.68	0.12
T-AOC, U/mgprot	5.26 ± 0.34	6.71 ± 0.78	0.01
CAT, U/mgprot	18.14 ± 4.75	24.71 ± 4.72	0.04
T-SOD, U/mgprot	265.40 ± 30.89	339.08 ± 40.55	0.01
GSH-Px, μmol/mgprot	63.95 ± 7.31	93.64 ± 18.69	0.01

MDA, malondialdehyde; CAT, catalase; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; CON, control; CHM, Chinese herbal medicine.

3.4 Serum and liver antioxidant capacity

Compared with the CON group (Table 6), the CHM group exhibited a significant increase in serum antioxidant parameters, specifically T-AOC ($p = 0.01$), T-SOD ($p = 0.02$) and GSH-Px ($p = 0.02$) content. In addition, the CHM group showed significantly increased T-AOC ($p = 0.01$), T-SOD ($p = 0.01$), CAT ($p = 0.04$) and GSH-Px activity ($p = 0.01$) in the liver tissues compared to the CON group.

TABLE 7 Effects of CHM on jejunal morphology and the mRNA expression of mucosa tight junction protein.

Items	CON	CHM	*p*-value
50 d villus height, μm	1066.44 ± 49.25	1237.66 ± 88.57	0.01
50 d crypt depth, μm	112.51 ± 11.99	113.83 ± 9.62	0.84
50 d V/C	9.71 ± 0.95	10.92 ± 1.00	0.06
ZO-1	0.99 ± 0.01	1.06 ± 0.44	0.77
MUC-2	1.01 ± 0.02	1.44 ± 0.91	0.38
Claudin-1	1.00 ± 0.03	2.28 ± 0.44	0.01
Occludin	1.01 ± 0.05	0.84 ± 0.39	0.41

V/C, villus height / crypt depth; ZO-1, Zonula occludens-1; MUC-2, Mucin-2; CON, control; CHM, Chinese herbal medicine.

3.5 Intestinal physical barrier function

As shown in Table 7, CHM supplementation significantly increased the height of the jejunal villi ($p = 0.01$) and upregulated the mRNA expression level of Claudin-1 ($p = 0.01$) in the jejunal mucosa compared to those in the CON group.

3.6 Gut microbiota

Figure 1 revealed that CHM supplementation affected broiler cecal microbiota. According to the Venn diagram, the overall number of operational taxonomic units (OTUs) was 1,827, with 442 OTUs common to both groups (Figure 1A). Notably, 426 OTUs were found to be unique to the CHM group. To depict the microbiome space of various groups, we performed non-metric multidimensional scaling (NMDS) using weighted UniFrac and Bray–Curtis distances. The composition of the gut microbiome differed between the two groups (Figure 1B). In contrast to that in the CON group, the inclusion of CHM significantly decreased chao 1 ($p < 0.01$) and observed_otus ($p < 0.01$) measures of alpha diversity (Table 8).

Figures 1C–E displays the distribution of the cecal microbiota at the phylum and genus levels. *Firmicutes* and *Bacteroidetes* were the most prevalent phyla in the two groups (Figure 1C). CHM significantly increased the relative abundance of Bacteroidetes ($p = 0.01$; Figure 1E) and decreased *Firmicutes* compared to those in the CON group ($p < 0.01$; Figure 1E). Similarly, at the genus level, CHM had a significant effect on the abundance of *Bacteroidales_unclassified* ($p = 0.01$; Figure 1E) and caused a significant decrease in the abundance of *Alistipes* ($p = 0.01$; Figure 1E).

Based on the effect size measurements (LEfSe), Figure 1F shows the identification of 19 biomarkers with linear discriminant analysis (LDA) values exceeding four. In addition, the CHM group exhibited enrichment of six bacterial taxa, namely unidentified *Bacteroidales* (species), unidentified *Bacteroidales* (family), unidentified *Bacteroidales* (genus), *Bacteroidota* (phylum), *Bacteroidales* (order), and *Bacteroidia* (class). Enrichment of the CON group was observed in various taxa including *Rikenellaceae* (family), *Alistipes* (genus), *Alistipes_unclassified* (species), *Clostridia* (class), *Alistipes_ihumii* (species), *Firmicutes* (phylum), *Oscillospirales* (order), *Incertae_sedis_unclassified* (species), *Incertae_sedis* (species), *Clostridiales* (order), *Bacteroides* (genus), *Bacteroides_sp__S461* (species), and *Bacteroidaceae* (family).

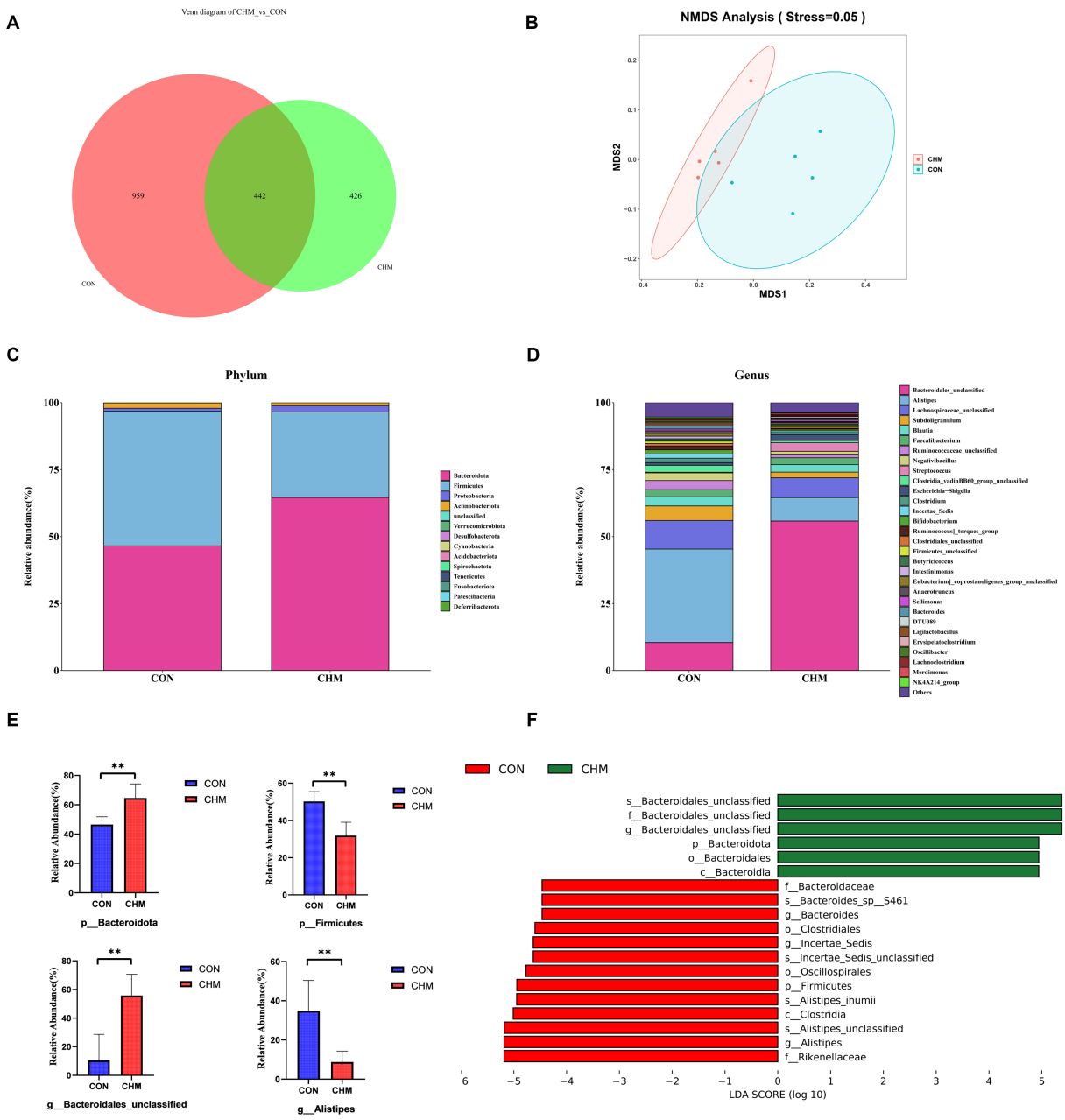

FIGURE 1
Relative abundance of the cecal microbiota. **(A)** Venn diagram of OTUs; number; **(B)** NMDS using Bray-curtis distance; **(C)** Phylum-level taxonomic composition of the cecal microbiota; **(D)** Genus-level taxonomic composition and relative abundance of the cecal microbiota; **(E)** The relative abundance of Phylum Firmicutes and Bacteroidetes and genus *Bacteroidales_unclassified* and *Alistipes*. **(F)** Lefse identified the most differential genera between the CON group and the CHM group. CON, control; CHM, Chinese herbal medicine. Significant differences are denoted by asterisks (*$p < 0.05$; **$p < 0.01$).

3.7 Correlation analysis of cecal microbiota, FCR and jejunal mucosal gene changes

Figure 2 displays the results of Spearman's correlation analysis, illustrating the relationship between the most prominent 20 genus and factors such as FCR, serum differential metabolites (DAO, HDL-C, LDH, UA, and endotoxin), and genes with significant differences in the jejunal mucosa. The abundance of *Bacteroidales_unclassified* had a positive correlation with the expression of IFN-β, TNF-α, Claudin-1, and IL-6 mRNAs, while it had a negative correlation with FCR, DAO, LDH, and endotoxin. However, *Alistipes* showed a negative correlation with the mRNA expression of IFN-β, and a positive correlation with the FCR, DAO, LDH, and endotoxin. Meanwhile, *Ruminococcaceae_unclassified* exhibited a negative correlation with the mRNA expression of IFN-γ, TNF-α, IL-6, and IL-10, while showing a positive correlation with FCR. Furthermore, *Clostridia_vadinBB60_group_unclassified* showed a positive correlation with FCR, LDH and UA and a negative correlation with IL-10 expression.

4 Discussion

FCR is used in animal production as a key indicator for evaluating feed utilization efficiency and production benefits. It can be used to assess chicken performance and is frequently used in meat-producing poultry (24). Birds with low FCR are considered as having high feed efficiency. FCR is affected by genetics, health, food, and the environment in which the chickens are raised (24–26). Neither of the two groups of our study differed significantly in BW, ADG, or ADFI; however, the BW and ADG were higher in the CHM group than that in the CON group. Therefore, supplementation with CHM reduced the FCR and improved the feed utilization efficiency. According to previous studies, the injection of AMT components into poultry muscle significantly alleviated the decrease in feed efficiency of lipopolysaccharide (LPS)-treated broilers (27). Other studies have shown that the addition of AMT polysaccharide and Glycyrrhiza uralensis polysaccharide reduced the FCR and increased ADG of broilers (28); however, the ADG between the two groups was not significantly different in our study. We speculate that AMT and CPO extracts contain numerous beneficial components, including polysaccharides, saponins and so on, which stimulate livestock digestion and absorption, enhance nutrient utilization, and reduce feed waste, thus increasing feed efficiency (27, 29–31).

Serum biochemical indexes partially reflect metabolism and health state of the body. UA levels in serum reflect both the production and excretion of UA. The present study indicated that CHM increased the level of HDL-C and reduced those of UA, LDH, DAO, and

TABLE 8 Alpha diversity indexes of cecal microbiota.

Items	CON	CHM	*p*-value
Chao 1	428.16 ± 48.69	307.68 ± 33.63	0.01
Shannon	4.85 ± 0.41	4.44 ± 0.28	0.11
Simpson	0.84 ± 0.06	0.87 ± 0.03	0.32
Goods_coverage	1.00	1.00	-
Pielou_e	0.55 ± 0.04	0.54 ± 0.03	0.45
Observed_otus	428.00 ± 48.56	307.60 ± 33.55	0.01

CON, control; CHM, Chinese herbal medicine.

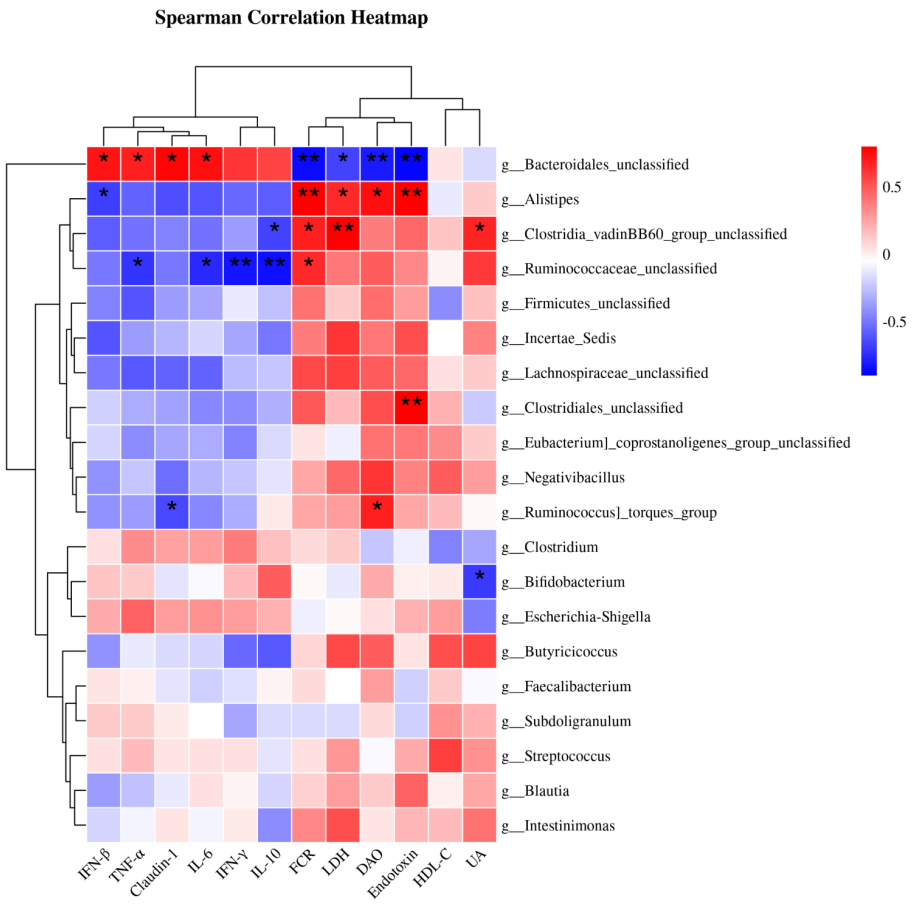

FIGURE 2
Spearman correlation analysis of cecal microbiota and FCR or serum differential metabolites or genes with significant differences in jejunal mucosa. Statistically significant associations ($p < 0.05$) were observed between certain indicators and bacterial genera. The red blocks indicate positive correlations, while the blue blocks indicate negative correlations. The intensity of the colors reflects the strength of the correlations. * $p < 0.05$ and ** $p < 0.01$ indicate significant differences compared to the CON group. IFN-β, interferon-β; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IFN-γ, interferon-γ; IL-10, interleukin-10; FCR, feed conversion ratio; LDH, lactate dehydrogenase; DAO, diamine oxidase; HDL-C, high-density lipoprotein cholesterol; UA, uric acid; CON, control; CHM, Chinese herbal medicine.

endotoxin in serum. LDH levels are primarily associated with the extent of cell necrosis and damage to the cell membrane (32). Higher levels of LDH indicate a greater degree of cell necrosis and damage. This increase in LDH levels can be attributed to the anti-inflammatory and antioxidant properties of the extract, as well as its role in regulating cell function. In addition, a positive correlation has been found between endotoxin levels and DAO activity in blood (33). Increased intestinal permeability can compromise intestinal barrier integrity, allowing bacteria and other harmful substances to enter the bloodstream more easily, thereby triggering inflammation and other health issues. Therefore, the addition of CHM can have a positive effect on body metabolism and intestinal barrier function by increasing HDL-C levels and decreasing endotoxin and LDH levels and DAO activity in the blood.

The antioxidant capacity of the body inhibits the production of free radicals and prevents chain reactions of free radicals, thus alleviating oxidative damage to the host. This improvement in antioxidant capacity is beneficial for the health and production performance of broilers (34). T-AOC comprehensively reflects the antioxidant capacity of both enzyme and non-enzymes defense systems (35). Antioxidative enzymes are composed of significant elements such as T-SOD, CAT, and GSH-Px, which have a critical function in eradicating superoxide anions and hydrogen peroxide. These enzymes protect cells and tissues against the harmful effects of oxidative stress (35–37). Wang, et al. (38) reported that diets supplemented with AMT increased sheep CAT, T-SOD, and T-AOC levels in the small intestinal mucosa and meat tissue, improving their antioxidant capacity. Similarly, adding AMT to quails increased their T-AOC, GSH-Px, and CAT activities (39). Our results showed increased serum and liver T-AOC, SOD, and GSH-Px, and liver CAT levels in the CHM group. This increase may be due to the bioactive compounds present in the CPO and AMT extracts mixtures, including polysaccharides, saponins, and flavonoids, which demonstrate significant antioxidant effects (40). Furthermore, research has revealed that these herbs can modulate the immune system, stimulate cell regeneration and repair, and support normal metabolism and physiological functions, while simultaneously counteracting free radical damage within the body (41–43). These effects may be because some compounds in CHM are antioxidants that fight free-radical damage in the body and boost antioxidant activity.

An intact intestinal morphology and a healthy intestinal epithelial barrier are important for maintaining animal health, improving immunity, protecting the host from pathogens, and supporting subsequent growth (44–46). The jejunum serves as the primary site for nutrient absorption, featuring a multitude of villi on the mucosa. The intestinal mucosa has a direct effect on the ability of the host to resist potentially invading pathogens. As villus height increases, both the intestinal surface area and the number of epithelial cells increase, resulting in an enhanced capacity for nutrient absorption (47). In broilers studies, AMT polysaccharide has been shown to increase villus height in the small intestine (48). Our results showed that CHM supplementation increased villus height and the mRNA level of Claudin-1 in the jejunal mucosa. Claudin-1 is an important intestinal tight junction protein that facilitates formation and reinforcement of intercellular junctions, resulting in improved cell epithelial barrier strength and stability (49, 50). These functions were further confirmed by the decreased levels of DAO and endotoxin in the CHM group. The mucosal immune system is an essential component of the immune system (51). In addition, cells of the innate immune system produce important cytokines that contribute to adaptive immunity (52). IL-6 and TNF-α are innate immunity-related cytokines with pro-inflammatory properties that are crucial for host defense, inducing inflammation and triggering apoptosis (53). In contrast, IL-10 is a vital anti-inflammatory cytokine. Elevated levels of IL-10 during inflammation help modulate and balance the inflammatory process, thereby maintaining homeostasis (54). A study demonstrated that CPO induced increased cytokine (IL-2 and IFN-γ) levels, upregulated the expression of the appropriate mRNA in mice, and improved the immune organ index (55). Another study reported that a polysaccharide derived from CPO could potentially have immunomodulatory effects. It was found to enhance the secretion of cytokines (IL-6 and TNF-α) in RAW 264.7 macrophages, without exhibiting any cytotoxic effects (56). Moreover, CPO has been implicated in promoting the production of IL-2, TNF, and IFN in mice upon extraction of pectic polysaccharide (57). Consistent with our present results, we found that the mRNA levels of cytokines IL-6, IL-10, IFN-β, IFN-γ, and TNF-α were elevated in the jejunal mucosa. Therefore, it can be speculated that herbal medicine's active ingredients act as immunostimulants, activating immune cells and promoting proinflammatory mediator secretion, potentially leading to upregulation of cytokines such as IL-6 and TNF-α, which may subsequently upregulate IFN-β and IFN-γ. Concomitantly, upregulation of the anti-inflammatory cytokine IL-10 maintains homeostasis by counteracting the inflammatory process. These findings indicate that CHM may enhance the gut barrier function and promote intestinal health in broilers.

The gut microflora is crucial for regulating intestinal motility, immune homeostasis, and nutrient absorption (58). Furthermore, research has shown that intestinal flora can assist the host in protecting against pathogens and inflammatory bowel diseases, thus enhancing the host's digestive system health (59). Chao 1 is a measure of the community richness. Our findings showed that the addition of CHM reduced microbial richness and the observed number of OUT's, which was different from other studies (28, 60). This may be associated with the ability of CHM to eliminate and impede pathogenic disease-causing microbes in the gut. Furthermore, NMDS analysis indicated that the microbial communities in the CON and CHM groups differed in composition. At the phylum level, our results indicated that *Firmicutes* and *Bacteroides* dominated the cecal microflora, which was consistent with the results of previous studies (61, 62). The ratio of *Firmicutes* to *Bacteroidetes* is an important indicator of gut microbiota health (63), whereas inflammation and gut permeability improve when the ratio of *Firmicutes* to *Bacteroides* decreases (64). Studies have suggested that the addition of herbal medicines, such as CPO and AMT, decreases the ratio of *Firmicutes* to *Bacteroidetes* in weaned piglets (17), which is consistent with our results. There is evidence that *Bacteroidetes* and *Firmicutes* contribute to broiler digestion (65), and *Bacteroides* genus displays a positive impact on host health and disease resistance (66). In addition, we observed a higher abundance of *Bacteroidales_unclassified* and lower abundance of *Alistipes* after CHM supplementation. A study previous found that inhibiting the proportion of *Alistipes* increased its metabolism (67). In addition, *Alistipes*, a microbe that produces indole, can disrupt the serotonin balance in the gut through excessive growth (68). Hence, adding AMT

and CPO extracts to broiler diets could enhance gut microbiota and health by increasing *Bacteroidetes* abundance and modulating the *Firmicutes* to *Bacteroides* ratio.

Spearman's correlation analysis revealed that the cecum microbiota correlated with the FCR, serum differential metabolites, and gene expression in the jejunal mucosa. *Bacteroides* are frequently associated with the decomposition of polysaccharides, particularly starch and glucan (69). We found that *Bacteroidales_unclassified* was positively correlated with immune factors and tight junction protein mRNA expression, whereas it was negatively correlated with the FCR. Similar with our results, previous studies have found a positive relationship between the *Bacteroidetes* phylum and the plasma level of the pro-inflammatory cytokine TNF-α (70). The genus *Bacteroides* was linked to the cytokine IL-6 produced by monocytes and maintained the integrity of the epithelial barrier by controlling intraepithelial lymphocytes (from which IL-6 is formed) (71, 72). *Bacteroides* spp. have been proven to induce macrophages and monocytes to release TNF-α through LPS-mediated pathways (73). It has also been reported that *Bacteroides fragilis* supplementation enhances the expression of the tightly wound response proteins Claudin-1 using real-time qPCR and immunofluorescence staining (74). In contrast to the genus *Bacteroidales_unclassified* in the correlation analysis, we found that the genus *Alistipes* showed a negative correlation with the mRNA expression of immune factors and tight junction protein factor mRNA and a positive correlation with the FCR, which was different from the findings of previous studies (75, 76). Given that *Alistipes* is a subbranch of *Bacteroidetes* that is relatively recent in terms of pathogenicity, comparative data suggest that *Alistipes* may cause colon cancer (77). Moreover, *Alistipes*, a possible pathogen, is implicated in the pathogenesis, which thrives in an inflammatory environment devoid of lipocalin 2, encouraging inflammation and tumor development (78). The present study showed that CHM supplementation increased *Bacteroidales_unclassified* abundance and decreased *Alistipes* abundance. Therefore, CHM supplementation improved feed efficiency and increased the mRNA expression of pro-inflammatory cytokines in the jejunal mucosa. Additionally, CHM supplementation decreased the level of endotoxin and activities of DAO and LDH in the serum. These effects may be associated with alterations in the broiler gut microbiota.

5 Conclusion

Dietary supplementation with 500 mg/kg CHM (AMT and CPO extracts) improved the FCR and antioxidant capacity in the serum and liver and decreased the levels of UA and endotoxin and activity of DAO. Moreover, dietary CHM raised the concentrations of IL-6, IFN-γ, IFN-β, TNF-α, and the anti-inflammatory cytokine IL-10, while it induced alterations in the microbial composition of the cecum in broiler chickens. Spearman correlation analysis identified a correlation between cecal microbiota composition and FCR or serum differential metabolites or genes with significant differences in the jejunal mucosa, which might be attributed to the increased *Bacteroidales_unclassified* abundance and decreased *Alistipes* abundance in broilers. Further research is needed to explore the effects and mechanisms of the active ingredients in CHM, primarily AMT and CPO extracts, on animal health.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/; PRJNA1023878.

Ethics statement

The animal study was approved by Animal Care and Use Committee of Foshan University (approval ID: FOSU#19-025). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SL: Data curation, Methodology, Writing – original draft, Writing – review & editing. GX: Data curation, Methodology, Writing – original draft. QW: Investigation, Software, Writing – original draft. JT: Investigation, Software, Writing – original draft. XF: Writing – review & editing. QZ: Investigation, Writing – original draft. LG: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Funding supports were obtained from National Natural Science Foundation of China (No.32202718), Guangdong Basic and Applied Basic Research Foundation (No.2020A1515110819) and Key Area Research and Development Program of Guangdong Province (No.2019B110209005).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 14 December 2023
DOI 10.3389/fvets.2023.1320469

OPEN ACCESS

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RECEIVED 12 October 2023
ACCEPTED 29 November 2023
PUBLISHED 14 December 2023

CITATION
Liu M, Huang J, Ma M, Huang G, Zhang Y, Ding Y, Qu Q, Lv W and Guo S (2023) Effects of dietary Chinese herbal mixtures on productive performance, egg quality, immune status, caecal and offspring meconial microbiota of Wenchang breeder hens. *Front. Vet. Sci.* 10:1320469. doi: 10.3389/fvets.2023.1320469

Effects of dietary Chinese herbal mixtures on productive performance, egg quality, immune status, caecal and offspring meconial microbiota of Wenchang breeder hens

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This study aimed to evaluate the effects of Chinese herbal mixtures (CHMs) on productive performance, egg quality, immune status, anti-apoptosis ability, caecal microbiota, and offspring meconial microbiota in hens. A total of 168 thirty-week-old Wenchang breeder hens were randomly divided into two groups, with each group comprising six replicate pens of fourteen hens. The groups were fed a basal diet (CON group) and a basal diet with 1,000 mg/kg CHMs (CHMs group) for 10 weeks. Our results showed that dietary supplementation with CHMs increased the laying rate, average egg weight, hatch of fertile, and offspring chicks' weight while concurrently reducing the feed conversion ratio (FCR) and embryo mortality ($p < 0.05$). The addition of CHMs resulted in significant improvements in various egg quality parameters, including eggshell strength, albumen height, haugh unit, and the content of docosatetraenoic acid (C20:4n-6) in egg yolk ($p < 0.05$). The supplementation of CHMs had a greater concentration of IgA and IgG while decreasing the content of IL-6 in serum compared with the CON group ($p < 0.05$). Addition of CHMs to the diet increased the expression of Bcl-2 and IL-4 in liver and ovary, decreased the expression of IL-1β, Bax, and Caspase-8 in jejunum and ovary, and decreased the expression of NF-κB in liver, jejunum, and ovary ($p < 0.05$). Moreover, dietary CHMs reduced the abundance of *Desulfovibrio* in caecal microbiota as well as decreased the abundance of *Staphylococcaceae_Staphylococcus* and *Pseudomonadaceae_Pseudomonas* in the offspring meconial microbiota ($p < 0.05$). In conclusion, the CHMs could improve productive parameters by enhancing immune status, anti-apoptosis capacity, and modulating the caecal microbiota of Wenchang breeder hens, as well as maintaining the intestinal health of the offspring chicks.

KEYWORDS

Chinese herbal mixtures, Wenchang breeder hens, egg quality, immune status, intestinal microbiota

1 Introduction

A number of announcements from many countries stated that the application of antibiotics in livestock feeds was either strictly restricted or completely prohibited. The production and health state of breeder hens are challenged by more stress factors, including impaired gut function (1) and immune stress (2), which contribute to a downward trend in egg production rate and egg quality. Therefore, it is critical to produce safe feed additives to improve animal health protection. Due to their rich ingredients, Chinese herbal medicines are gaining popularity as dietary supplements for animals (3). Chinese herbal remedies are natural ingredients that have been used in livestock production as safe feed additives to preserve animal health and prevent sickness (4, 5).

A previous study found that *Epimedium* is a flavonoid-rich herb with a range of potential biological activities, such as antioxidant and antibacterial qualities (6). Angelica roots are primarily composed of chemical constituents such as ferulic acid, Z-ligustilide, butylidenephthalide, and various polysaccharides (7). Among these compounds, ferulic acid has anti-inflammatory and immunomodulatory effects (8). *Rehmanniae radix preparata* possesses several pharmacological properties, such as antioxidative (9) and those that enhance immunity (10). The primary components of Radix bupleuri extract consist of saikosaponins, flavonoids, polyacetylenes, and lignans (11), as well as having also been utilized as a remedy for immunomodulatory and antioxidative effects in human and animal species (12). The effective active substances in *Pericarpium citri reticulatae* include flavonoids, total phenols, carotenoids, and polysaccharides (13). The major components of *Leonurus japonicus* include alkaloids, diterpenes, and flavones (14). The pharmacological activities of Yimucao, such as lipid-lowering, anti-inflammatory, anti-oxidative, immunomodulatory, and anti-cancer activities (14, 15). *Paeoniflorin*, a major bioactive constituent in *Radix paeoniae* (16). A study conducted on mice using *Paeonia lactiflora* extract revealed its antioxidant, immunological, and anti-inflammatory effects (17). *Eucommia bark* is abundant in bioactive compounds, including lignans, phenolics, and iridoids. It has demonstrated anti-osteoporosis (18), anti-inflammatory, and antioxidant effects (19). *L. barbarum* polysaccharides are vital bioactive constituents found in *L. barbarum*, known for their diverse range of bioactivities, including antioxidant and immunomodulatory effects (20), as well as liver protection (21). The polysaccharide called *codonopsis pilosula* has generated a lot of attention owing to its prebiotic, antioxidant, anti-tumor, immunomodulatory, and anti-fatigue qualities (22). *Astragalus polysaccharide* has been shown to exhibit antioxidant, immune-regulating, and cardiovascular disease-alleviating effects (23). Chinese herbal medicines contain a variety of active ingredients, including polysaccharides, alkaloids, amino acids, vitamins, and more (24). These components can enhance animal performance by boosting the body's anti-apoptotic (25), anti-inflammatory, and antioxidant capabilities (26), as well as by modulating microbial composition (27). Previous research has shown that adding herbal mixture (*Paeonia lactiflora, licorice, dandelion*, and *tea polyphenols*) can enhance growth performance, boost immunity, and alter the composition of the intestinal microbiota in weaning pigs (28). Certain studies conducted on pigeons have discovered that the addition of AEF (*Astragalus, Epimedium*, and *Ligustrum lucidum*) extract to their drinking water resulted in improved intestinal health and enhanced growth performance, especially under conditions of stress pigeons (29). A herbal mixture made up of numerous herbs includes multiple active components that may be more effective biologically than a single herb, as well as having a wider range of applications and better production results (28, 42).

In the animal body, the intestinal microbiota not only governs the digestion and absorption of nutrients (30) but also influences immune homeostasis and chronic diseases (31). It's worth noting that the gut microbiota can be passed from the mother to her offspring through vertical transmission (32). The transmission of the microbiota is influenced by the microbiome composition of maternal hens' feces, embryos, and chicks' ceca. Maternal nutritional strategies play a critical role in regulating the phenotypic traits of animal offspring, and ensuring proper maternal nutrition is essential for early embryonic development (33). Furthermore, the nutrients in eggs are necessary for the early growth and development of chicks. There is a hypothesis that alterations in the maternal diet might affect the nutrient constituents of the eggs, consequently influencing the nutritional requirements and gut microbiota of chicken offspring (34).

To date, the combination of CHMs (*Epimedium, Angelica sinensis, Rehmanniae Radix Preparata, Radix Bupleuri, Pericarpium citri reticulatae, Leonurus japonicus, Radix Paeoniae Alba, Eucommia ulmoides, Lycii fructus, Codonopsis pilosula*, and *Astragalus membranaceus*) has not been extensively studied in Wenchang Breeder Hens. Furthermore, the potential influence of maternal supplementation with CHMs on the meconium microbiota of offspring has yet to be fully understood. The objective of this study was to explore the potential benefits of maternal supplementation with CHMs on various aspects, including productive performance, egg quality, immune status, anti-apoptosis ability, and intestinal microbiota, and to investigate the potential influence on the meconial microbiota structure in offspring chicks.

2 Materials and methods

2.1 Preparation of Chinese herbal mixtures

The raw materials of Chinese herbal mixtures were purchased from Hebei Anguo Qi'an Pharmaceutical Co. Ltd. in China, and then the herbs were crushed into fine powder, sifted through an 80-mesh sieve, and mixed thoroughly in proportion and stored at room temperature (25°C) for later use. The CHMs has the following contents per 100 g: *Epimedium* 10.5 g, *Angelica sinensis* 10.5 g, *Rehmanniae Radix Preparata* 5.3 g, *Radix Bupleuri* 5.3 g, *Pericarpium citri reticulatae* 5.3 g, *Leonurus japonicus* 15.8 g, *Radix Paeoniae Alba* 5.3 g, *Eucommia ulmoides* 10.5 g, *Lycii fructus* 10.5 g, *Codonopsis pilosula* 10.5 g, and *Astragalus membranaceus* 10.5 g in the ratio 2:2:1:1:1:1:3:1:2:2:2:2:2:2. The proximate chemical composition of CHMs was determined using AOAC (Association of Official Analytical Chemists) procedures (35), which included crude protein,

Abbreviations: CHMs, Chinese herbal mixtures; FCR, feed conversion ratio; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; IgA, immunoglobulin A; IgM, immunoglobulin G; IL-6, interleukin-6; Bax, BCL2-Associated X; BCL-2, B-cell lymphoma-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; IFN-γ, interferon-γ; MyD88, Myeloid Differentiation Factor 88; NF-κB, Nuclear factor-kappa B; TLR4, Toll-like receptor 4.

TABLE 1 The nutritive composition and active ingredients of CHMs powder.

Nutritive ingredients	Content (%)	Effective active ingredients	Content (mg/g)
Crude protein	8.93	Total polysaccharides (%)	12.65
Crude fat	2.7		
Crude fiber	15.4		
Ash	6.5		
Ca	0.64		
P	0.21		

TABLE 2 The ingredient and nutrient composition of the basal diet (% as fed basis).

Ingredients	Content (%)
Corn	57.80
Soybean meal	23.90
Fish meal	3.40
Soybean oil	1.60
Limestone	6.85
Gypsum Powder	0.65
Calcium hydrogen phosphate	1.20
Uniform chaff	3.60
Premixes[a]	1.00
Total	100
Nutrient composition[b]	
Digestible energy (MJ/kg)	11.31
Crude protein (%)	17.03
Calcium (%)	3.14
Available phosphorus (%)	0.36
SID-Lys (%)	0.94
SID-Met (%)	0.41
SID-Cys (%)	0.26

[a]The premix per kilogram of feed contains vitamin A 16500 IU, vitamin D 36250 IU, vitamin E 75 IU, vitamin K3 10 mg, vitamin B1 5 mg, vitamin B2 15 mg, vitamin B6 15 mg, vitamin B12 0.05 mg, vitamin C 186 mg, folic acid 2.5 mg, D-biotin 0.375 ng, nicotinamide 100 mg, DL-tocopheryl acetate 40 mg, Fe 200 mg, Cu 16.66 mg, Mn 184 mg, Zn 150 mg, I 0.834 mg, Se 0.416 mg, choline chloride 0.75 g, DL-methionine 1.188 g, DL-lysine 0.591 g, $NaHCO_3$ 1.485 g, NaCl 2.39 g, phytase 1,500 IU, xylanase 1,500 IU, cellulase 250 IU, acid protease 125 IU, Amylase 25,000 IU, β-mannanase 4,500 IU, β-glucanase 1,500 IU.
[b]The nutrient levels were calculated from data provided by Feed Database in China.

ether extract, crude fiber, ash, total phosphorus, and calcium. The total polysaccharides in CHMs were determined using the phenol-sulfuric acid method with glucose as the standard (36). The active ingredients and nutrient composition of CHMs are shown in Table 1.

2.2 Animals, diet, and experimental design

All experimental protocols were approved by the Animal Care and Use Committee of the South China Agricultural University (approval number: SYXK 2019-0136, Guangzhou, China).

A total of 168 thirty-week-old Wenchang breeder hens were randomly assigned to two groups, each consisting of six replicates, with fourteen hens in each replicate. These Wenchang breeder hens were sourced from Enping Jilong Industrial Co., Ltd. (Jiangmen, China) and were housed and fed at the Enping Jilong hens production facility. The groups were fed a basal diet (CON group) and a basal diet with 1,000 mg/kg CHMs (CHMs group) for 10 weeks. The photoperiod was maintained at 16 h of light and 8 h of darkness throughout the study. Each hen received 88 g of feed per day to prevent overfeeding and had unlimited access to fresh water. Breeder hens underwent artificial insemination, with 35 μL of pooled semen administered to each bird every 3 days, following the procedure outlined by Liu et al. (37). The ingredient and nutrient composition of the basic diets was shown in Table 2.

2.3 Sample collection

At the end of the feeding period, one hen per replicate (a total of six hens) was randomly selected and subjected to a 12 h fasting period for sample collection. Blood samples were drawn from the wing vein into tubes and allowed to stand at room temperature for 20 min. Subsequently, the tubes were centrifuged at 3000 × g for 10 min at 4°C to collect the serum. These serum samples were stored at −20°C for future analysis. Hens were euthanized by exsanguination and necropsied, and the liver, ovary, and mucosal samples of the middle jejunum were collected immediately and quickly frozen at −80°C for further analysis.

In the final week of the experiment, eggs from each group were collected and subsequently incubated using the same incubator. After hatching, meconium samples were collected from the offspring chicks by gently massaging their abdominal regions. Both cecal digesta and meconium samples were aseptically collected and then stored at −80°C for 16S rRNA analysis.

2.4 Measurement of the laying and hatching performance and egg quality

Laying performance was assessed throughout the experiment by daily recording of egg production, egg weight, and the number of qualified eggs. Laying rate, average egg weight, qualified egg rate, and feed conversion rate were calculated based on the data collected from each replicate.

At the conclusion of the experimental period, a random selection of 10 eggs from each replicate was used to evaluate egg quality. We measured both the horizontal and vertical diameters of these selected eggs using a Vernier caliper (530-101, Mitutoyo, Japan) to calculate the egg shape index. Eggshell strength was determined with an eggshell strength tester (ESG-1, Yaoen, Nanjing, China), while eggshell thickness and weight were measured separately using a Vernier caliper and an electronic balance (FB224, Hengping, Shanghai, China), respectively. Furthermore, egg yolk color, albumen height, and the Haugh unit were assessed using an automatic egg quality tester (EA-01, Orka, Israel).

For hatching performance assessment, all eggs were incubated in the same incubator (Bengbu Sanyuan Incubation Equipment Co., Ltd., Anhui, China) during the experimental period. The incubator maintained a temperature range of 37.2°C to 38.0°C and a relative

humidity of 60 to 75%. Eggs were manually turned 12 times a day throughout the incubation period and were lightly sprayed with water once daily, starting from the 15th day of incubation until they hatched (38). The parameters, including fertility, hatching of fertile eggs, hatchability of set eggs, embryo mortality, and the weight of the hatched chicks, were recorded and calculated.

2.5 Measurements of fatty acid content of egg yolk

At the conclusion of the experimental period, five eggs per replicate were randomly selected to assess the fatty acid content in the egg yolk. The fatty acid composition of the samples was determined using gas chromatography. The mean level of each fatty acid was then used to calculate the total content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Fatty acid measurement services were provided by Waltek Testing Group (Foshan) Co., Ltd.

2.6 Analysis of biochemical components in serum

The activities of caspase-8 (caspase-8; Detection range 83.75 pmol/L ~ 120 pmol/L), the serum concentration of immunoglobulin A (IgA; Detection range 10 μg/mL ~ 320 μg/mL), immunoglobulin G (IgG; Detection range 75 μg/mL ~ 2,400 μg/mL), and interleukin-6 (IL-6; Detection range 1 pg./mL ~ 32 pg./mL) were examined by Enzyme-linked immunosorbent assay (ELISA). All ELISA kits were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (China). Serum concentrations of IgA, IgG, IL-6, and Caspase 8 activity were measured following the instructions of the ELISA kit.

2.7 Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissue samples (liver, ovary, and jejunal mucosa) using the RNA isolator Total RNA Extraction Reagent kit (Vazyme). Subsequently, total cDNA was synthesized using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme) with 1 μg of total RNA, followed by RT-qPCR amplification using the ChamQ universal SYBR qPCR Master Mix (Vazyme). Gene primers for qRT-PCR were designed by Primer Premier 6.0 software (Premier Biosoft International, United States), synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China), and used in this study (Table 3). The relative expression of the target gene was analyzed using the $2^{-\Delta\Delta Ct}$ method, normalized against the geometric mean of the expression of β-actin and GAPDH (39).

TABLE 3 Laying performance of breeder hens.

Gene	Primer sequence (5′ → 3′)	Accession number
β-actin	Forward: GAGAAATTGTGCGTGACATCA	L08165.1
	Reverse: CCTGAACCTCTCATTGCCA	
Bax	Forward: GGTGACAGGGATCGTCACAG	XM_422067
	Reverse: TAGGCCAGGAACAGGGTGAAG	
Bcl-2	Forward: GCTGCTTTACTCTTGGGGGT Reverse: CTTCAGCACTATCTCGCGGT	NM_205339.2
Caspase8	Forward: TAAAATGACCAGCCGACCCC	NM_204592.4
	Forward: TCTGCATCCACATGTGTCCC	
GAPDH	Reverse: ACCTCTGTCATCTCTCCACA	K01458
	Reverse: GGCAGAGCTCAGTGTCCATT	
IL-1β	Forward: CAGCCTCAGCGAAGAGACCTT Reverse: ACTGTGGTGTGCTCAGAATCC	NM_204524.2
IL-4	Forward: CTTCCTCAACATGCGTCAGC	AJ621735
	Reverse: TGAAGTAGTGTTGCCTGCTGC	
MyD88	Forward: ATCCGGACACTAGAGGGAGG	NM_001030962.1
	Reverse: GGCAGAGCTCAGTGTCCATT	
NF-κB	Forward: GTGTGAAGAAACGGGAACTG	NM_205129
	Reverse: GGCACGGTTGTCATAGATGG	
TLR4	Forward: AGGCACCTGAGCTTTTCCTC	NM_001030693.1
	Reverse: TACCAACGTGAGGTTGAGCC	

2.8 Cecal and meconial microbiota analysis in breeder hens and offspring chicks

This trial followed the established procedures of previous researchers (40). To isolate total microbial DNA from the cecal and meconial content samples, we utilized the QIAamp DNA Stool Kit (Qiagen, Valencia, United States). The V3–V4 region of the bacterial 16S rRNA gene was amplified through PCR with the following primers: forward primer 5′-ACTCCTACGGGAGGCAGCA-3′ and reverse primer 5′-GGACTACHVGGGTWTCTAAT-3′. PCR products were then purified using Vazyme VAHTS™ DNA Clean Beads (Vazyme) and quantified with a PicoGreen dsDNA Assay Kit (Invitrogen, United States). Subsequently, we conducted 16S rRNA sequencing

TABLE 4 Laying performance of breeder hens.

Items	CON	CHMs	SEM	Value of *p*
Laying rate, %	61.92	65.62*	0.081	0.014
Average egg weight, g	43.09	44.37*	0.311	0.032
Percentage of qualified eggs, %	94.82	95.70	0.030	0.155
Feed conversion ratio, g/g	3.32	3.08*	0.055	0.015

*Significant differences in comparison with the CON group are expressed as *$p < 0.05$ and **$p < 0.01$ ($n = 6$).

on the Illumina Novaseq_PE250 platform (Illumina), with sequencing services provided by Personal Biotechnology Co., Ltd. in Shanghai, China. Data collection and analysis were carried out using the Genescloud Platform[1].

2.9 Statistical analysis

All data were initially organized using Excel software and subsequently subjected to statistical analysis with SPSS 20 and GraphPad Prism 7.0 software. For the comparison of data between two groups, a two-tailed unpaired Student's *t*-test was employed. The data are presented as the means ± SEM. Significance was determined at $p < 0.05$.

3 Results

3.1 Effects of dietary CHMs on laying performance of breeder hens

Regarding the laying performance of breeder hens, as presented in Table 4, there was no significant difference was observed in the percentage of qualified eggs between the CON and CHMs groups. However, the CHMs group showed a significant increase in laying rate and average egg weight ($p < 0.05$) compared to the CON group, along with a decrease in the feed conversion ratio ($p < 0.05$).

3.2 Effects of dietary CHMs on hatchability of breeder hens

The fertilizing capacity and hatchability results were presented in Table 5. The embryo mortality of the CHMs group was significantly decreased ($p < 0.05$) than that of the CON group. In addition, supplementing CHMs to the diet led to a significant increase in the hatching of fertile eggs and the weight of offspring chicks compared to the CON group ($p < 0.05$).

3.3 Effects of dietary CHMs on egg quality of breeder hens

Concerning the egg quality indices shown in Table 6, the CHMs in diet did not affect egg shape index, eggshell thickness, eggshell ratio, egg yolk colour and yolk percentage at 10 weeks. However, CHMs significantly improved eggshell strength ($p < 0.05$), albumen height ($p < 0.01$) and haugh unit ($p < 0.05$).

3.4 Effects of dietary CHMs on fatty acid content in egg yolk

The fatty acid contents of egg yolk from breeder hens fed with CHMs were presented in Table 7. Regarding SFA, compared to the CON group, CHMs significantly increased margaric acid (C17:0) in the yolk ($p < 0.05$), and we also observed a tendency to increase behenic acid (C22:0, $p = 0.086$). Furthermore, there was a significant increase in cis-11-Eicosenoic acid (C20:1), a type of monounsaturated fatty acid (MUFA), in the CHMs group ($p < 0.05$). In the case of polyunsaturated fatty acids (PUFAs), CHMs-supplemented diets resulted in a significant increase in the content of dihomo γ-linolenic acid (C20:3n-6) and docosatetraenoic acid (C20:4n-6) in egg yolk ($p < 0.05$).

3.5 Effects of dietary CHMs on immunoglobulins and inflammatory factors of breeder hens

To further investigate the impact of CHMs on immune function, we assessed the levels of immunoglobulins and inflammatory factors in breeder hens (Figure 1). The serum concentrations of IgA and IgG in the CHMs group were significantly higher than those in the CON group ($p < 0.01$ and $p < 0.05$, respectively). With the introduction of CHMs, the serum levels of the proinflammatory cytokine IL-6 in breeder hens were significantly lower than those in the CON group ($p < 0.05$). Moreover, dietary supplementation with CHMs led to increased mRNA expression of IL-4 ($p < 0.05$ and $p < 0.05$, respectively) and reduced mRNA expression of NF-κB ($p < 0.05$ and $p < 0.01$, respectively) in both the liver and ovary. It also resulted in decreased mRNA expression of IL-1β in the ovary of breeder hens compared to the CON group ($p < 0.05$). In the jejunal mucosa of breeder hens, CHMs feed supplementation significantly decreased the relative mRNA levels of MyD88, NF-κB, and IL-1β ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) compared to the CON group.

3.6 Effects of dietary CHMs on apoptosis-related factors of breeder hens

As shown in Figure 2, in the serum of breeder hens fed with dietary CHMs, there was a trend towards lower Caspase-8 concentrations ($p = 0.087$). Additionally, a significant decrease in the mRNA expression of Caspase-8 was observed in the jejunal mucosa and ovary of the CHMs group ($p < 0.05$). In comparison to the CON group, the mRNA expression of the anti-apoptotic gene Bcl-2 showed a significant increase ($p < 0.05$), and the Bax/Bcl-2 ratio was

1 www.genescloud.cn

TABLE 5 Fertilizing capacity and hatchability of breeder hens.

Items	CON	CHMs	SEM	Value of *p*
Embryo mortality, %	7.61	3.16*	1.173	0.047
Fertility of set eggs, %	93.00	94.00	1.067	0.667
Hatch of fertile eggs, %	92.39	96.84*	1.173	0.045
Hatchability of set eggs, %	86.00	90.00	1.675	0.143
Offspring chick's weight, g	31.93	32.90*	0.230	0.023

*Significant differences in comparison with the CON group are expressed as $*p < 0.05$ ($n = 6$).

TABLE 6 Egg quality of breeder hens.

Items	CON	CHMs	SEM	Value of *p*
Egg shape index	1.33	1.31	0.010	0.534
Eggshell thickness, mm	0.32	0.32	0.004	0.937
Eggshell strength, kg/cm^2	3.76	4.30*	0.121	0.023
Eggshell ratio, %	13.11	13.20	0.248	0.850
Albumen height, mm	2.51	3.21**	0.139	0.009
Yolk colour	5.38	5.88	0.205	0.227
Haugh unit	48.07	56.42*	1.732	0.013
Yolk percentage, %	32.47	32.88	0.713	0.695

*Significant differences in comparison with the CON group are expressed as $*p < 0.05$ and $**p < 0.01$ ($n = 12$).

TABLE 7 Fatty acid content in egg yolk of breeder hens.

Items	CON	CHMs	SEM	*p*-value
C14:0	0.040	0.045	0.002	0.341
C15:0	0.004	0.005	0.001	0.253
C16:0	1.830	2.118	0.157	0.390
C17:0	0.013	0.023*	0.003	0.046
C18:0	1.338	1.574	0.137	0.421
C22:0	0.013	0.019	0.002	0.086
Total SFA	3.238	3.784	0.293	0.382
C14:1	0.007	0.007	0.0006	0.919
C16:1	0.317	0.332	0.026	0.815
C17:1	0.006	0.008	0.001	0.450
C18:1n-9	5.698	5.414	0.540	0.810
C20:1	0.049	0.065*	0.004	0.019
C24:1	0.035	0.036	0.006	0.925
Total MUFA	6.112	5.862	0.567	0.840
C20:2	0.016	0.018	0.001	0.616
C18:3n-3	0.022	0.024	0.002	0.635
C20:3n-3	0.234	0.208	0.043	0.782
C22:6n-3	0.155	0.221	0.024	0.186
C18:2n-6	1.964	2.328	0.171	0.314
C18:3n-6	0.017	0.021	0.002	0.317
C20:3n-6	0.006	0.012*	0.001	0.022
C20:4n-6	0.067	0.156*	0.022	0.028
Total PUFA	2.481	2.987	0.219	0.273

*SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Significant differences in comparison with the CON group are expressed as $*p < 0.05$ ($n = 5$).

significantly down-regulated ($p < 0.05$) in the liver of breeder hens in the CHMs group. Additionally, in the jejunal mucosa, the CHMs group exhibited a higher relative mRNA expression of the pro-apoptotic gene Bax compared to the CON group ($p < 0.05$). In the ovary of breeder hens, the CHMs group had higher expression of Bcl-2 ($p < 0.05$) and lower expression of Bax ($p < 0.05$) compared to the CON group, resulting in a highly significant downregulation of the Bax/Bcl-2 ratio ($p < 0.01$).

3.7 Description of the 16S rRNA gene sequencing data

To explore the effects of dietary supplementation with CHMs on the gut microbiota, we performed 16S rRNA gene sequencing on the cecal content and meconium of breeder hens and offspring chicks, respectively. The diversity of the cecal and meconial microbiota was shown in Figure 3. Venn diagram analysis revealed that 1,699 OTUs were overlapped among the two groups, and 12,341, and 10,151 specific OTUs were unique to the CON and CHMs groups on the cecal microbiota, respectively (Figure 3A). Concerning the meconial microbiota of offspring chicks, a Venn diagram analysis revealed 48 shared OTUs between the CON and CHMs groups. The CON group exhibited 396 unique OTUs, while the CHMs group had 188 unique OTUs (Figure 3B). Based on the PCA (principal component analysis) scatterplot, a clear separation was observed between the cecal samples of the CON and CHMs groups, with no overlapping clusters (Figure 3C). However, there was no obvious separation between the meconium samples of CON and CHMs groups (Figure 3D). Then, we employed Chao 1, Observed_species, Shannon index, and Simpson index to assess the alpha diversity of the cecal and meconial microbiota. The Shannon and Simpson diagrams indicated that the α diversity of the cecal microbiota in breeder hens was visibly lower in the CHMs group than that in the CON group ($p < 0.05$) (Figure 3E). Furthermore, in comparison to the CON group, the CHMs group exhibited a decrease in the Shannon indices in the meconial microbiota ($p < 0.05$) (Figure 3F). No significant differences were observed in the Chao 1 and Observed_species indices of the cecal and meconial microbiota between the two groups ($p > 0.05$).

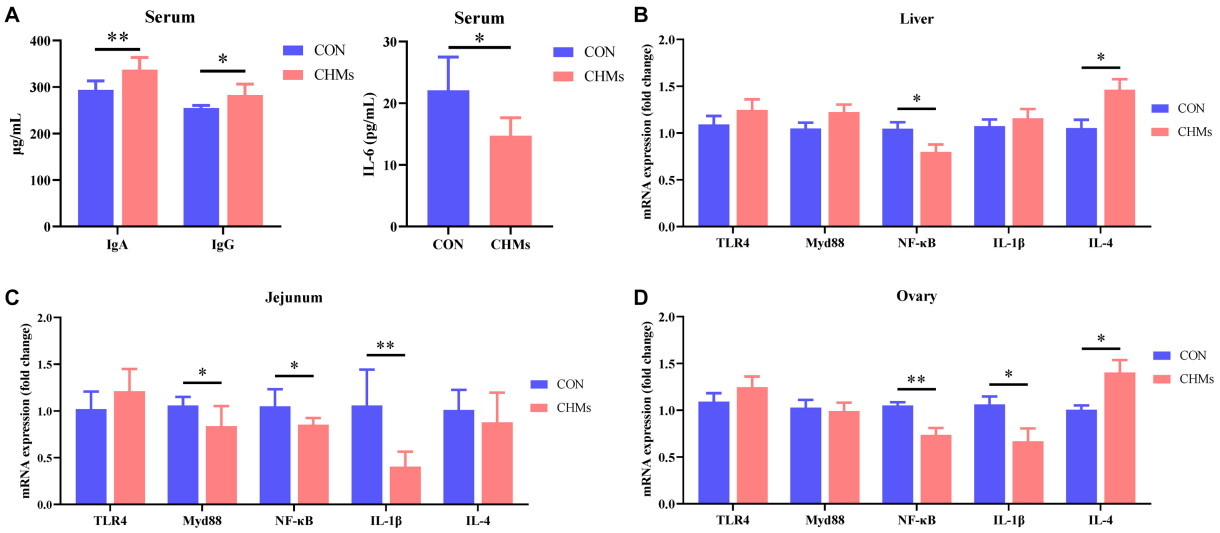

FIGURE 1
Effects of dietary CHMs on immunoglobulins and inflammatory factors of breeder hens. **(A)** Serum IgA, IgG; **(B)** Serum IL-6; **(C,D)** The expressions of inflammatory genes (TLR4, MyD88, NF-κB, IL-1β, IL-4) in liver, jejunum and ovary, respectively. The data were expressed as mean ± SEM ($n = 6$). *$p < 0.05$ and **$p < 0.01$ vs. CON group.

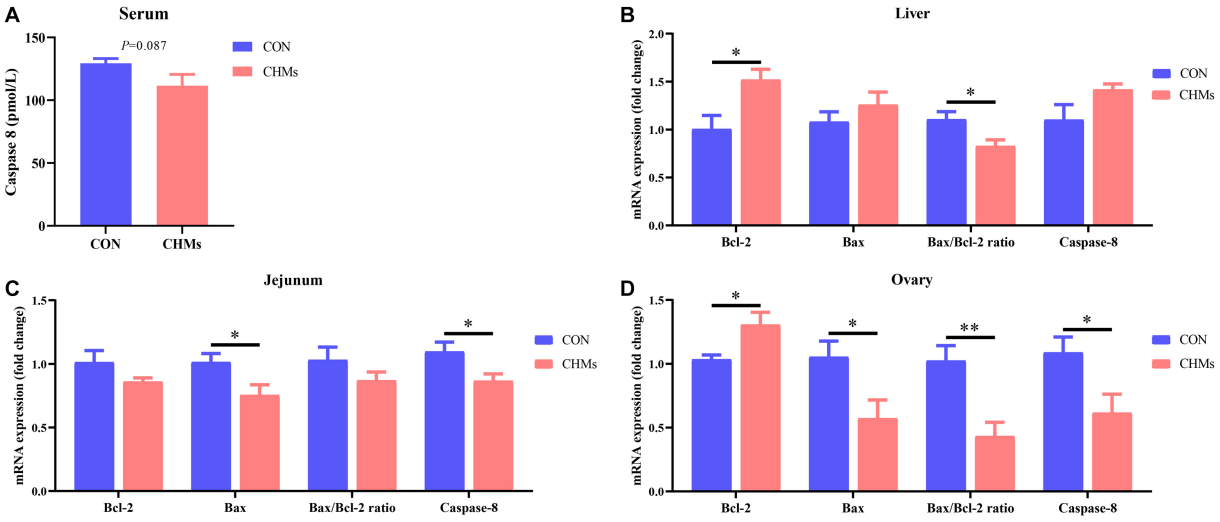

FIGURE 2
The impact of dietary CHMs on apoptosis-related factors in breeder hens. **(A)** Serum Caspase-8 concentration; **(B–D)** Relative mRNA expression of apoptosis-related genes in the live, jejunum and ovary, including Bcl-2, Bax, Bax/Bcl-2 ratio and Caspase-8. The data were expressed as mean ± SEM ($n = 6$). *$p < 0.05$ and **$p < 0.01$ vs. CON group.

3.8 Changes in cecal and meconial microbiota communities

To explore how maternal nutrition interventions, immunity, and microbes may play roles in maternal effects, we analyzed the changes in the composition of the cecal and meconial microbiota communities at the phylum and genus levels (Figure 4). In the cecal microbiota of breeder hens, the three most abundant bacterial phyla were *Bacteroidetes, Firmicutes*, and *Proteobacteria* (Figure 4A). The dominant genera included *Bacteroides*, *Oscillospira*, and *Lactobacillus*. At the phylum level, analysis revealed significantly lower abundances of *Proteobacteria* in the cecal microbiota of breeder hens in the CHMs group compared to the CON group ($p < 0.05$) (Figure 4C). However, the abundances of *Bacteroidetes* and *Spirochaetes* exhibited an upward trend in the CHMs group, respectively ($p = 0.079$, $p = 0.071$). Notably, at the genus level of the cecal microbiota, the intervention of CHMs significantly reduced the relative abundance of *Desulfovibrio* ($p < 0.05$) (Figure 4E). Compared to the CON groups, there was a decreasing trend in the relative abundance of *Bacteroides* in the CHMs group ($p = 0.071$), while the relative abundance of *Ruminococcaceae_Ruminococcus* showed an upward trend in the CHMs group ($p = 0.087$).

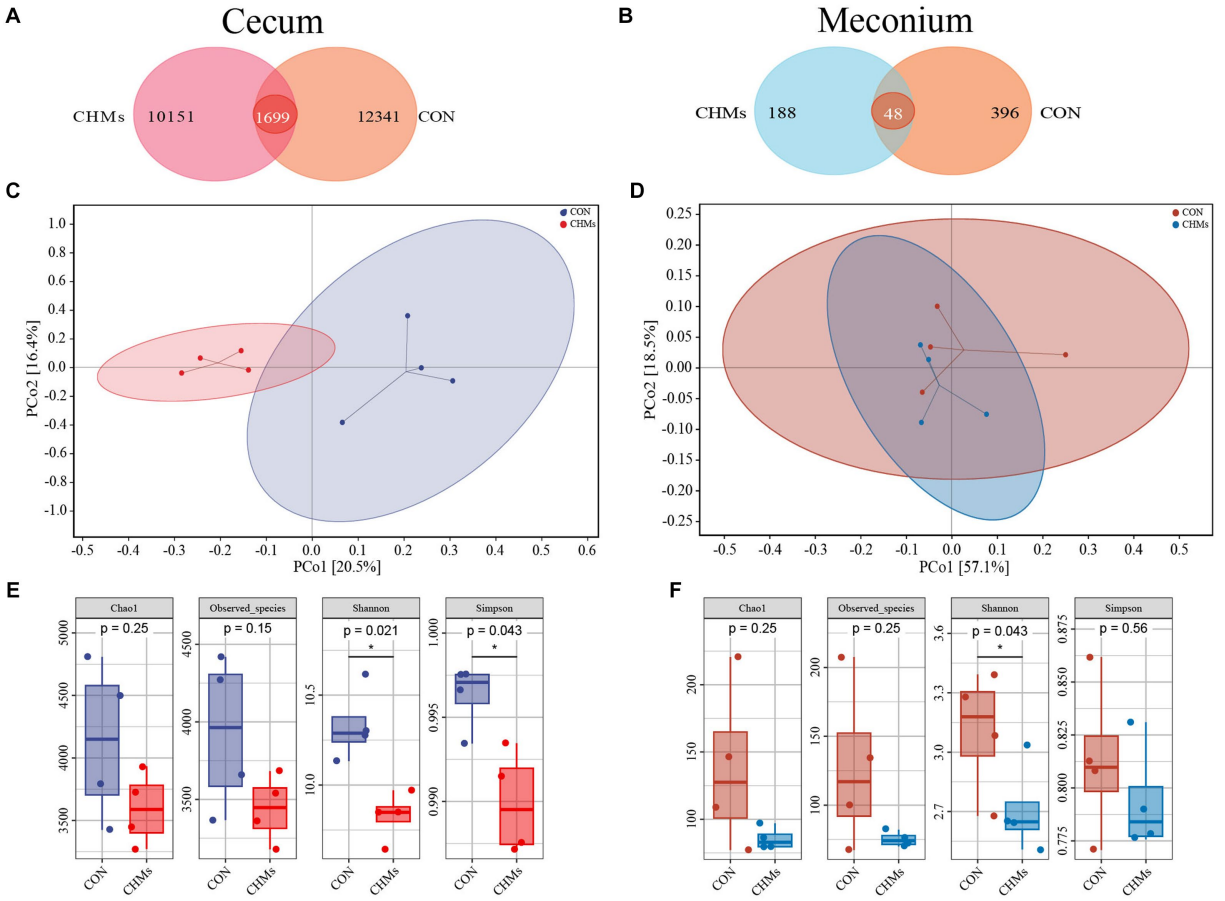

FIGURE 3
Gut microbiota diversity of breeder hens and offspring chicks. **(A,B)** Venn diagram of caecal and meconial microbiota on OTUs level. **(C,D)** PCoA two-dimensional figure based on Bray_Curtis distance analysis. **(E,F)** Alpha diversity, including Chao 1, Observed_species, Shannon index and Simpson index. The data were expressed as mean ± SEM ($n = 4$). *$p < 0.05$, vs. CON group.

Next, we investigated the composition and structure of the meconial microbiota based on some maternal effects benefiting offspring fitness. In the meconial microbiota of offspring chicks, *Firmicutes* and *Bacteroidetes* emerged as the two most abundant bacterial phyla in both the CON and CHMs groups, accounting for over 98% of the total phyla (Figure 4B). At the genus level, *Sphingomonas* and *Ralstonia* were the dominant genera in the meconial microbiota, followed by *Caulobacter* and *Clostridiaceae_Clostridium*. No significant differences were observed in the bacterial phyla between the two groups ($p > 0.05$) (Figure 4D). In comparison to the CON group, the CHMs group exhibited a lower relative abundance of *Staphylococcaceae_Staphylococcus* and *Pseudomonadaceae_Pseudomonas* ($p < 0.05$) (Figure 4F). The relative abundance of Ralstonia showed an upward trend in the CHMs group ($p = 0.068$).

The Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was employed to identify the major microflora with significant differences between the CON group and CHMs group. The LEfSe taxonomic cladogram of cecal microbes revealed that *Spirochaetes* (class), *Treponema* (genus), and *Spirochaetaceae* (family) exhibited higher abundance in the CHMs group (Figure 4G). While the *Proteobacteria* (phylum), *Desulfovibrionaceae* (family), and *Deltaproteobacteria* (class) were the most significant abundance of the CON group. Regarding the LEfSe analysis of meconial microbes, some bacterial groups, including the *Ralstonia* (family), *Betaproteobacteria* (class) and *Oxalobacteraceae* (family) in CHMs group had a higher score, whereas some other bacterial groups, such as *Bacilli* (class), *Bacillales* (order), *Staphylococcaceae* (family) in CON group had a higher score (Figure 4H).

3.9 Correlation analysis of altered intestinal microbe and different indicators

Potential correlations between the meconial and cecal microbiota alterations with many different indicators were evaluated employing a Spearman correlation analysis (Figure 5). The genera *Desulfovibrio* and the phylum *Proteobacteria* were negatively correlated with laying performance ($r = -0.88, -0.83$, $p < 0.05$) but positively correlated with the level of serum IL-6 ($r = 0.95, 0.81$, $p < 0.05$) (Figure 5A). Additionally, the level of serum IgG and laying performance were positively correlated with the phyla Bacteroidetes ($r = 0.76, 0.90$, $p < 0.05$). As shown in Figure 5B, the weight of offspring chicks had a connection positively with the abundances of *Ralstonia* ($r = 0.76$; $p < 0.05$), but

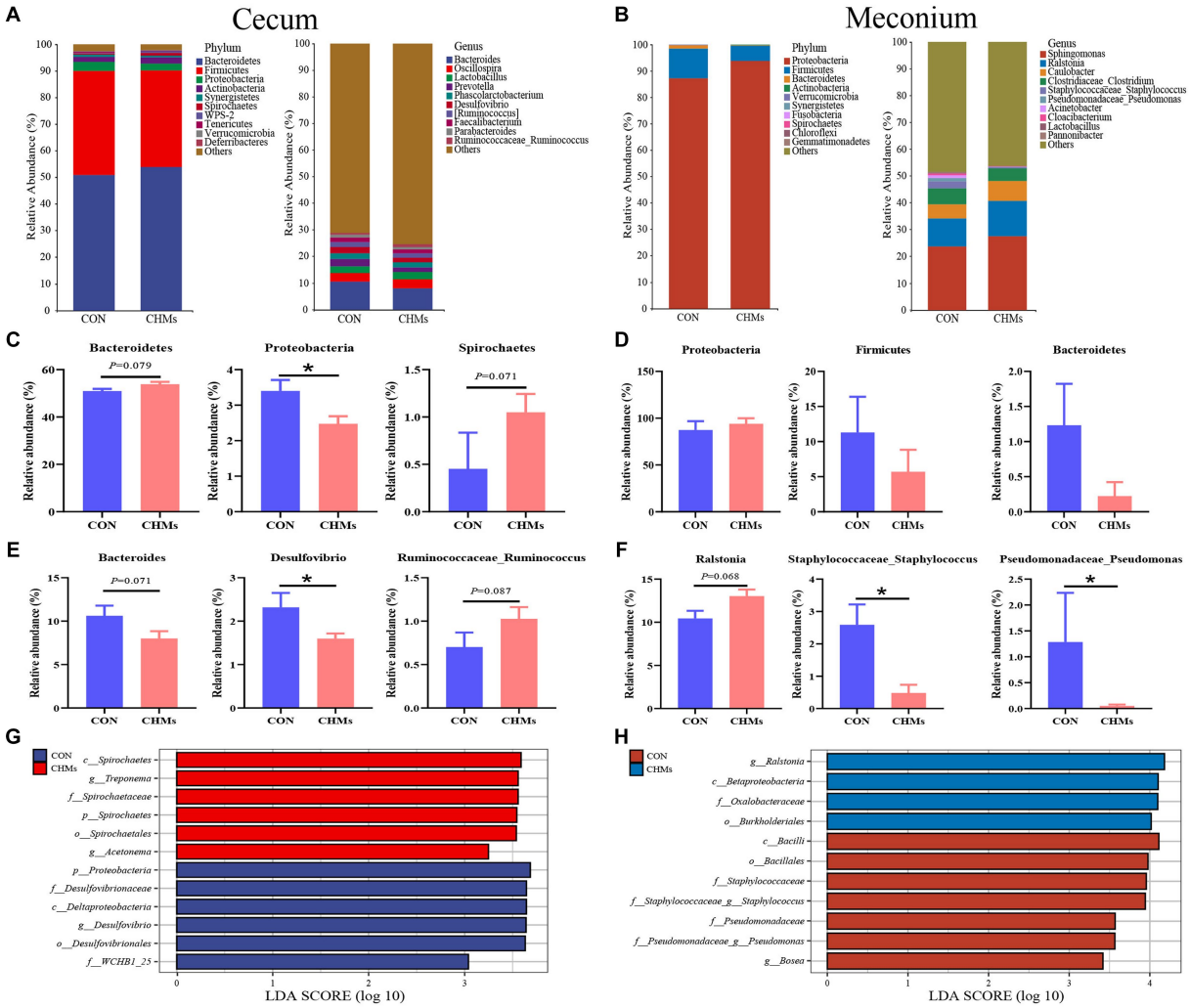

FIGURE 4
Gut microbiota structure of breeder hens and offspring chicks. **(A,B)** Relative abundance of caecal and meconial microbiota on phylum and genus level. **(C–F)** The significant difference genera and phylum between two groups on caecal and meconial microbiota, respectively. **(G,H)** LEfSE taxonomic cladogram between two groups on caecal and meconial microbiota (LDA > 3). The data were expressed as mean ± SEM ($n = 4$). *$p < 0.05$, vs. CON group.

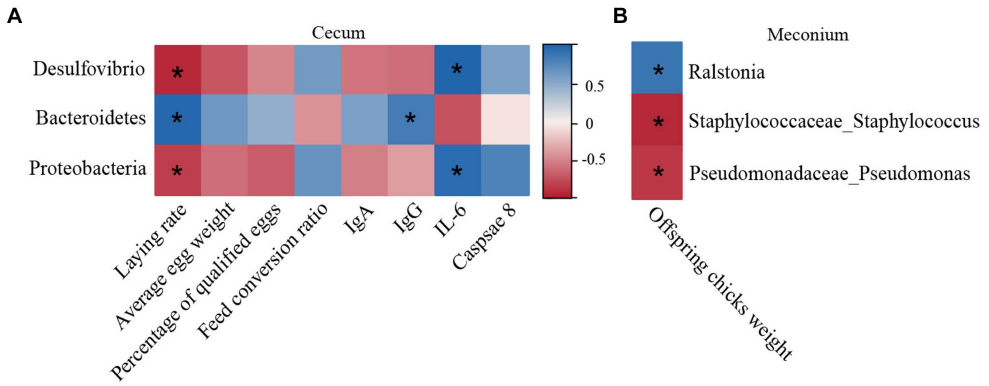

FIGURE 5
Matrix diagram of the correlation analysis. **(A)** Spearman correlation analysis among the cecal microbiota, laying performance, and serum indices. **(B)** Spearman correlation analysis between the meconial microbiota and weight of offspring chicks. Positive and negative correlations are shown by the blue and red matrices, respectively. *$p < 0.05$ ($n = 4$).

it was negative correlated with *Staphylococcaceae_Staphylococcus* and *Pseudomonadaceae_Pseudomonas* ($r = -0.81, -0.74, p < 0.05$).

4 Discussion

In the current experiment with Wenchang Breeder Hens, we found that dietary supplementation with CHMs beneficially affected laying rate and average egg weight, although it reduced feed conversion rate. Despite the different combinations of Chinese herbal mixture (*Pine needle* and *Artemisia annua*), Li et al.'s study also reported that CHM increased egg production rates in hens but decreased feed conversion rates, which may be related to the regulation of cholesterol metabolism by CHM (41). Epimedium in CHMs has been shown to have multiple regulatory functions, including sexual dysfunction, hormone metabolism, immune function, and antioxidant properties (6). In our study, these great potentials were confirmed in the growth performance of laying hens. CHMs reduced embryonic mortality while increasing the hatching of fertile eggs and offspring chicks' weight in this study. Therefore, the increase in egg production and improvement in hatching performance may be attributed to the improved health status of laying hens fed a diet supplemented with CHMs. However, due to the complexity of CHMs and the limitations of this study, further research is still needed to investigate their impact on animals.

Extracts of plant-derived polyphenols and polysaccharides demonstrate strong antioxidant activity, which effectively prevent protein breakdown by lowering oxidation of proteins and lipids (42). Early studies have suggested that the addition of Chinese herbs, specifically a combination of *R. astragali*, *S. miltiorrhiza Bunge*, and *C. monnieri*, led to a significant improvement of the albumen height and haugh unit in eggs (43). In terms of egg quality, dietary supplementation with CHMs significantly increased eggshell strength, albumin height, and haugh unit in this study. The CHMs contain a combination of polysaccharides and polyphenols derived from various Chinese herbs. It is plausible that the cumulative effects of these compounds contributed to the observed improvement in egg quality in this study. In addition, a significant increase was also observed in the content of margaric acid (SFA), cis-11-Eicosenoicacid (MUFA), dihomo γ-linolenic acid (n-6 PUFA), and docosatetraenoic acid (n-6 PUFA) in egg yolk. Previous studies have shown that the lipids in eggs, such as egg yolk oil, phospholipids, and fatty acids, are high-quality nutrients with anti-inflammatory, antioxidant, cardiovascular protection, memory improvement, and physiological homeostasis functions (44). Particularly, polyunsaturated fatty acids (PUFAs) are regarded as one of the most crucial components that influence the normal development and physiological functions of the body (45). Numerous studies have shown that C20:3n-6, a long-chain polyunsaturated fat, is considered one of the most important components of breast milk (46). The high level of C20:3n-6 makes eggs from hens treated with CHMs a better choice for pregnant women and infants. In general, the intervention of CHMs improved the external and internal quality of eggs, which is a benefit for subsequent production or marketing.

Numerous clinical studies have found that CHMs have pleiotropic mechanisms of action in reproductive tract diseases, regulating the expression of estrogen receptors, reactive oxygen species, inflammatory factors, and apoptosis-related proteins (47). Inflammatory damage and cell apoptosis caused by reproductive tract infections are common causes of reproductive dysfunction in laying hens (48, 49). The reproductive function of hens is closely related to the levels of inflammatory factors and apoptosis-related genes in the body, which is particularly important for layer production. Li et al.'s study found that Shaoyao decoction improves tissue fibrosis and treats radiation enteritis by reducing inflammation and apoptotic responses (50). The findings of this study suggest that CHMs tend to decrease the activity of caspase-8 enzyme in hens' serum. The relative expression of genes for apoptosis-related proteins in liver, jejunum, and ovarian tissues was also determined in our study. CHMs regulated the expression of mRNAs including Bax, Bcl-2, Caspase-8 in the above three tissues and activated the anti-apoptotic responses in hens. In a study conducted by Yan et al., lotus leaf extract was observed to upregulate the mRNA expression levels of the Caspase-8, Caspase-9, and Caspase-10 in laying hens. Simultaneously, it downregulated the mRNA expression levels of Caspase-3 and Caspase-7. This regulation of caspases had an impact on cell apoptosis and immune function, ultimately resulting in an improvement in salpingitis among laying hens (51). CHMs diet could regulate the ability of ovarian cell apoptosis in breeder hens.

In recent years, the immunomodulatory, anti-inflammatory, and anti-apoptotic effects of Chinese medicines have been widely studied. In our study, CHMs treatment increased the concentrations of serum immunoglobulins IgA and IgG in hens, which are considered to potent inducers for promoting neutrophils against pathogens (52). In addition, CHMs decreased the concentration of the pro-inflammatory factor IL-6 in hens' serum. The relative expression of genes for inflammation-related factors in liver, jejunum, and ovarian tissues was also determined in our study. CHMs regulated the expression of mRNAs including NF-κB, IL-4, IL-1β, and MyD88 in the above three tissues and activated the anti-inflammatory responses in hens. IgA, IgM, and IgG are the major serum immunoglobulins of the body as well as their concentrations in plasma have a significant relationship with immunity in laying hens (53). The interleukin (IL) family plays a crucial role in regulating inflammation, with IL-1β and IL-6 assuming vital roles in maintaining intestinal structural integrity, modulating the intestinal immune systems, and being involved with various apoptotic and inflammatory responses (54). Chinese herbal medicines (CHMs) contain numerous bioactive substances, including polysaccharides, volatile oils, flavonoids, alkaloids, and glycosides, which can enhance the body's immunity (28). Therefore, we hypothesize that CHMs possess potent anti-inflammatory properties and the ability to regulate apoptosis protein-related genes, ultimately bolstering the immunity and health status of breeder hens.

It is widely known that the cecum of chickens contains a rich gut microbiota. Several studies have shown that herbal supplements or extracts have a beneficial effect on the composition of the intestinal microbiota in chickens. Evidently, the modulation of gut microbial structure by CHMs is intricately linked to the enhancement of growth performance, reproductive performance, and immune function in the organism (55, 56). The study by Song et al. demonstrated that dietary supplementation with astragalus polysaccharides significantly decreased the population of *Clostridium perfringens* in the cecum of broilers with necrotizing enteritis (57). In our study, CHMs altered the structure of the cecum microbiota in hens. Despite a decrease in the alpha diversity of the microbiota, there was an increase in beneficial bacteria (like *Ruminococcus*) and a decrease in pathogenic bacteria

(like *Desulfovibrio*) in the cecum of hens. In the cecum of hens treated with CHMs, there was a significant decrease in *Desulfovibrio* (58), which is considered an intestinal noxious bacterium, while *Ruminococcus* (59), known for its capacity to degrade complex polysaccharides and transform them into a range of nutrients for their hosts, exhibited an inclination to increase. The fermented Chinese herbal compound containing Bupleurum chinense can reduce the abundance of *Desulfovibrio* in broiler cecum (27). Peng et al.'s (60) findings confirmed that supplementation of the diet with *Eucommia ulmoides* extract might simulate an immune response by altering gut microbial populations. On the one hand, this could be owing to the key bioactive ingredients of CHMs, such as polysaccharides, which have anti-inflammatory and immunomodulatory actions that enhance gut bacterial abundance. Another hand, and it could be the outcome of interactions between gut microbiota after CHMs intake.

Prior to egg laying, all the necessary components to support the growth of the offspring must be completely passed from the mother to the egg. We infer that CHMs produce favorable adaptive maternal effects to ensure the growth and intestinal health of the offspring by enhancing maternal immunity and anti-apoptotic effects as well as maintaining intestinal health, but their maternal effects in the offspring need to be investigated in depth. The immunity and health of the hen during laying influence the safety and development of the offspring embryo (61). Ding et al. (62) revealed that maternal microbial colonization occurs throughout embryonic development and claimed that the microbes present in the egg are transported from the oviduct during egg formation (despite the fact that they related the maternal fecal microbiota to the embryonic microbiota). Furthermore, consistent with the findings of the present study, previous research has demonstrated that meconium samples were predominantly composed of *Proteobacteria* and *Firmicutes*, with a lesser abundance of the phyla *Actinobacteriota* and *Bacteroidota* (63). *Staphylococcus* and *Pseudomonas* are both common foodborne pathogens and the most common causative agents of human septicemia (64, 65). The CHMs treatment led to a significant reduction in the relative abundance of *Staphylococcus* and *Pseudomonas* in the meconium of offspring. Gut microbes exert a profound impact on various physiological functions of the host, encompassing aspects from energy metabolism to immune responses, especially those related to the gut system of immunity (66). The gut microbes of newly hatched chicks were sourced from the maternal magnum, which was transferred into egg whites for colonization of the embryonic gut (61). Before long, environmental, genetic, and other factors shape the unique gut microbiota of offspring chicks. Current studies on *Sphingomonas* and *Ralstonia* have focused on botany and environmental soils since it can help plants to combat stressful environments (67, 68), but its role in the poultry gut microbiota remains to be investigated.

We performed Spearman correlation analysis of the significantly changed gut microbiota with phenotypic and physiological indicators. We observed a significant correlation between the hen's egg production rate and the hen's cecum microbiota, as well as body weight and meconium bacteria of offspring chicks. The reduction of harmful bacteria by the intervention of CHMs was negatively correlated with laying rate and chick body weight, which is consistent with the widely prevailing view (42). Therefore, we concluded that the addition of CHMs to diets maintains hen health by modulating the structure of the gut microbiota and reducing the relative abundance of opportunistic pathogens. Meanwhile, the resulting adaptive maternal effect maintains the initial weight and intestinal health of the offspring.

5 Conclusion

To summarize, this study showed that the addition of CHMs to laying hen diets can improve laying rate, hatchability, and eggs' external and nutritional quality. We suggest that these positive effects are related to the expression of mRNAs for immunoglobulins, apoptosis-related proteins, and inflammatory factors in hens, along with changes in the gut microbiota. Notably, CHMs significantly reduced the relative abundance of opportunistic pathogens in the hens' cecum and offspring's meconium, which may account for the improved performance and egg quality, immunity, anti-apoptosis level, and anti-inflammatory level as well as further drive the maternal effects of Wenchang breeders by CHMs. In conclusion, these findings suggest that CHMs are a valuable feed additive for breeder hens.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number can be found below: https://www.ncbi.nlm.nih.gov/sra/PRJNA1033218.

Ethics statement

The animal studies were approved by Animal Care and Use Committee of the South China Agricultural University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ML: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JH: Conceptualization, Visualization, Writing – original draft. MM: Investigation, Methodology, Writing – review & editing. GH: Investigation, Methodology, Software, Writing – review & editing. YZ: Data curation, Investigation, Writing – review & editing. YD: Investigation, Methodology, Software, Writing – review & editing. QQ: Project administration, Supervision, Writing – review & editing. WL: Conceptualization, Project administration, Supervision, Writing – review & editing. SG: Conceptualization, Funding acquisition, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by grants from the National Key Research and Development Program (2022YFD1801103), Hainan Province's Key Research and

Development Project (ZDYF2023XDNY067) and "Quancheng Scholars" Construction Project (2021).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 19 December 2023
DOI 10.3389/fvets.2023.1305129

OPEN ACCESS

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RECEIVED 30 September 2023
ACCEPTED 07 November 2023
PUBLISHED 19 December 2023

CITATION
Kılınç G (2023) Can tree leaves be used as an alternative source of synthetic antioxidants? Use of jujube leaf extract in laying hens. *Front. Vet. Sci.* 10:1305129. doi: 10.3389/fvets.2023.1305129

Can tree leaves be used as an alternative source of synthetic antioxidants? Use of jujube leaf extract in laying hens

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This study was carried out to determine the antioxidant activity of jujube (*Zizyphus jujuba* Mill.) leaf extract (JLE) and to evaluate the effects of its use as an extract in laying hen diet (Nick Brown; 32 weeks old) on performance, egg quality and lipid peroxidation. A total of 4 groups (24 replicates/group), one control (JLE-0) and three experimental groups (JLE-1, JLE-2, JLE-3), were formed and the hens were individually distributed in cages. The groups were fed with 0, 45, 90 and 135 mg/kg extract, respectively. The total phenolic content of the extract was 118.60 g gallic acid aquivalent/kg (GAE/kg) and the IC50 value was determined as 332.01 as a result of the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity assay. Performance parameters except for feed conversion ratio (FCR) were not affected by the extract in the diet. Compared to the control group, FCR decreased ($p < 0.05$) and feed conversion improved in all experimental groups. The linear effect was significant for egg production (EP) ($p < 0.05$). Furthermore, egg quality parameters except for the albumen index (AI), Haugh unit (HU), shell weight (SW), and shell thickness (ST) were not affected by the extract in the diet. The highest AI and HU were in the JLE-2 group ($p < 0.05$). Besides, SW was found to increase in all experimental groups ($p < 0.001$). The highest ST was in JLE-1 ($p < 0.001$). The addition of the extract was found to slow down lipid oxidation by decreasing Thiobarbituric Acid Reactive substances (TBARs) levels on days 0 and 28 ($p < 0.05$). In conclusion, JLE can be used as a natural extract in laying hen diets.

KEYWORDS

antioxidants, egg quality, jujube leaf, laying hens, lipid oxidation, performance

1 Introduction

Some synthetic antioxidants (butylated hydroxyanisole, butylated hydroxytoluene, ethoxyquin) have been used in poultry nutrition to improve product quality (1, 2) but these products have been reported to have potential toxicological effects (3, 4). This has increased the interest in natural antioxidants as an alternative to synthetic antioxidants (5). Among these, plant-derived antioxidants have been reported to have an important place (6–8). Plants are known to contain phenolic compounds (9). It has been reported that phenolic compounds can reveal their antioxidant properties by scavenging some radical species (ROS/RNS), suppressing their (ROS/RNS) formation and protecting the antioxidant defence mechanism (10). In a study, it was reported that jujube leaves contain a good amount of phenolic substances, especially rutin and apigeni-7-glucoside (11). Jujube is a medicinal plant in the Rhamnaceae family (12). Studies (13, 14) have shown that the leaves of this plant are important as antioxidants. There are a limited number of studies on the use of *Zizyphus jujuba* leaves in animal nutrition. In various studies

(15–17), the potential uses of different parts (fruit, seed meal, and powder) of the *Zizyphus jujube* in poultry (quail and broiler) were investigated. In some other studies (18–23) the use of different species of the Zizyphus genus (*Zizyphus mauritiana, Zizyphus sipina, and Zizyphus vulgaris*) in poultry (laying hens, broiler chickens, and quail) diets has been evaluated. In one of these studies (24), it was reported that the leaf extract of the *Zizyphus mauritiana* species had an immunostimulatory effect on broilers. In another study (21), it was reported that the *Zizyphus mauritiana* leaf extract (0.24 mL) added to the drinking water of quails increased the blood hemoglobin level, and it was emphasized that the extract at the level of 0.24 mL could be a good alternative source of antioxidants in quails. In another study (23), it was reported that *Zizyphus spina* leaf extract (7.5 mL kg^{-1} dose) could be used as a safe natural antioxidant to improve the reproductive status of heat-stressed chickens.

There are also different studies on the use of Zizyphus species in the nutrition of rabbits (25), fish (26), lambs (27), and goats (28).

In the literature review, there is no study investigating the effects of *Zizyphus jujuba* leaf extract on egg lipid oxidation and egg quality in laying hens. Therefore, the aim of the study was to determine the effects of three different levels of *Zizyphus jujuba* leaf extract (45, 90 and 135 mg/kg) on performance, egg quality and egg lipid peroxidation in laying hens.

2 Materials and methods

2.1 Ethical statements

This study was approved by The Ondokuz Mayıs University Animal Ethics Committee of (2018/40).

2.2 Collection of jujube leaves

Jujube (*Zizyphus jujuba* Mill.) leaves used in this study were collected from Harmanağalı Village, Suluova, Amasya Province, Türkiye in the spring (Figure 1).

2.3 Determination of volatile oil profile

Volatile oil was obtained by distillation method from jujube leaf powder. Volatile oil profile was performed by gas chromatography coupled to mass spectrometry (GC-MC) (Agilent: 6890 MS: 5973, New Jersey, United States). Component mass spectra were identified by comparing the retention indices of the components defined in the Flavor2, W8N05ST, and HPCH1607 libraries. Volatile components with an evaluation of less than 50% confidence were not taken into account in the library evaluation (29).

2.4 Preparation of jujube leaf extract

The collected leaves were washed with tap water, rinsed with distilled water and left to dry in the shade. The dried leaves were ground in a laboratory blender (Waring Laboratory Blender, United States) and sieved. Extraction was performed with ethanol solution (80%) in an ultrasonic bath (Caliskan, ultrasonic cleaner, Türkiye) at a temperature (25°C) and time (30 min) determined by preliminary experiments. The resulting mixture was filtered twice through coarse filter paper. Then, the solvent was removed in a rotary evaporator at 50°C and the extracts were stored at +4°C until use (Figure 2).

2.5 Determination of DPPH free radical scavenging activity of the extract

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity was determined using the modified method of Singh et al. (30). Accordingly, 0.01 mM DPPH solution prepared with methanol was added to solutions prepared from the extract and mixed with a vortex (IKA vortex 4 basic) for 15 s. The samples were kept in the dark for 60 min. At the end of this period, the absorbance of the samples was measured at 515 nm wavelength on a spectrophotometer (Genesys 10S UV–VIS, Therma Scientific, United States). The % reduction values were determined using the following formula (31). The results were given as IC50 values.

$$DPPH\ free\ radical\ scavenning\ activity\ (\%) = \left[\frac{1 - AS}{AC}\right] x\,100$$

AS: Sample absorbance, AC: Control absorbance.

2.6 Determination of total phenolic content of the extract

The total phenolic content of the extract was determined according to the method of Singleton and Rossi (32). To 40 µL of solutions prepared from the extract, 2.4 mL distilled water, 200 µL Folin-Ciocalteau, 600 µL Na_2CO_3, and 760 µL distilled water were added, respectively. The tubes were kept in the dark for 2 h. At the end of this period, the absorbance of the samples was measured in a spectrophotometer (Genesys 10S UV–VIS, Therma Scientific, United States) at a wavelength of 765 nm. The results were expressed as g GAE/kg (GAE: Gallic Acid Equivalent).

2.7 Experimental design and diet

In the study, 4 groups were formed, one as control (JLE-0) and the other three as experimental groups (JLE-1, JLE-2, JLE-3), and a total of 96 chickens (Nick Brown, 32 weeks old), 24 chickens in each group, were individually distributed in 4-storey cages. G*Power 3.1. statistical program and "F tests-ANOVA Fixed effects, one-way" module were used to determine the sample size (number of chickens). The study by Radwan-Nadia et al. (33) was used as a reference for the calculation of the effect size.

The homogeneity of the chickens in terms of body weight (BW) was tested before they were placed in the cages ($p > 0.05$). An adaptation period of 1 week was applied before the start of the experiment. During the 10-week experiment, feed and water were given as *ad-libitum* and a photoperiod of 16 h of light and 8 h of dark was applied daily. Moreover, the temperature was controlled with a mini temperature data logger (datalogger; testo 174 T, Germany).

The basal diet used in the experiment was obtained from a private feed mill (laying cage hens 1st period). The nutrient composition of the

FIGURE 1
Jujube (*Zizyphus jujuba* Mill.) trees (Harmanağalı Village, Amasya, Türkiye).

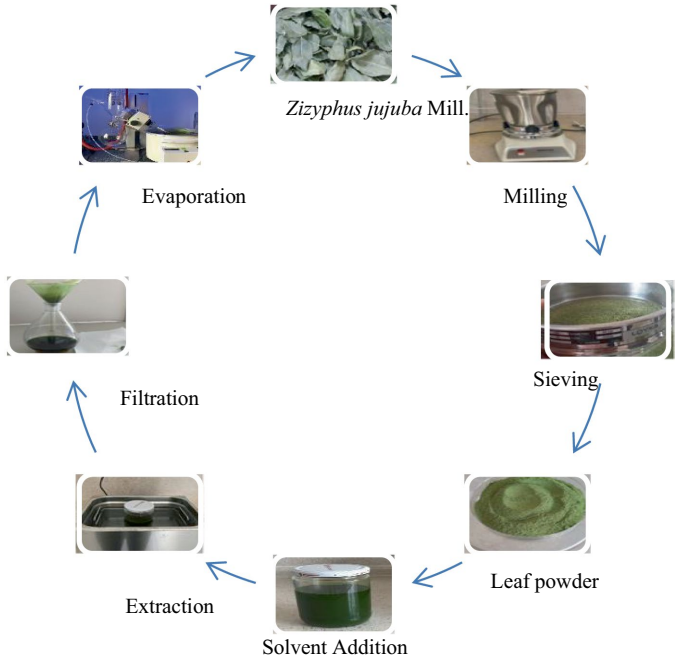

FIGURE 2
The preparation of jujube leaf extract.

diet was determined according to the method reported in AOAC (34). The content and chemical composition of the diet are given in Table 1.

The extracts obtained were first added to the diet little by little to form premixes and then added to the whole diet at 45, 90 and 135 mg/kg levels.

2.8 Parameters determined in the *in-vivo* study

Performance parameters (initial body weight, final body weight, body weight gain, egg weight, egg production, feed intake, and feed conversion ratio) were determined every 2 weeks. For this purpose, data (number of eggs, daily feed intake) were recorded daily. Eggs and feed, which were left in front of the animals, were weighed every 2 weeks.

$$Body\ weight\ gain = Final\ body\ weight - Initial\ body\ weight$$

$$Egg\ production = \frac{Number\ of\ eggs\ per\ 2\ weeks}{Number\ of\ annimals}$$

$$Feed\ intake = \frac{Amount\ of\ feed\ given\ 2\ weeks - Amount\ of\ feed\ remaining\ per\ 2\ weeks}{Day}$$

TABLE 1 Chemical composition of basal diet used in the experiment.

Ingredients	kg/ton	Chemical composition	%
Maize	325.00	Dry matter	89.19
Wheat	170.576	Ether extract	4.68
Triticale	100.00	Crude fibre	5.22
Full-fat soybean (34%)	115.474	Crude protein	17.50
Sunflower meal (34%)	92.11	Crude ash	12.60
Soybean meal (46%)	20.00	D-Lysine	0.650
Hazelnut meal (42%)	28.45	D-Methionine	0.271
Corn gluten (60%)	34.04	Calcium	3.650
Vegetable oil	9.00	Phosphorus	0.375
Limestone	88.03	Sodium	0.150
Dicalcium phosphate	8.47		
Salt	3.16		
Lysine sulphate	2.10		
Premix**	2.40		
Toxin binder	1.20		
Nutrient content (analyzed) (%)			
Crude protein	Ether extract	Crude ash	Crude fibre
17.13	4.44	13.72	5.77
Calculated ME (kcal/kg)*			
2,770			

* Metabolic Energy (ME) value was calculated according to the method developed by Carpenter and Clegg (35). **Supplied the following per 2.5 kg of diet: 10.000.000 IU Vitamin A, 3.000.000 IU Vitamin D3,25.000 mg Vitamin E, 3.000 mg Vitamin K3, 3.000 mg Vitamin B1, 6.000 mg Vitamin B2, 40.000 mg Vitamin B3, 10.000 mg Vitamin B5, 4.000 mg Vitamin B6, 20 mg Vitamin B12, 50 mg Biyotin, 300.000 mg Choline, 1.000 mg Vitamin B9, 60.000 mg Fe, 5.000 mg Cu, 80.000 mg Mn, 60.000 mg Zn, 1.500 mg I. 150 mg Se, 2.500 mg Canthaxanthin (red), 1,000 mg Beta-Apo-8 (yellow), 650.000 mg DL-Methionine, 150.000 mg Optiphos 5.000 CT, 500.000 mg Kemzym, 237.908 Sepiolite.

$$Feed\ conversion\ ratio = \frac{Feed\ intake}{Egg\ mass}$$

Egg quality characteristics (egg weight, shape index, yolk index, albumen index, Haugh unit, shell weight, shell thickness and yolk L*, a*, b* values) were determined once every 2 weeks. For this purpose, a total of 80 eggs (20 eggs from each group) were analyzed. Egg weight and shell weight were weighed with a precision balance with a sensitivity of 0.01. Egg width and length, yolk diameter, albumen width and length were measured with an insize digital caliper. Yolk and albumen heights were measured using a tripod micrometer (Mitutoyo, 0.01 mm, Japan); shell thickness was measured using a micrometer (Mitutoyo, 0.001 mm, Japan) and yolk L*, a*, b* values were measured using a colorimeter (PCE-CSM 4).

The relevant parameters were determined using the following equations (36, 37).

$$Shape\ index = \frac{Egg\ width}{Egg\ length} x\,100$$

$$Albumen\ index = \frac{Albumen\ height}{\frac{Average\ of\ albumen\ length\ and\ albumen\ width}{}} x\,100$$

$$Yolk\ index = \frac{Yolk\ height}{Yolk\ diameter} x\,100$$

$$Haugh\ unit = 100 \log\left[H + 7.57 - 1.7W^{0.37}\right]$$

H = Albumen height, W = Egg weight.

Egg yolk TBARs values were determined at the end of the experiment (day 0) and in eggs collected at the end of the experiment and stored at +4°C for 28 days (day 28). A total of 48 eggs were analyzed for each storage period, 12 eggs from each group. Egg yolk TBARs were determined spectrophotometrically (Genesys 10S UV–VIS, Therma Scientific, United States) using the method reported by Kılıç and Richards (38). Accordingly, 12 mL of TCA (trichloroacetic acid) solution (7.5% TCA, 0.1% EDTA, 0.1% propyl gallate) was added to egg yolk samples (2 g) and homogenized at ultra-turrax for 20–25 s and filtered through Whatmann 1 filter paper. Three ml of the filtrate was taken into glass tubes and 3 mL of 0.02 M TBA (thiobarbituric acid) solution was added. These tubes containing the solution were kept in a water bath at 100°C for 40 min and then cooled under tap water. The tubes were centrifuged at 2000 rpm for 5 min and the absorbance values were read at 530 nm wavelength in a spectrophotometer. TBARs values were calculated using the formula below and the results were given as μmol MDA/kg egg.

$$TBARs = \left[\left(\frac{absorbance\ /}{k\left(0.06\right) x\ 2\ /\ 1000}\right) x\ 6.8\right] x\,1000\ /\ sample\ weight$$

k = Value obtained from the standard curve.

2.9 Statistical analysis

Analysis of variance (one-way ANOVA), comparison of groups (Duncan test) and effects of increasing levels of JLE (polynomial analysis) of the data obtained from the study were performed using the SPSS 20.0 package program. The effects of groups were evaluated at $p < 0.05$ level (39).

3 Results

3.1 Characteristics of dried jujube (*Zizyphus jujuba* mill.) leaf

The results of the GC/MS analysis of essential oil isolated from the jujube leaves indicated that 24 there are compounds (Table 2). Major compounds are phytol, 2-pentadecanone, myristic acid, and alpha-damascone.

3.2 Antioxidant activity of the extract

DPPH free radical scavenging activity and total phenolic content of jujube leaf extract are given in Table 3.

The total phenolic content of the extract was 118.60 g GAE/kg and the IC50 value was 332.01 as a result of the DPPH free radical scavenging activity assay.

3.3 Effect of extract on performance parameters

The effect of JLE on performance parameters is given in Table 4.

In the laying hen diet, the effect of JLE on initial BW, FBW, BWG, egg weight and feed intake among performance parameters was insignificant ($p > 0.05$), while its effect on FCR was significant ($p < 0.05$). Compared to the control group, FCR decreased in all experimental groups, therefore, FCR improved ($p < 0.05$). As a result of polynomial analysis, linear and quadratic effects were found to be significant in terms of FCR ($p < 0.05$). Additionally, it was determined that as the JLE dose increased, a statistically insignificant numerical increase occurred in egg production.

3.4 Effect of the extract on egg quality characteristics

The effect of JLE on egg quality characteristics is given (Table 5).

While the effect of JLE on egg weight, shape index, yolk index and yolk L*, a*, b* values was insignificant ($p > 0.05$), it was significant for albumen index ($p < 0.05$), Haugh unit ($p < 0.05$), shell weight ($p < 0.001$) and shell thickness ($p < 0.001$).

The highest albumen index (9.63%) and Haugh unit (86.39%) values were observed in the JLE-2 group. Compared to the control group, shell weight was higher in all experimental groups ($p < 0.001$). Furthermore, it was determined that 45 and 135 mg/kg levels of JLE in the diet increased shell thickness ($p < 0.001$). In the polynomial analysis, the linear effect was significant in terms of shell weight ($p < 0.05$).

TABLE 2 Volatile oil profile of jujube (*Zizyphus jujuba* Mill.) leaves.

Volatile oils g/100 g volatile oil		Volatile oils g/100 g volatile oil	
Isoelemicine	1.3	Benzoic acid	1.0
Myristic acid	5.3	Palmitic acid	1.8
1-Naphthalenol	0.9	14-Beta-H-Pregna	0.3
6-Octen-1-ol	0,9	Pyridine-3-carboxamide	0.5
2-Pentadecanone	11.2	n-Hexadecanol	0,5
1-3-Dimethyl thiepin	0.6	Manoyl Oxide	0.6
Isophytol	0.7	6,9-Pentadecadien-1-ol	0.5
2-Ethylcycloheptanone	0.5	9,12,15-Octadecatrienoic acid	1.0
Spiro (Methylenecyclopropane)	0.5	Phytol	63.8
1-Hexadecene	0.7	1-Octadecene	0.9
Alpha-Damascone	3.9	Eicosane	1.0
Methyl Hexadecanoate	0.7	n-tetracosane	0.5

3.5 Effect of the extract on egg yolk TBARs

The effect of JLE on egg yolk TBARs value is given in Table 6.

There was a significant difference between the groups in terms of egg yolk TBARs values at both day 0 and day 28 ($p < 0.05$). It was determined that 45, 90, and 135 mg/kg JLE in laying hen diets decreased the egg yolk TBARs on days 0 and 28, thus, improved egg shelf life. Moreover, in the polynomial analysis, linear and quadratic effects were found significant ($p < 0.05$).

4 Discussion

It was determined that JLE added to laying hen diets at different levels significantly affected only FCR among the performance parameters ($p < 0.05$). The decrease in FCR, which is one of the important parameters of performance in laying hens, in JLE-1, JLE-2 and JLE-3 groups shows the positive effect of JLE addition. In the polynomial analysis, the linear effect was significant in terms of FCR ($p < 0.05$). It is thought that this improvement in FCR is due to the bioactive components in the jujube leaf extract. When the essential oil profile of jujube leaf was evaluated in the current study, it was determined that a large proportion of it was phytol (63.8 g/100 g). It is known that phytol is used as an antibacterial, antiviral, and anti-inflammatory agent (40). It is thought that this effects on the gut microflora due to phytol may have improved FCR.

Although the difference between the groups in terms of EP was not significant, the linear effect was significant according to the polynomial analysis. It was found remarkable that egg production increased numerically with the increasing level of JLE in the diet ($p > 0.05$).

There are a limited number of studies on the use of *Zizyphus jujuba* in poultry nutrition. Son (15) reported that *Zizyphus jujuba* seed meal (0.3%, 0.6%, 0.9%) did not affect BWG and FCR.

Basiryan et al. (16) reported that the addition of *Zizyphus jujube* powder (0.75%) to broiler diets did not affect growth performance and internal organ relative weights except liver.

In another study in broilers, Yusup et al. (22) investigated the effects of different levels (10%, 15%, 20%) of bidara (*Zizyphus spina-christi* L.) leaf extract in drinking water on production and mortality and reported that bidara leaf extract in drinking water increased body weight gain, final body weight, feed intake, and water consumption. In the same study, it was determined that this extract in drinking water improved feed conversion.

Abdulameer et al. (19) reported that hydroalcoholic *Zizyphus mauritiana* leaf extract added to broiler drinking water at different levels (3, 7, 10 mL/L) did not affect body weight gain. In the same study, it was observed that feed intake was higher in the group with a 10 mL/L level of extract added to drinking water, and FCR was lower in the group with a 3 mL/L level of extract added compared to the control group, so feed conversion was better in this group.

Egg weight, shape index, yolk index, and yolk L*, a*, b* values were not affected by JLE in diet ($p > 0.05$), while albumen index, Haugh unit, shell weight and shell thickness were affected. The highest albumen index and Haugh unit were in the JLE-2 group, while the highest shell thickness was in the JLE-1 group. Compared to the control group, shell weight was higher in all experimental groups ($p < 0.001$).

Özgan (41) in a study investigating the possibilities of using grape seed oil in laying hens, reported that they thought that β-ovomucin in the structure of albumen was protected thanks to the antioxidant properties of this oil and accordingly, albumen height could increase.

TABLE 3 Antioxidant activity of jujube leaf extract.

Total phenolic content (g GAE /kg)	IC50
118.60	332.01

GAE, gallic acid equivalent.

Many studies (13, 14, 42, 43) have reported that jujube leaf extract has antioxidant properties. Based on this information, it can be said that JLE increased the albumen index and Haugh unit in the present study due to its antioxidant properties.

Dietary strategies are valuable options to improve nutritional value and oxidative stability of poultry products (44). The addition of JLE at different levels to the laying hen diet decreased yolk TBARs levels on day 0 and day 28 ($p < 0.05$), indicating that JLE delayed lipid oxidation. In addition, both linear and quadratic effects were significant at day 0 and day 28. This positive result is thought to be due to the antioxidant properties of jujube leaves. Volatile oil of jujube

TABLE 4 Effects of jujube leaf extract on performance parameters.

Parameters	Groups				SEM	*p* values		
	JLE-0	JLE-1	JLE-2	JLE-3		C	L	Q
IBW	1853.83	1830.83	1878.42	1855.48	18.83	0.854	0.757	0.998
FBW	1921.00	1962.00	1964.21	1918.38	18.89	0.729	0.974	0.258
BWG	67.17	131.17	85.79	62.79	15.44	0.385	0.673	0.162
EW	64.27	64.75	64.83	63.56	0.191	0.073	0.226	**0.022**
EP	83.60	84.90	85.52	85.73	0.315	0.073	**0.013**	0.387
FI	117.46	117.15	116.43	117.24	0.541	0.918	0.777	0.607
FCR	2.235^{a}	2.149^{b}	2.107^{b}	2.163^{ab}	0.015	**0.020**	**0.049**	**0.016**

Different superscript letters (a, b) within the same row indicate significant differences between groups ($p < 0.05$). C, combined; L, linear; Q, quadratic. JLE-0, JLE-1, JLE-2, JLE-3: groups, which were fed with the addition of 0, 45, 90 and 135 mg/kg jujube leaf extract to the diet, respectively. IBW, initial body weight, g; FBW, final body weight, g; BWG, body weight gain, g; EW, egg weight, g; EP; egg production, %; FI, feed intake, g/day/hen; FCR, feed conversion ratio, g feed/g egg. SEM, standard error of mean.
The bold values are $p < 0.05$.

TABLE 5 Effects of jujube leaf extract on egg quality parameters.

Parameters	Groups				SEM	*p* values		
	JLE-0	JLE-1	JLE-2	JLE-3		C	L	Q
EW (g)	63.36	64.60	64.97	63.82	0.229	0.053	0.392	**0.009**
SI (%)	78.71	79.12	78.56	79.01	0.129	0.380	0.757	0.947
YI (%)	42.91	42.38	42.36	42.33	0.115	0.220	0.087	0.267
AI (%)	9.07^{b}	9.05^{b}	9.63^{a}	9.15^{b}	0.066	**0.004**	0.165	0.081
HU	84.86^{b}	84.51^{b}	86.39^{a}	84.82^{b}	0.258	**0.044**	0.440	0.237
SW (g)	6.68^{b}	7.03^{a}	6.88^{a}	7.01^{a}	0.029	**0.000**	**0.001**	0.048
ST (μm)	366^{b}	378^{a}	365^{b}	373^{a}	0.001	**0.000**	0.527	0.415
L*	59.63	59.48	59.89	58.32	0.265	0.162	0.137	0.180
a*	12.14	12.06	12.12	12.10	0.138	0.997	0.951	0.911
b*	41.88	41.39	42.57	41.10	0.242	0.150	0.588	0.309

Different superscript letters (a, b) within the same row indicate significant differences between groups ($p < 0.05$). JLE-0, JLE-1, JLE-2, JLE-3: groups, which were fed with the addition of 0, 45, 90 and 135 mg/kg jujube leaf extract to the diet, respectively. EW, egg weight, g; SI, shape index, %; YI, yolk index, %; AI, albumen index, %; HU, haugh unit, %; SW, shell weight, g; ST, shell thickness, μm; L*, lightness; a*, redness; b*, yellowness. SEM, standard error of mean. C, combined; L, linear; Q, quadratic.
The bold values are $p < 0.05$.

TABLE 6 Effects of jujube leaf extract on egg yolk TBARs.

Parameters	Groups				SEM	*p* values		
	JLE-0	JLE-1	JLE-2	JLE-3		C	L	Q
TBARs (day 0)	0.179^{a}	0.140^{b}	0.121^{b}	0.143^{b}	0.0062	**0.006**	**0.013**	**0.009**
TBARs (day 28)	0.203^{a}	0.157^{b}	0.149^{b}	0.158^{b}	0.0059	**0,002**	**0,004**	**0,010**

Different superscript letters (a, b) within the same row indicate significant differences between groups ($p < 0.05$). JLE-0, JLE-1, JLE-2, JLE-3: groups, which were fed with the addition of 0, 45, 90 and 135 mg/kg jujube leaf extract to the diet, respectively. TBARs, Thiobarbituric acid reactive substances, μmol MDA/kg egg. SEM, standard error of mean. C, combined; L, linear; Q, quadratic.
The bold values are $p < 0.05$.

leaves contains 63.8 g/100 g phytol (Table 2). Phytol is known to have a strong antioxidant effect in preventing the formation of thiobarbituric acid reactive (45).

According to the current literature review, there is no study investigating the effect of *Zizyphus jujuba* leaf extract on egg yolk lipid oxidation levels in laying hens.

However, Cellat et al. (17) reported that *Zizyphus jujuba* fruit decreased serum and breast muscle MDA levels, hence, showed a positive effect on MDA levels in their study of quails.

In addition, in another study investigating the effects of different levels of *Zizyphus jujuba* extract (500, 1,000 ppm) on the shelf life and meatball quality during storage, Gök and Bor (46) reported that the extract reduced TBARs in meatballs.

5 Conclusion

In this study in which the possibilities of using JLE as a natural antioxidant in laying hen diets were investigated, it was determined that JLE had a positive effect on FCR, which is one of the performance parameters. Higher albumen index and Haugh unit and lower yolk TBARs levels in eggs obtained from hens fed with a JLE-supplemented diet compared to the control group showed that JLE contributed to the preservation of egg freshness due to its antioxidant properties. Moreover, the effect of JLE on shell weight and shell thickness, which are the external quality characteristics of eggs, was also significant. In conclusion, it is thought that JLE can be used as a natural antioxidant in laying hens, but more studies should be conducted on the subject.

Data availability statement

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

Ethics statement

This study was approved by The Ondokuz Mayıs University Animal Ethics Committee of (2018/40). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Funding

The author declares financial support was received for the research, authorship, and/or publication of this article. This research was supported by Amasya University Scientific Research Projects Coordination Unit (project no: FMB-BAP 18-0336).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 03 January 2024
DOI 10.3389/fvets.2023.1325115

OPEN ACCESS

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RECEIVED 20 October 2023
ACCEPTED 04 December 2023
PUBLISHED 03 January 2024

CITATION
Zhang Z, Shi B, Lv X, Dong Y, Li L and Xia Z (2024) Effects of silybin supplementation on growth performance, serum indexes and liver transcriptome of Peking ducks. *Front. Vet. Sci.* 10:1325115. doi: 10.3389/fvets.2023.1325115

Effects of silybin supplementation on growth performance, serum indexes and liver transcriptome of Peking ducks

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As an emerging feed additive extracted from the traditional herb milk thistle, silybin has few applications and studies in Peking ducks. The aim of this study was to explore the practical significance of silymarin application in Peking ducks and to provide more theoretical support for the application of silymarin in livestock and poultry production. A total of 156 1-day-old healthy Peking ducks were randomly divided into four groups and supplemented with 0mg/kg (control group), 400mg/kg (S400), 800mg/kg (S800) and 1,600mg/kg (S1600) of silybin in the diets at day 14, to investigate the effects of silymarin on the growth, serum indexes and liver transcriptome of Peking ducks. The whole experiment lasted until day 42, and the sample collection was scheduled to take place in the morning. A substantial inprovement in average daily gain (ADG) and a decrease in feed conversion ratio (FCR) occurred in the S1600 group on days 14–28 compared to the control group ($p < 0.05$). The FCRs of other additive groups in the same period showed the same results. Supplementation of diets with silybin significantly increased serum IgA levels and when 1,600mg/kg of silybin was given, levels of TNF-α and IL-6 were also significantly decreased ($p < 0.05$). In addition, we observed that the S1600 group had a significantly lower ($p < 0.05$) glutamine transaminase and an increased ($p < 0.05$) T-SOD level in the S400 group ($p < 0.05$). Liver transcriptome sequencing showed that 71 and 258 differentially expressed genes (DEGs) were identified in the S400 and S1600 groups, respectively, compared with the control group. DEGs related to cell composition and function, antigen processing and presentation were up-regulated, while DEGs related to insulin resistance and JAK–STAT were down-regulated. Conclusively, silybin can be used as a feed additive to improve the growth performance and health status of Peking ducks.

KEYWORDS

silybin, growth performance, immunity levels, liver, transcriptome

1 Introduction

With a history of more than 400 years, Peking ducks are not only an important part of China's livestock and poultry breeding industry, but also a world-famous meat duck breed, which occupies a pivotal position in both domestic and international duck breeding industries. With the passage of time, the waterfowl production industry continues to develop and

progress, and the global duck meat production is increasing year by year (1). Asia is the largest meat duck producing region in the world, and China is the main meat duck-producing country (2). That means there is a higher demand on the productivity of broiler ducks, as well as higher demands on nutritional conditions, feeding management and other factors (3). Although the use of growth-promoting antibiotics can significantly improve the growth performance of birds and the corresponding efficiency, considering the subsequent adverse effects caused by the use of antibiotics, many countries and regions have proposed and implemented measures to reduce the use of antibiotics in recent years (4, 5). The importance of finding and researching the use of green and safe alternatives is particularly urgent. Traditional Chinese medicines (TCM) and their extracts are getting more and more attention due to their excellent performance in food, medicine, farming, and amelioration of pathogen infections.

Silybin is a flavonoid extracted from the herb *Silybum marianum* of the Asteraceae family, which is effective in the treatment of Hepatitis, Cirrhosis, Viral Liver Disease, Alcoholic and Non-Alcoholic liver disease, and other liver diseases, which can play a role in antioxidant by scavenging free radicals and inhibiting lipid peroxidation (6–12). Previous studies have found that silybin reduces the formation of free radicals in injured cells, which could also serve as an explanation for the hepatoprotective properties of silybin (13). Furthermore, silybin has a protective effect on the liver of ducks fed diets containing mycotoxin (14). Some scholars have found that silybin activates the body's antioxidant system by increasing sulfhydryl concentration and reducing power (15). Studies on silybin in different animal bodies have gradually increased in recent years, but there are fewer relevant studies on the application of silybin in Peking ducks. Having considered the different reactions that may occur in different animal organisms after the introduction of silybin into the body, we believed that studies related to the effects of silybin on Peking ducks were necessary.

The liver is not only an important immune organ of the body, but also an important hub of many physiological processes (16). It has a high capacity for repair and regeneration and can usually respond quickly to external stimuli (17). The transcriptome is an important tool for studying the dynamics of gene expression, analyzing and identifying disease markers, and facilitating the discovery of new targets (18). RNA sequencing (RNA-Seq) is a common method of genomic analysis with high resolution, high throughput, and high sensitivity, through which the technology can reveal more new transcribed regions or capture the transcriptome more accurately in different environments or over time (19, 20). Currently, no relevant studies have been found on the effect of feeding diets containing silybin on gene expression in the liver of Peking ducks. Therefore, the application of transcriptomics to study the expression of hepatic genes in Peking ducks fed diets containing silybin is beneficial to further understanding the effects of silybin on the growth and development of Peking ducks, and may also provide further information on the application of silybin in animal diets.

In order to clarify the effects of dietary silybin supplementation on Peking ducks, we set up different silybin supplementation levels to carry out relevant experiments, then investigated the growth performance, serum biochemical levels, antioxidant levels, immune factor levels, inflammatory factor levels, and hepatic transcript levels of Peking ducks.

2 Materials and methods

2.1 Animal ethics and reagents

All procedures were carried out in accordance with the provisions of the Animal Welfare and Animal Experimental Ethics Review Committee of China Agricultural University. The animal ethics number is AW61303202-2-3. Animal experiments were conducted in Nankou Pilot Test Base of the Chinese Academy of Agricultural Sciences. Silybin was purchased from Shaanxi Haokang Biotechnology Co., LTD., with a concentration of 98%.

2.2 Animals, diets, and experimental design

One hundred and fifty-six 1-day-old healthy Jing Dian Peking ducks were randomly divided into four treatment groups with three replicates of 13 ducks each. 0.400 mg/kg, 800 mg/kg, and 1,600 mg/kg silybin were added to the diets of the four treatment groups from 14 days of age. The basic diet was formulated in accordance with NY/T 2122–2012 "Feeding Standards for Meat Ducks," and the composition and nutritional levels of the basic diet were shown in Tables 1, 2. The weight of all ducks was recorded at 14 days of age, and again on the 28th and 42nd day of the experiment, then the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated. Before the experiment begins, the duck house should be cleaned and disinfected thoroughly. Clean the manure at 7 a.m. every day, and clean the sink

TABLE 1 Composition of basal diets (air-dry basis, %).

Items	Day 1–14	Day 15–42
Corn	56.00	60.24
Soybean meal	32.69	24.67
Wheat midding	5.00	9.00
Soybean oil	2.10	1.80
Phytases	0.02	0.02
$CaHPO_4$	1.00	1.60
Limestone	1.50	1.20
DL-Met	0.15	0.12
L-Lys	0.20	0.10
Vitamin premix[a]	0.02	0.02
Trace mineral premix[b]	0.20	0.20
NaCl	0.35	0.30
Cholie chloride (50%)	0.24	0.20
Ethoxyquin (33%)	0.03	0.03
Maifanite	0.50	0.50
Total	100.00	100.00

[a]The vitamin premix provided the following per kilogram of diet: vitamin A, 12,500 IU; vitamin D3, 3,500 IU; vitamin E, 20 IU; vitamin K3, 2.65 mg; thiamin, 2.00 mg; riboflavin, 6.00 mg; pyridoxin, 3.00 mg; VB12, 0.025 mg; biotin, 0.0325 mg; folic acid, 12.00 mg; pantothenic acid, 50 mg; nicotinic acid, 50.00 mg. [b]The mineral premix provided the following per kilogram of diet: Cu, 6 mg; Fe, 80 mg; Zn, 40 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.

TABLE 2 Nutrient levels of basal diets (calculated value).

Items	Day 1–14	Day 15–42
ME	12.31	12.53
CP	19.52	16.83
Lys	1.12	0.87
Met	0.46	0.39
Ca	0.88	0.89
AP	0.29	0.39
TP	0.54	0.62
Met+Cys	0.79	0.69

ME, Metabolizable energy; CP, Crude protein; Lys, Lysine; Met, Methionine; Ca, Crude ash; AP, Available phosphorus; TP, Total phosphorus; Cys, Cysteine.

and trough once a week. During the experiment, ducks were able to feed and drink freely, and performed immune procedures normally.

2.3 Sample collection

After fasting for 12 h in advance, three Peking ducks were randomly taken from each replicate group at 7:00 am on day 28 and day 42 of the experiment, respectively, and were labeled and weighed. 10 mL of blood was collected from the jugular vein and centrifuged at 4000 r for 10 min at 4°C, and the serum was stored at −20°C for further testing. The liver tissues of mung bean grain size were taken in 2 mL RNA-Free enzyme lyophilization tubes and stored in liquid nitrogen first, and then transferred and stored at −80°C.

2.4 Analysis of serum component

Serum ALP, ALT, ALB, TP and γ-GT levels were measured by an automatic biochemical analyzer. The levels of serum IgA, IgG and IgM, inflammatory factors IL-1β, IL-4, IL-6, IL-10 and TNF-α were determined using an enzyme marker; the levels of antioxidant indicators T-SOD, T-AOC, CAT and GSH-PX were determined using a spectrophotometer. The assay kits used for all the above indicators were provided by Beijing Kangjia Hongyuan Biotechnology Co. All of the above indicators were measured in accordance with the instructions.

2.5 Total RNA extraction and sequencing

The total RNA in liver tissue was extracted by trizol, and the Illumina Stranded mRNA Library Prep Kit was used to construct a library with 1 μg total RNA of each sample. The mRNA isolated from total RNA by Oligo dT should first be randomly interrupted with buffer under suitable conditions, and a strand of cDNA should be synthesized using the mRNA as a template under the action of reverse transcriptase using random primers. Subsequently, the synthesis of the second chain was carried out. The uneven end of the synthesized cDNA is repaired and an "A" base is added to the 3′ end. The above products were purified and sorted. PCR amplification was performed with the sorted products, and after quantification with Qubit 4.0, sequencing was performed using the Illumina NovaSeq 6000 platform.

2.6 Data processing and analysis

The raw sequencing data was filtered using fastp software to obtain high quality sequencing data. The raw data after quality control was then compared to the reference genome using HiSat2, and the results were also compared for quality assessment. Then the expression levels of genes and transcripts were quantitatively analyzed by RSEM.[1] In order to identify differentially expressed genes among different samples and study their functions, the data were screened and analyzed using the software DESeq2. A gene was considered to be differentially expressed gene (DEG) when it satisfied both FDR < 0.05 & $|\log2FC| \geq 1$. Subsequent analysis of the data was performed using the Gene Ontology (GO)-based Functional Enrichment and Annotation tool and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.7 Statistical analysis

Excel 2021 was used for enter and initially organization of experimental data, then the data were subjected to one-way ANOVA analysis using SPSS 26.0. When the *p*-value below 0.05 were considered to be significantly different. Images were processed by GraphPad Prism 9.0.

3 Results

3.1 Growth performance

The effects of dietary supplementation with silybin on the growth performance of Peking ducks were shown in the result (Table 3 and Figure 1). From day 14 to day 28, ADG and FCR of Peking ducks in the S1600 group were significantly increased ($p < 0.05$); however, there were no significant differences in the ADG, ADFI and FCR among the treatment groups from day 28 to day 42. From day 14 to day 42, there was a decreasing trend in ADFI and FCR in S400 and S1600 groups compared with the control group, but the differences were not significant.

3.2 Serum indicators

The results of the serum index tests were shown in the figures (Figures 2, 3). At day 42, IgA content in the silybin-added group were all significantly elevated compared with the control group ($p < 0.05$); IgG in S1600 group was also elevated, with significant differences ($p < 0.05$) (Figure 2A). The levels of IL-6 and TNF-α in S1600 group were both decreased significantly ($p < 0.05$) and IL-10 was increased. But there was no effect on the levels of IL-1β and IL-4 at day 42, while IL-10 in S400 group was increased at day 42 with a non-significant difference (Figure 2B). Compared with the control group, the content of T-SOD in S400 group was significantly increased ($p < 0.05$), while T-AOC and CAT were also increased, but there was no significant difference in the content of GSH-PX in the additive group compared

1 http://deweylab.biostat.wisc.edu/rsem/

TABLE 3 Growth performance during the growing season of adding silybin.

Items	Groups				*p* value
	C	S400	S800	S1600	
Day 14–28					
Average daily gain, g/d (ADG)	90.698[a]	99.896[ab]	99.023[ab]	102.430[b]	0.185
Average daily feed intake, g/d (ADFI)	219.381	229.469	233.022	219.505	0.616
Feed conversion rate (FCR)	2.422[a]	2.297[a]	2.351[a]	2.142[b]	0.007
Day 28–42					
Average daily gain, g/d (ADG)	111.746	99.444	100.000	102.698	0.483
Average daily feed intake, g/d (ADFI)	204.190	197.106	200.275	188.150	0.458
Feed conversion rate (FCR)	1.831	2.022	2.013	1.832	0.595
Day 14–42					
Average daily gain, g/d (ADG)	99.833	104.353	99.512	103.123	0.194
Average daily feed intake, g/d (ADFI)	223.745	219.288	227.521	213.276	0.335
Feed conversion rate (FCR)	2.243[abc]	2.102[a]	2.286[b]	2.068[ac]	0.062

In the same row, values with different superscript indicate a significant difference ($p < 0.05$).

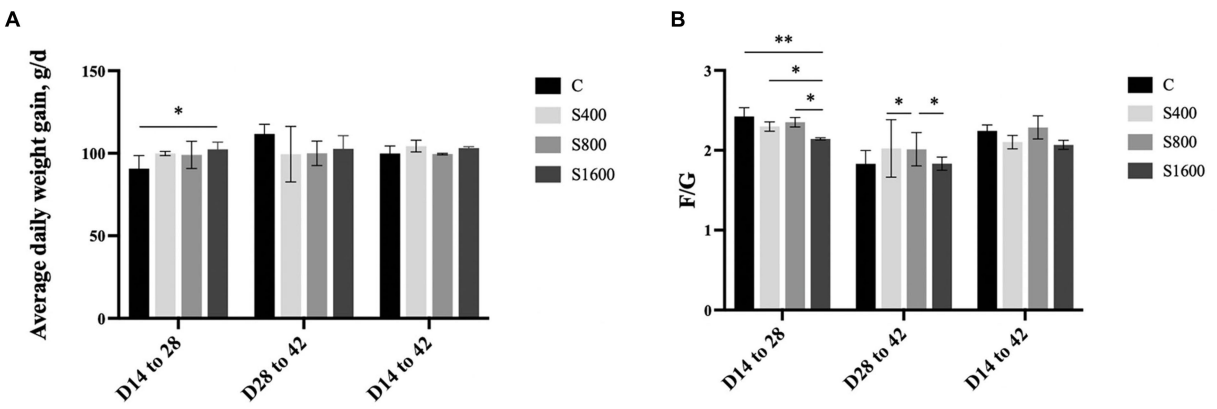

FIGURE 1
Effect of silybin on average daily gain and feed conversion rate. **(A)** Average daily weight gain in different stages. **(B)** Feed conversion rate in different stages. *$p < 0.05$; **$p < 0.01$.

with the control group (Figure 3A). ALT was observed to be significantly different between groups S1600 and control ($p < 0.05$), and there was a tendency for a decrease in γ-GT in S400 group (Figure 3B).

3.3 RNA sequencing data statistics

A total of nine samples were completed for transcriptome analysis, and 66.1 Gb Clean Data was obtained, with Clean Data of each sample reaching more than 6.04 Gb, and the percentage of Q30 bases was above 93.63%. The details of these sequencing data are reflected in Table 4.

3.4 Statistics of differentially expressed genes (DEGs)

The differentially expressed genes between different treatment groups and control group were analyzed, respectively. The results showed that a total of 258 differentially expressed genes were identified between the S1600 group and the control group, of which 166 DEGs were up-regulated and 92 DEGs were down-regulated; 71 DEGs were identified between the S400 group and the control group, of which 26 were up-regulated and 45 were down-regulated (Table 5; Figure 4). Gene clustering analysis of the expression models of DEGs in the two grouping scenarios showed that the gene expression patterns were similar within each grouping scenario, suggesting good reproducibility of the samples between the groups and a high degree of gene set reliability (Figure 5). Details of the expression of differentially expressed genes are shown in Supplementary Tables S1, S2.

3.5 Differentially expressed genes (DEGs) annotation and enrichment analysis

The results of GO analysis showed that 258 DEGs between S1600 group and control group were annotated to the three major categories

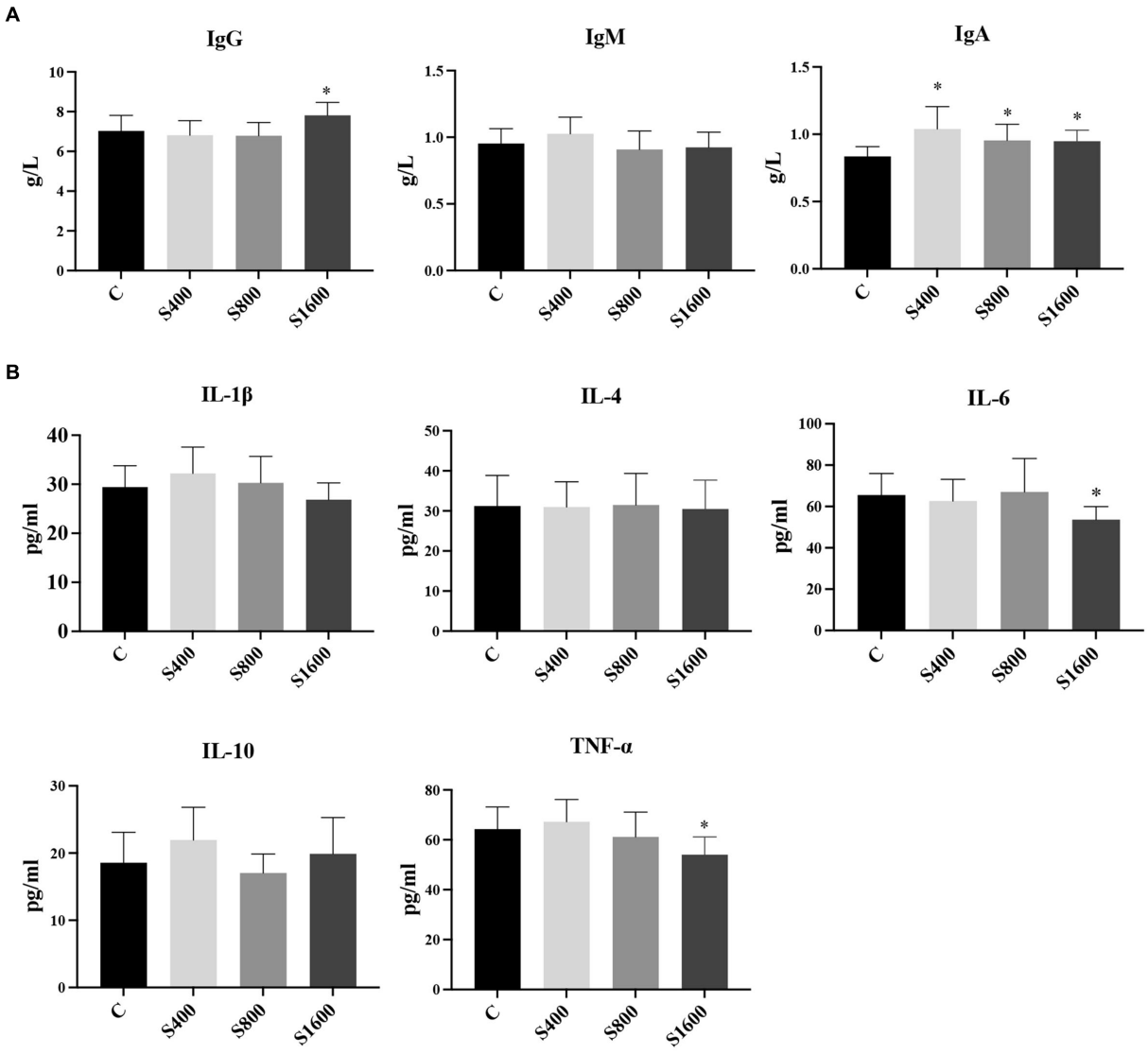

FIGURE 2
Serum immune levels and inflammatory indices affected by silybin. **(A)** Effect of silybin on IgG, IgA and IgM. **(B)** Effect of silybin on the expression of inflammatory factors. *$p < 0.05$; **$p < 0.01$.

of biological processes, cellular components, and molecular functions, with the largest number of DEGs being annotated to the biological process term (Figure 6A). The most abundant GO Terms in biological processes is cellular processes (GO: 0009987), with 118 DEGs annotated under this item; the cellular component (GO: 0044464) and organelle (GO: 0043226) entries in the cellular component were annotated to the most DEGs, with 119 and 72 annotations, respectively. Binding (GO: 0005488) function is the most abundant GO Terms in molecular function. KEGG enrichment analysis of 258 differentially expressed genes showed that up-regulated DEGs were significantly enriched in antigen processing and presentation, phagosome, and complement pathways (Figure 6C); while down-regulated genes were mainly enriched in pyruvate metabolism, insulin resistance, and polyunsaturated fatty acid synthesis in stem cells (Figure 6D). GO analysis showed that cellular fractions were annotated to the maximum of the number of differentially expressed genes in the S400 and control groups. The entries with the highest number of differentially expressed genes annotated to the three broad categories were consistent with the former (Figure 6B). These two groups of up-regulated differentially expressed genes were significantly enriched in amino acid metabolism and synthesis, and drug metabolism pathways (Figure 6E); whereas down-regulated differentially expressed genes were enriched in unsaturated fatty acid synthesis pathways and JAK–STAT signaling pathway (Figure 6F). Specific information on the results of KEGG enrichment analysis of differentially expressed genes is shown in the Supplementary Tables S3, S4.

4 Discussion

Due to the irrational use of antibiotics in livestock and poultry farming, drug residues and the emergence of drug-resistant bacteria have seriously jeopardized human health (21, 22). Currently, one of

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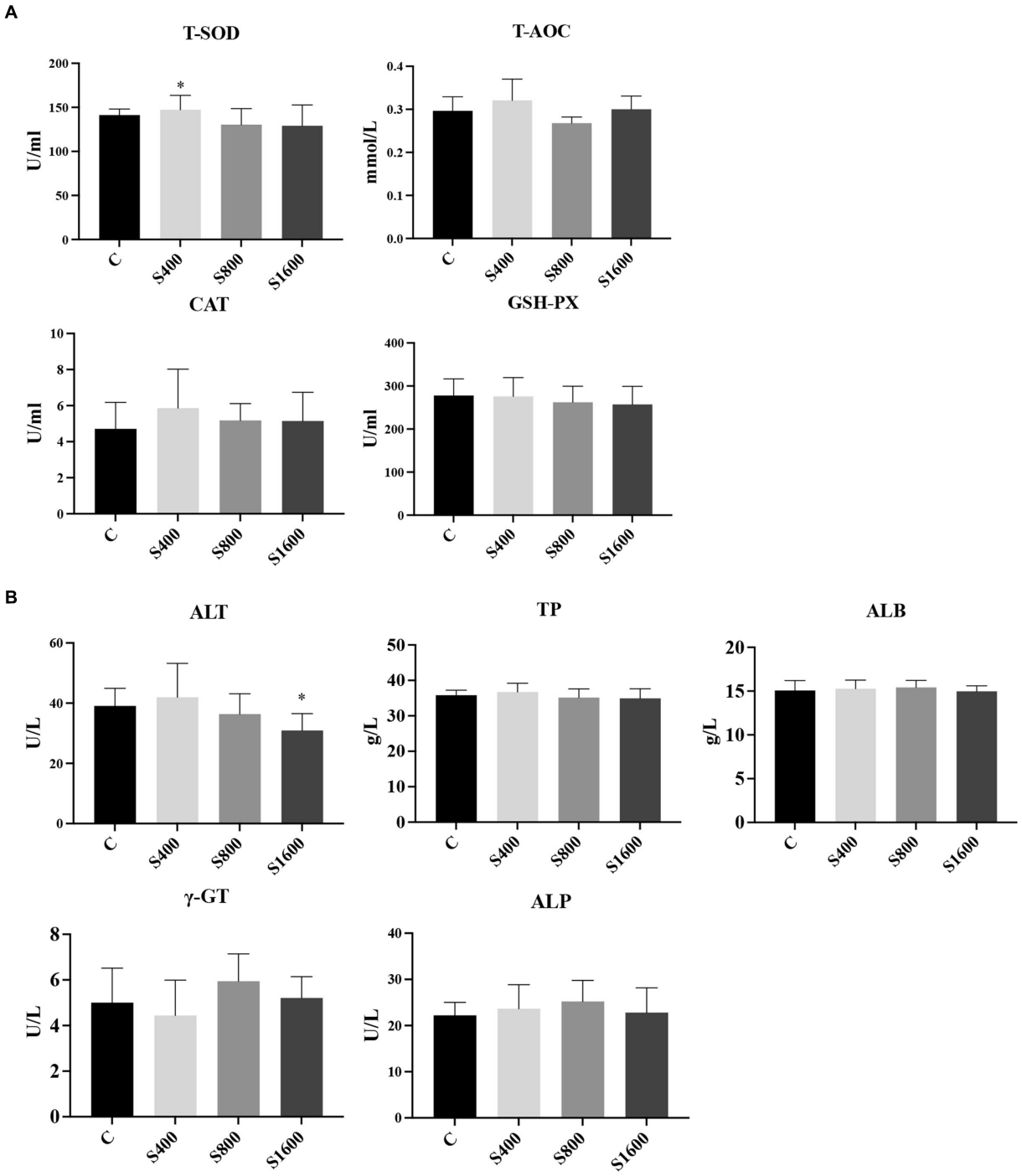

FIGURE 3
Serum biochemical and antioxidant as influenced by silybin. **(A)** Effect of silybin on serum antioxidant levels. **(B)** Effects of silybin on biochemical levels. $^{*}p < 0.05$; $^{**}p < 0.01$.

the main measures to deal with this situation is to reduce and gradually abandon the use of some antibiotics, thus finding suitable and effective alternatives has become a major trend today (23). It has been proved that the application of herbs and related substances such as Chinese herbs, Chinese herbal extracts, Chinese herbal ferments and other related substances in livestock and poultry production can play a role in lowering the FCR, improving the meat quality, enhancing the intestinal barrier function, promoting the metabolism of proteins and other effects, then lowering the cost of feeding (24–26). Silybin is extracted from the traditional plant milk thistle, which has outstanding anti-inflammatory and antioxidant effects. Among the many extracts, silybin has important biological effects and can be used in almost all common liver diseases (27). This study aims to investigate the effects of dietary silybin on the growth performance and liver transcriptome of Peking ducks, and to provide a theoretical basis for the application of silybin in feed additives. The results showed that dietary silybin

TABLE 4 RNA sequencing data statistics.

Sample	Raw reads	Raw bases	Clean reads	Clean bases	Q20 (%)	Q30 (%)
QK5	55,234,502	8,340,409,802	54,751,870	8,115,627,975	98.12	94.55
QK6	63,316,286	9,560,759,186	62,681,984	9,300,686,701	98	94.24
QK5_6	45,726,180	6,904,653,180	45,312,194	6,741,760,126	97.98	94.17
QK10	53,815,644	8,126,162,244	53,379,050	7,951,670,230	98.01	94.23
QK12	45,793,940	6,914,884,940	45,352,738	6,731,750,720	98.05	94.37
QK10_12	51,914,606	7,839,105,506	51,372,750	7,633,608,369	97.91	94.04
QK13	50,235,290	7,585,528,790	49,724,084	7,385,028,335	97.75	93.63
QK14	41,112,750	6,208,025,250	40,708,590	6,039,480,909	97.94	94.12
QK13_14	42,377,138	6,398,947,838	41,949,394	6,196,310,790	98.13	94.59

supplementation can significantly reduce the FCR of Peking ducks aged 14–28 days, and the addition of 1,600 mg/kg also significantly increased the average daily gain of Peking ducks at the same period, similar findings were found in Shanmugam's study (28), where they observed that dietary supplementation of milk thistle extract can increase body weight and improve growth performance of broilers. The research results of Tedesco also confirmed that plant extracts such as magnolol and Astragalus polysaccharide can significantly improve the growth performance, enhance meat quality and reduce the FCR in poultry animals such as chickens and ducks (29–31). Therefore, we believe that silybin has a positive regulatory effect on improving the growth performance of animals. Interestingly, however, in our study, silybin had no significant effect on the growth performance of Peking ducks at day 28–42. Similarly, it was found that the addition of *Cornus officinalis* extract did not significantly affect its FCR and growth performance in the application of broilers (32). There is a strong correlation between the growth performance of birds and the development of internal organs, in which the development of the intestine plays an important role, and the digestion and absorption of nutrients mainly occur in the small intestine. Therefore, we hypothesized that the regulatory effect of silybin on animal growth performance might be related to its regulatory mechanism on the intestine.

As one of the hallmarks of the acquired immune system, immunoglobulins have been rapidly and extensively explored and studied for their immunomodulatory and anti-inflammatory properties, and their levels correlate with the level of immunity in the body (33, 34). Studies have confirmed that Chinese herbal extracts can significantly improve the body's immunity (35). In this study, silybin significantly increased serum IgA levels compared to controls, and high levels of addition can also significantly improve the IgG content. The results showed that the addition of silybin in the ration can improve the immunity level and enhance the disease resistance of Peking ducks. Inflammatory factors play an important role in the regulation of pathogenic infection and immune homeostasis. Interleukin-6 (IL-6) is an important inflammatory factor involved in a variety of inflammatory responses or inflammatory disease processes (36); Tumor necrosis factor-α (TNF-α) has a strong pro-inflammatory capacity and can establish an inflammatory response through activation of the MAPK and NF-κB cascade signaling pathways (37). We found that serum levels of IL-6 and TNF-α were significantly reduced by the addition of 1,600 mg/kg silybin to the ration, which indicates that silybin can improve the anti-inflammatory ability of the organism and achieve the effect of organismal defense. This is similar to previous studies, also confirming the anti-inflammatory properties of silybin (14, 38). Silybin has excellent antioxidant ability, maintaining the REDOX balance of the body by scavenging free radicals, activating the body's antioxidant system and preventing the formation of lipid peroxides (13, 15, 27). We observed that a significant increase in serum levels of total superoxide dismutase (T-SOD) occurred in the group supplemented with 400 mg/kg of silybin in the ration, and the same finding was observed in another study (39). T-SOD is the most important and optimal free radical scavenger in organisms, protecting the organism from oxidative damage by specifically scavenging superoxide anion (40). Furthermore, by reducing the expression of inflammatory response markers, silybin can further regulate the expression activity of downstream genes/pathways, thereby inhibiting the expression of iNOS and regulating the redox state of the body (10, 41). Therefore, we hypothesized that the antioxidant capacity of silybin may be achieved by scavenging free radicals or anti-inflammation, while the incomplete representation of the results may be related to the sample ingestion time, ample source and other factors. The liver is the largest and most important metabolic organ, and the health status of the liver can be roughly understood through the content changes of liver enzymes such as ALT and AST in serum. The increase of liver enzymes in serum often indicates liver disease, bile duct disease and other diseases. Compared with the control group, the serum ALT content in the S1600 group in this study was significantly reduced, indicating that liver function was improved, which was consistent with the results of other studies. Compared with the control group, the serum levels of ALT and AST were significantly reduced in the NAFLD model group mice after silymarin treatment. The liver function of the mice was significantly improved (42). In addition, in a randomized controlled clinical trial in human medicine, it was found that the serum ALT and AST levels of patients with silymarin were reduced by 0.26 IU/mL and 0.53 IU/mL, respectively, both of which were statistically significant (43). Therefore, we believe that the addition of silybin to the ration can have a protective effect on the liver of Peking ducks.

To investigate the effects of silybin on physiological processes and metabolic pathways in Peking ducks, transcriptomic analysis was performed on the liver of 42-day-old Peking ducks. KEGG enrichment showed that the differentially expressed genes in the S1600 group and the control group were significantly up-regulated in the antigen processing and presentation pathways. In our results, a significant increase in the level of immunity occurred in the S1600 group, and the

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TABLE 5 Differentially expressed gene statistics.

DIFF group	Total DEGs	Up	Down
S1600 vs. C	258	166	92
S400 vs. C	71	26	45

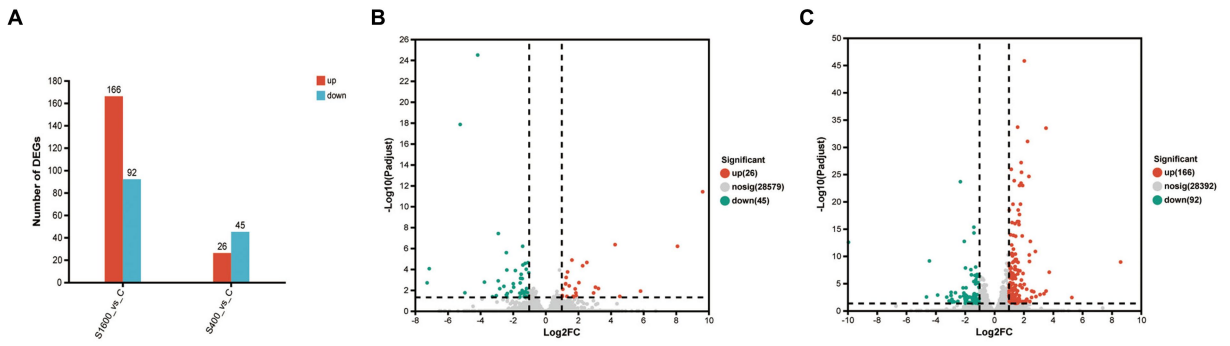

FIGURE 4
Up-regulation and down-regulation of differentially expressed genes. **(A)** The expression of differentially expressed genes between S1600 and C group, and S400 and C group. **(B)** Volcano plot of differentially expressed genes between S400 and C group. **(C)** Volcano plot of differentially expressed genes between S1600 and C group.

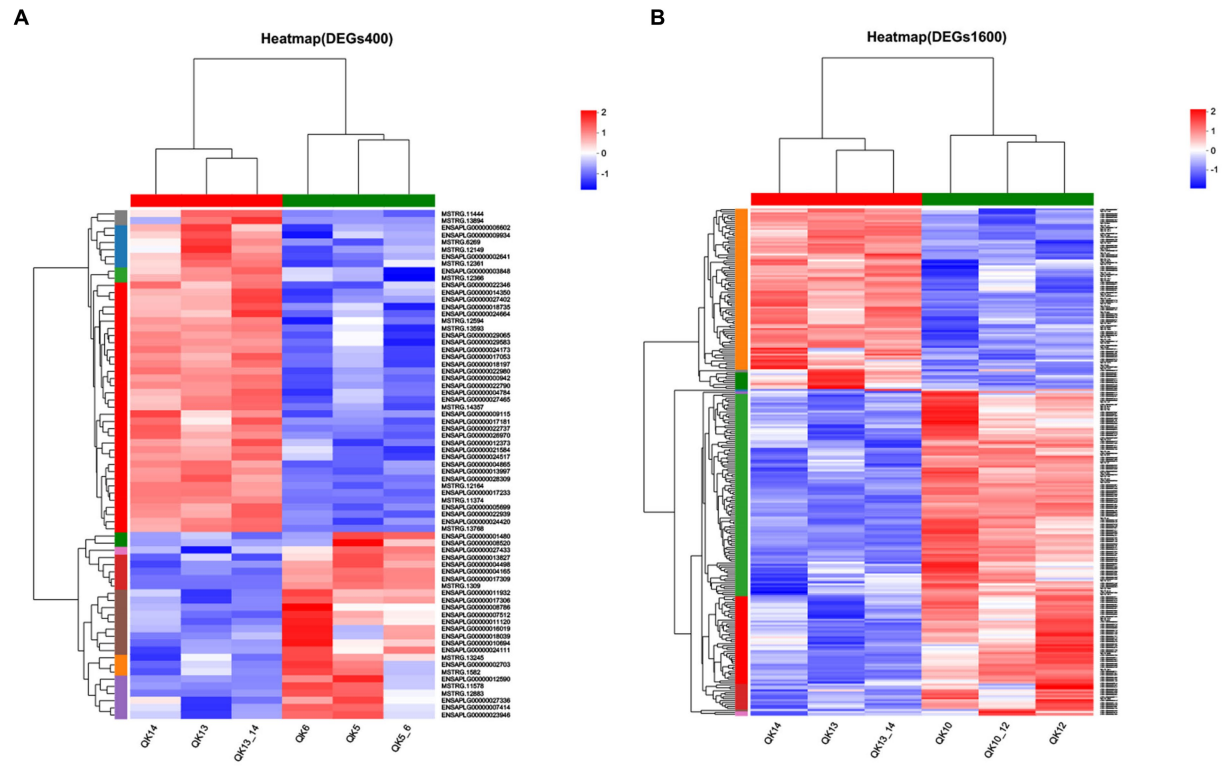

FIGURE 5
Heatmap of gene expression between silybin-added and control groups. **(A)** Heatmap of gene expression in S400 and C groups. **(B)** Heatmap of gene expression in S1600 and C groups.

resistance to disease was strengthened. Processing and presentation of antigens is an important basis of acquired immunity. As an important immune organ of the body, liver plays an important role in antigen presentation. Disturbance of liver homeostasis can lead to a variety of liver diseases, such as autoimmune hepatitis and cirrhosis. Moreover, when the organism recognizes a pathogen or its own antigenic

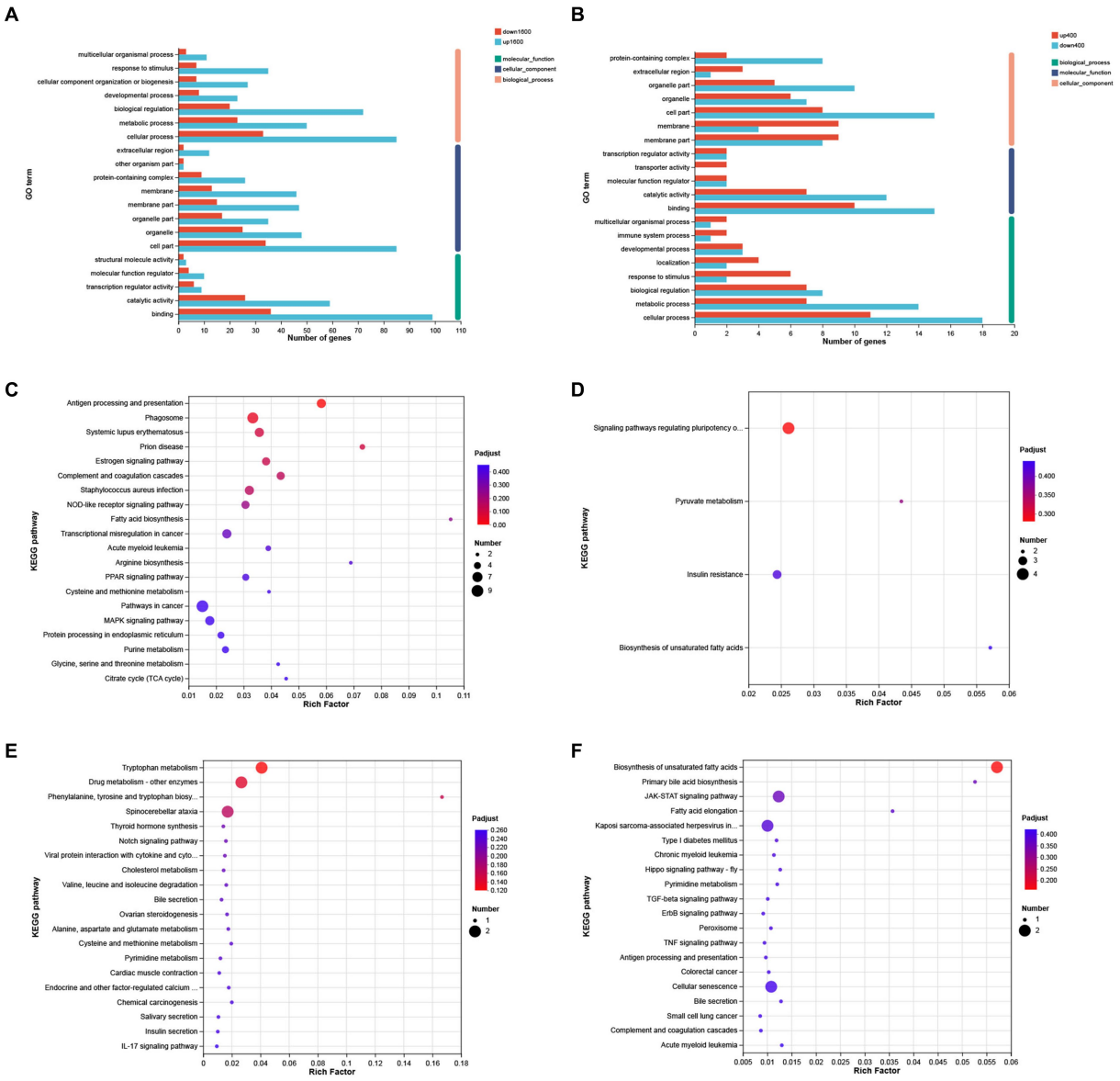

FIGURE 6
GO annotation and KEGG enrichment analysis of differentially expressed genes. **(A)** GO annotation analysis of differentially expressed genes in S1600 and C groups. **(B)** GO annotation analysis of differentially expressed genes in S400 and C groups. **(C)** KEGG enrichment analysis of differentially expressed genes up-regulated in S1600 and C groups. **(D)** KEGG enrichment analysis of differentially expressed genes down-regulated in S1600 and C groups. **(E)** KEGG enrichment analysis of differentially expressed genes up-regulated in S400 and C groups. **(F)** KEGG enrichment analysis of differentially expressed genes down-regulated in S400 and C groups. The vertical axis represents different GO terms **(A,B)** or pathways **(C–F)**, and the horizontal axis represents rich factor.

response, the cells in the liver can respond rapidly, exhibiting excellent antigen-presenting properties (44). We also observed that differentially expressed genes in the insulin resistance pathway were significantly downregulated in the S1600 and control groups. Insulin resistance is associated with many pathologic conditions of the liver, influenced by genetic and environmental factors, and is present in many metabolically related diseases (27, 45). What has been shown is that pro-inflammatory factors can induce the development of insulin resistance. A study found that the insulin resistance of mice was significantly improved after the application of neutralizing IL-6 antibody (46). Similarly, silybin treatment reduced the expression levels of TNF-α and NF-κB in high-fat mice, reducing body weight and insulin resistance (47). This is in agreement with our previous findings that the addition of 1,600 mg/kg silybin significantly reduced serum levels of the pro-inflammatory factors IL-6 and TNF-α.

5 Conclusion

In this experiment, we investigated the effects of feed supplementation with silybin on growth performance, serum inflammatory factors, immune levels, antioxidant levels, serum biochemistry and liver transcriptome of Peking ducks. It was discovered that the addition of 1,600 mg/kg of silybin could greatly enhance the growth performance of Peking ducks, improve their immune ability and reduce the inflammatory response. The

transcriptome results suggest that it might be achieved by regulating antigen processing and presentation, amino acid metabolism and synthesis, and JAK–STAT pathways.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information repository, accession number PRJNA1027007. The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/sra/PRJNA1027007.

Ethics statement

The animal study was approved by the Animal Welfare and Animal Experimental Ethics Review Committee of China Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZZ: Formal analysis, Methodology, Project administration, Visualization, Writing – original draft. BS: Data curation, Formal analysis, Writing – review & editing. XL: Funding acquisition, Project administration, Resources, Writing – review & editing. YD: Investigation, Resources, Writing – review & editing. LL: Conceptualization, Writing – review & editing. ZX: Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by a sub-theme of the National Key Research and Development Program of China (2021YFD1300300).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1325115/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 11 January 2024
DOI 10.3389/fvets.2023.1321351

OPEN ACCESS

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RECEIVED 13 October 2023
ACCEPTED 21 December 2023
PUBLISHED 11 January 2024

CITATION
Gómez-Verduzco G, Arce-Menocal J, López-Coello C, Avila-González E, Márquez-Mota CC, Polo J and Rangel L (2024) Feeding spray-dried plasma to broilers early in life improved their intestinal development, immunity and performance irrespective of mycotoxins in feed.
Front. Vet. Sci. 10:1321351.
doi: 10.3389/fvets.2023.1321351

Feeding spray-dried plasma to broilers early in life improved their intestinal development, immunity and performance irrespective of mycotoxins in feed

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Introduction: Fungi that produce mycotoxins can grow on certain food products, such as grains and feed, and can cause a variety of health issues if consumed by animals, including chickens. The use of spray-dried plasma (SDP) is one strategy for combating the health problems caused by mycotoxins.

Materials and methods: In the present study, Ross 308 chickens ($n = 960$) were divided into four treatment groups. T1 group was given a control diet (corn–soybean meal), T2 group was given a control diet +2% SDP, T3 group was given a control diet +2% SDP + mixture mycotoxins and T4 group was givena control diet + mycotoxin mixture.

Results: The presence of SDP resulted in weight gain and decreased feed efficiency, whereas mycotoxins resulted in weight loss and increased feed efficiency. SDP increased the thymus' relative weight. The presence of mycotoxins increased the heterophile/lymphocyte ratio. The presence of mycotoxins reduced the production of IL-2 and macrophage inflammatory protein-3 Alpha (MIP-3a), whereas the presence of SDP increased the production of macrophage colony-stimulating Factor (M-CSF). SDP resulted in higher IgA concentrations in the intestinal and tracheal washes than mycotoxin. Finally, adding SDP to broiler diets boosts weight gain, feed efficiency, and immune system development.

Discussion: Our results provide information supporting that SDP is a promising tool for improving poultry immunity and performance.

KEYWORDS

feed additive, intoxication, poultry, health, nutrition, growth, Immunity

1 Introduction

Grain consumption by the agricultural industry has increased significantly in recent years because of rising demand for high-quality animal protein to meet the needs of the world's expanding population (1) and consistent increase is expected in the future. Grain storage and transport to various geographically distant regions have been linked to the development of mycotoxins that can be consumed by animals (2, 3).

The main ingredients used in poultry feed in intensive production systems are soybean meal mixed with several cereal grains, which are susceptible to mycotoxin contamination (4). Mycotoxin contamination in cereal grain is a major problem; it is estimated that more than 70% of cereal-based diets contain at least one mycotoxin (5). In most countries, the quantity of grains produced for animal feed is insufficient to satisfy their needs. As a result, grains are acquired from other regions or countries with long delivery periods, which favors the growth of fungi and the subsequent production of secondary metabolites such as mycotoxins (6–8). Mycotoxin poisoning in chickens has been associated with economic losses due to cross-border rejection in the local and global markets, which affects access to macro-and micronutrients, particularly in poor countries. Mycotoxins are produced by a variety of fungal species, including Aspergillus, Fusarium, and Penicillium. Among the well-known agricultural mycotoxins are aflatoxins (AF), fumonisins (FUM), zearalenone (ZEN), T-2 toxin (T-2), deoxynivalenol (DON), and ochratoxin A (OTA) (9, 10). Patulin, citrinin, sterigmatocystin, ergot alkaloids, and trichothecenes are harmful mycotoxins that can negatively impact animals, food, humans, and plants (11). These toxins pose a threat to animal health, leading to reduced productivity, impaired growth, reproductive problems, and, in severe cases, death. It is crucial to address the presence of these mycotoxins to safeguard the well-being of animals and ensure the safety of food and crops. Particularly in avian diets can negatively impact the immune system, gastrointestinal tract, liver, and other organs, resulting in decreased productivity and, in extreme situations, death (12). Furthermore, mycotoxin contamination in animal feed can result in the transfer of these toxins to animal products such as milk and meat, posing a risk to human health. Therefore, understanding the types of mycotoxins and their impact on animal health is crucial for ensuring food safety and maintaining the well-being of both animals and humans. Furthermore, the fact that mycotoxins are difficult to eliminate with thermal, chemical, or physical treatments is another cause for concern (4).

Therefore, the poultry industry needs feed additives that help to counteract the negative effects of mycotoxins in animal feed (13). Among these additives is spray-dried plasma (SDP), which is a complex mixture of functional proteins with antibacterial properties such as albumin, transferrin, immunoglobulins and glycoproteins, bioactive peptides, growth factors, amino acids, and other molecules of biological interest that, in addition to improving farm animal productivity, has positive effects on animal health and welfare (14, 15). The poultry industry has been characterized as a highly efficient production industry for converting protein of plant origin into protein of animal origin, and those in the industry constantly seek continuous improvement in all production processes. The biosafety protocols currently used in this industry represent an opportunity for improvement (5).

In murine models, rats treated with *Staphylococcus aureus* enteroxin B, an immune system activator, developed intestinal inflammation. (16). When rats were fed SDP (8%), they showed an improvement in intestinal health, which was characterized by stronger intestinal barrier integrity, which was associated with a decrease in proinflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) and an increase in anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor beta 1 (TGB-β1) (17). IL-10 is an anti-inflammatory cytokine that suppresses the activation and function of immune cells, as well as the expression of pro-inflammatory cytokines (18–20). In contrast, TGB-β1 regulates the magnitude and type of the immune response (21).

Recent studies have shown that the use of SDP in the feed of farm animals such as pigs (22, 23) and poultry (24) increases the total systemic antibody titers. Therefore, the use of SDP may be a suitable strategy to improve the humoral immune response induced by vaccines. Based on these reports, the purpose of this study was to examine the use of SDP in broilers that received a balanced feed with or without extra addition of mycotoxins during the first 7 days of the chick's life and to evaluate their productive performance, intestinal health, and humoral and cellular immune responses.

2 Materials and methods

2.1 Facilities and care of experimental animals

The experiment was carried out in accordance with the guidelines of the Official Mexican Standard (NOM-033-SAG/ZOO-2014) for animal welfare, and the experimental protocols were approved by the Institutional Committee for the Care and Use of Animals of the School of Veterinary Medicine of the National Autonomous University of Mexico (CI-CUAE-FMVZ-UNAM MC-2017/1-14). The experiment was carried out on an experimental farm located in the state of Michoacán, Mexico, at a height of 1940 meters above sea level.

2.2 Experimental design and handling of animals

Nine hundred sixty mixed chicks (50% male, 50% female) of the Ross 308 lineage from the same hatchery and same breeder were used, all the chicks against Marek's disease in the hatch. The chicks were kept in production until 42 days of age. The chicks were completely randomized into four experimental treatments, with 8 replicates and 30 birds per replicate. The T1 (−P, −M) group was fed a control diet (corn–soybean meal), the T2 (+P, −M) group was fed a control diet +2% SDP, the T3 (+P, +M) group was fed a control diet +2% SDP + mycotoxin mixture and the T4 (−P, +M) group was fed a control diet + mycotoxin mixture. The dosage of SDP was determined according to previous reports (25) and was supplemented in the diet. The mixture of mycotoxins added to the feed in treatment groups T3 and T4 was 2.5 parts per billion (ppb) aflatoxin, 4.14 ppb T-2, 0.66 ppb ochratoxin, 19.32 ppb zearalenone, 5.83 ppb fumonisin and 3.6 ppb deoxynivalenol (DON). The SDP was donated by APC proteins ®. Only the feed administered on the first 7 days (preliminary diet) was supplemented with mycotoxins. The feeding of the chicks was divided into four phases: starting: 1 to 7 days, growing: 8 to 21 days, finisher 1 22 to 35 days, and finisher 2 36 to 42 days, and they had *ad libitum* access to feed and water. Table 1 shows the formulation of the experimental treatments.

To evaluate the systemic immune response, chickens were simultaneously vaccinated with a live virus vaccine against Newcastle

10.3389/fvets.2023.1321351

TABLE 1 Composition of the experimental diets.

Ingredients	Preliminary control	Preliminary plasma	Starter	Finisher 1	Finisher 2
	(0 – 7 days)	(0 – 7 days)	(7 – 21 days)	(21–35 days)	(35 – 42 days)
Yellow corn (7.5%)	604.7	634.1	630.5	650.6	674.8
Soy-bean meal (48%)	351.8	314.6	326.7	302.6	277.7
Spray-dried plasma (78%)	0.0	20.0	0.0	0.0	0.0
Limestone (40%)	11.4	11.7	10.9	10.1	9.3
Dical phos (21/17)	9.0	8.5	7.7	6.9	6.2
Pigment 30 g/kg	0.0	0.0	0.0	2.0	2.7
Soy oil	11.3	1.2	13.3	17.9	20.5
NaCl	3.7	2.6	3.8	3.8	3.8
DL-Methionine (99%)	2.9	2.7	2.6	2.0	1.4
Vit/Min premix**	3.0	3.0	3.0	3.0	3.0
Lysine HCl (78.6%)	1.2	0.8	1.0	0.5	0.1
Nicarbazine	0.5	0.5	0.5	0.0	0.0
Salinomycin	0.0	0.0	0.0	0.5	0.5
L-Threonine (99%)	0.4	0.1	0.0	0.0	0.0
Phytase Dupont	0.1	0.1	0.1	0.1	0.1
Total (kg)	1,000	1,000	1,000	1,000	1,000
Calculated composition					
Metabolizable energy(kcal/kg)	3,050	3,050	3,100	3,100	3,200
Crude protein (%)	22	22	21	20	19
Total calcium (%)	0.70	0.70	0.65	0.60	0.550
Available phosphorus (%)	0.45	0.45	0.42	0.4	0.380
Digestible Lys (%)	1.18	1.18	1.1	1.0	0.900
Digestible Met (%)	0.606	0.576	0.555	0.488	0.419
Digestible TSAA (%)	0.92	0.92	0.86	0.78	0.700
Digestible Trp (%)	0.227	0.236	0.214	0.202	0.189
Digestible Thr (%)	0.8	0.8	0.725	0.689	0.654
Digestible Ile (%)	0.959	0.939	0.911	0.863	0.815
Digestible Val (%)	0.995	1.023	0.949	0.902	0.856
Linolenic acid (%)	1.839	1.359	1.986	2.253	2.425
Total sodium (%)	0.2	0.2	0.2	0.2	0.200

Dical Phos: dicalcium/monocalcium phosphate, Vit/Min: Mineral and vitamin premix provided: vitamin A 12,000,000 IU, vitamin D3 2,500,000 IUP, vitamin E 15,000 IU, vitamin K3 2000 mg/kg, vitamin B1 2,250 mg/kg, vitamin B2 7,500 mg/kg, vitamin B3 45,000 mg/kg, vitamin B5 12,500 mg/kg, vitamin B6 3,500 mg/kg, vitamin B12 20 mg/kg, folic acid 1,500 mg/kg, biotin 125 mg/kg, iodine 300 mg/kg, selenium 200 mg/kg, cobalt 200 mg/kg, iron 50,000 mg/kg, copper 12,000 mg/kg, zinc 50,000 mg/kg, manganese 110,000 mg/kg. ** BHT(1.2%) and BHQ (9%).

disease through the intraocular route and an inactivated virus vaccine against Newcastle disease through the subcutaneous route (La Sota® Newcastle strain Laboratorios Avilab, SA and Newcastle Plus®, Laboratorios Avilab, S. A de CV, Tepatitlán de Morelos, Jalisco, Mexico.)

2.3 Determination of mycotoxins in treatments

The mycotoxin content was determined according to VICAM technology as previously reported (26). Table 2 shows the mycotoxin content in the treatments.

2.4 Productive performance

Chickens and feed were weighed weekly until Day 42 of age. Weight gain, feed consumption and feed conversion index were determined. Mortality was recorded daily.

2.5 Morphometric index

The morphometric index of the spleen, thymus, bursa of Fabricius and liver was determined on Days 21 and 42 by weighing each organ of 8 chickens from each treatment group with a scale (precision

TABLE 2 Mycotoxin content in experimental treatments.

Treatments	Aflatoxin	Toxin T-2	Ochratoxin	Zearalenone	Vomitoxin	Fumonisin
	ppb					
T1 (−P, −M)	3.63	27.05	1.90	126.28	415	790
T2(+P, −M)	4.44	28.93	1.81	162.66	335	880
T3(+P, +M)	5.36	37.07	3.95	213.17	422	980
T4(−P, +M)	5.48	46.00	4.73	256.03	540	960
MTV	15.0	150.0	75.0	35,000	15,000	7,500

T1: control diet (corn–soybean meal), T2: control diet + 2% SDP, T3 control diet + 2% SDP + mycotoxin mixture, T4 control diet + mycotoxin mixture. MTV: maximum tolerable value Waters Corporation ™- VICAM; UK.

balance FPRS223, Thermo Fisher Scientific Inc., Germany.). The morphometric index was calculated as previously reported (27).

2.6 Evaluation of intestinal contact surface area

At 21 days of age, 8 birds were taken from each treatment group and the jejunum and ileum were isolated and analyzed. The data were obtained and processed according previously reported methods (28) with support from the Motic Images Plus 2.0 program (Routine Software Series, Motic Asia, Hong Kong).

2.7 Measurement of serum interleukin and chemokine levels

The serum levels of IL-2, M-CSF, and MIP-3α were determined at 14 days of age with a commercial ELISA test (Cat GCYT1-16 K. Millipore Corporation Merck; Darmstadt, Germany). Blood samples were taken from the left radial vein of the birds; 1 bird per replicate. The sera were handled as previously reported (29).

2.8 Measurement of total tracheal and intestinal IgA concentrations and serum IgY concentration

To quantify the total and nonspecific production of IgA in the epithelia of the trachea and jejunum, a commercial antigen capture ELISA chicken IgA quantification kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used following the manufacturer's recommendations. At 21 and 42 days of age, 8 broilers were sacrificed by anesthesia overdose. Ten-centimeter sections of jejunum and 3 cm of trachea were removed from each broiler. This process was performed according to what was previously reported (30). Serum samples were collected on day 14, 28 and 42 to measure IgY using the commercial available ELISA kit (Neo Biolab, Cambridge MS, Catalog AB157693) following the manufacturer's recommendations.

2.9 Blood heterophil/lymphocyte index (H/L)

The H/L index in blood was determined on Days 25 and 40. Blood from 3 birds/treatment was collected from the radial vein of the wing and stored in EDTA-coated tubes, and then these samples were processed as previously reported (31).

2.10 Evaluation of the systemic humoral immune response

The humoral immune response was determined by the quantification of antibodies against Newcastle disease through the ELISA method at 14 days of age.

On Day 10 of age, the first immunization was carried out, and on Day 17 of age, a second immunization was carried out, only with the emulsified vaccine. Two milliliters of serum of eight chickens were taken from each treatment. Serum samples were collected and frozen at −20°C to determine the specific serum antibody titers against the Newcastle virus by the hemagglutination inhibition test (32).

2.11 Statistical analysis

Data were analyzed to verify the fulfillment of normality and homogeneity of variance assumptions (33). The results were analyzed using a completely randomized design, with a factorial arrangement of 2 × 2 treatments. One factor was the diet with and without 2% plasma (from 1 to 7 days of age), and the other factor was the diet with and without a mixture of mycotoxins. The comparison of means was performed by the Tukey test (StatSoft. Statistica version 10.0, 2011) and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Body weight development, feed intake, feed conversion and mortality

As shown in Table 3, the addition of 2% SDP caused an increase in body weight on Day 7 ($p < 0.021$) and on Day 42 ($p < 0.001$), the T4 group had the lower ($p < 0.001$) body weight in comparison with T1, T2 and T3.Mycotoxins in the feed resulted in a decrease in body weight that could be observed from 7 days of age until 42 days of age ($p < 0.05$). As shown in Table 3, the inclusion of 2% SDP did not affect chicken feed intake in the period from 7 to 42 days, while the inclusion of mycotoxins caused an increase in feed intake ($p < 0.032$) at the end of the study (Day 42). At 7 days old, animal feed with T4 showed a higher feed conversion ($p < 0.001$) in comparison with T1, T2 and T3. Inclusion of 2% SDP causes a lower feed conversion on Days 7 and 42.

TABLE 3 Body weight development, feed intake, feed conversion at 7 and 42 days, and mortality at 42 days of age of male and female chickens fed 2% plasma and mycotoxins.

Factor		Body weight (Kg)		Feed intake (Kg)		Feed conversion		Mortality (%)
		7 days	42 days	7 days	42 days	7 days	42 days	42 days
Plasma (P)								
+ P		0.152[a]	3.058 [a]	0.124	4.896	1.108[b]	1.622[b]	5.42
−P		0.145[b]	3.008[b]	0.129	4.89	1.227[a]	1.648[a]	6.04
Probability **		0.021	0.001	0.064	0.836	0.001	0.016	0.555
Mycotoxin (M)								
+ M		0.148	3.019[b]	0.128	4.925[a]	1.199[a]	1.653[a]	5.63
−M		0.150	3.046[a]	0.125	4.860[b]	1.136[b]	1.617[b]	5.83
Probability **		0.48	0.023	0.157	0.032	0.023	0.001	0.844
−P	− M	0.151[a]	3.025	0.125	4.841	1.125 [b]	1.621	6.25
− P	+ M	0.140[b]	2.99	0.133	4.938	1.329 [a]	1.674	5.83
+ P	− M	0.148 [ab]	3.067	0.124	4.879	1.147 [b]	1.612	5.42
+ P	+ M	0.156 [a]	3.048	0.124	4.912	1.069 [b]	1.633	5.42
Probability **		0.001	0.472	0.162	0.278	0.001	0.128	0.844
Mean		0.149	3.033	0.127	4.893	1.167	1.635	5.73
SEM***		0.002	0.007	0.001	0.015	0.021	0.006	0.501

P, SDP; M, mycotoxins, +, added, − without. Results of a 2 × 2 factorial arrangement; one factor was the diet with and without 2% plasma (from 1 to 7 d of age) and the other factor was the diet with and without mixed mycotoxins. When there were significant differences (5%), between the treatments, the comparison of means was made by Tukey's test. Values with different sides are significantly different according to the probability indicated in the standard table. a>b.

Interestingly, feed conversion increased ($p < 0.05$) during the entire production cycle when mycotoxins were included in the food (Table 3). No differences in mortality were observed in the chickens in the present study (Table 3).

Morphometric and H/L indices On Day 42, the addition of 2% SDP resulted in a 1.09-fold increase in the relative weight of the thymus when compared to no SDP inclusion (Table 4). There were no differences in the relative weights of the spleen, bursa, or liver with any of the interventions. The hemogram results revealed that the heterophil/lymphocyte index (H/L) was higher ($p < 0.001$) in the serum of birds given 2% SDP (T2) compared to birds fed mycotoxins (T3 and T4) or no mycotoxins (T1) at 25 days of age (Table 4). The presence of mycotoxins increased the H/L index at 40 days ($p < 0.001$).

3.2 Evaluation of the intestinal contact area surface in the duodenum, jejunum, and ileum

As shown in Table 5, in chickens treated with 2% SDP, the surface capacity of the duodenum contact area increased 4.0% ($p < 0.033$) compared with that in chickens not treated with SDP. Interestingly, the surface capacity of the duodenal contact area decreased by 6% in chickens that were fed a diet with high mycotoxin levels ($p < 0.004$) compared to chickens that were fed a diet with low mycotoxin levels.

As shown in Table 5, supplementation with 2% SDP did not alter the contact area of the jejunum, while the jejunum contact area of chickens that were fed a diet with mycotoxins decreased by 8.5% ($p < 0.001$) compared to that of chickens fed a diet without mycotoxins.

As observed in Table 5, supplementation with 2% SDP increased ($p < 0.001$) the surface capacity of the ileum by 5.9% compared to non-supplemented diets, while diets with mycotoxins decreased the surface capacity by 13.5% ($p < 0.001$) compared to diets that did not have mycotoxins.

3.3 Serum interleukin and chemokine levels

Table 6 shows that there are no differences in IL-2 and MIP-3 levels between chickens that received mycotoxins in their feed and those that did not. M-CSF concentration was increased in chickens treated with 2% SDP ($p < 0.001$).

3.4 Assessment of the systemic humoral immune response

The results of the hemagglutination inhibition test on the 14th day of life show that T2 and T3 increased total serum antibody response by 2.0 and 1.8-fold, respectively, when compared to T4 (Figure 1).

3.5 Measurement of total tracheal and intestinal IgA and serum IgY concentration

On Days 21 and 42, it was observed that the inclusion of 2% SDP (T2 and T3) significantly increased ($p < 0.001$) the concentration of IgA in intestinal and tracheal washes. Interestingly, in this same period, the inclusion of mycotoxins (T4) decreased ($p < 0.01$) the IgA titers both in the intestine and in the trachea (Figures 2A,B). The inclusion of mycotoxin (T4) caused a decrease ($p < 0.001$) in the concentration of serum IgY on Days 14, 28 and 42 (Figure 3).

10.3389/fvets.2023.1321351

TABLE 4 Relative weight expressed in (%) of the spleen, thymus, bursa of fabricio and liver with respect to live body weight at 42 days of age of male and female chickens fed with 2% plasma and mycotoxins.

FACTOR		Relative weight (%)				Heterophile/lymphocyte ratio	
		Spleen	Thymus	Bursa	Liver		
Plasma (P)		42 days				25 days	40 days
+ P		0.126	0.187[a]	0.115	1.733	2.39[a]	1.93
− P		0.124	0.171[b]	0.106	1.799	0.59[b]	1.88
Probability **		0.719	0.033	0.266	0.089	0.001	0.85
Mycotoxin (M)							
+ M		0.119	0.173	0.107	1.763	0.80[b]	2.43[a]
− M		0.132	0.185	0.114	1.769	2.18[a]	1.38[b]
Probability **		0.056	0.128	0.34	0.874	0.002	0.001
− P	− M	0.132	0.172	0.107	1.815	0.29[b]	1.29
− P	+ M	0.116	0.169	0.106	1.783	0.89[b]	2.47
+ P	− M	0.131	0.198	0.122	1.723	4.07[a]	1.47
+ P	+ M	0.121	0.177	0.108	1.743	0.70[b]	2.39
Probability **		0.693	0.223	0.375	0.505	0.001	0.652
Mean		0.125	0.179	0.111	1.766	1.49	1.91
SEM***		0.003	0.004	0.004	0.019	0.47	0.197

And heterophil/lymphocyte (H/L) ratio at day 25 and 40 of age. P, SDP; M: mycotoxins, +, added, − without. Results of a 2 × 2 factorial arrangement; one factor was the diet with and without 2% plasma (from 1 to 7 d of age) and the other factor was the diet with and without mixed mycotoxins. When there were significant differences (5%), between the treatments, the comparison of means was made by Tukey's test. Values with different sides are significantly different according to the probability indicated in the standard table. a>b.

TABLE 5 Surface capacity contact area of duodenum, jejunum, and ileum from male and females chickens at 21 days of age fed 2% plasma and mycotoxins.

Factor		Surface capacity contact area (Micron^2)		
		Duodenum	Jejunum	Ileum
Plasma (P)				
+ P		9.9[a]	6.8	6.9[a]
− P		9.5[b]	6.6	6.5[b]
Probability **		0.033	0.272	0.001
Mycotoxin (M)				
+ M		9.4[b]	6.4[b]	6.4[b]
− M		10.0[a]	7.0[a]	7.0[a]
Probability **		0.004	0.001	0.001
− P	− M	9.2	6.9[abc]	6.4[bcd]
− P	+ M	9.6	6.4[bc]	7.0[abc]
+ P	− M	9.9	6.9[abc]	7.5[a]
+ P	+ M	10.3	7.0[abc]	6.5[bcd]
Probability **		0.653	0.001	0.001
Mean		9.7	6.7	6.7
SEM**		0.106	0.073	0.067

P, SDP; M, mycotoxins, +, added, − without. Results of a 2 × 2 factorial arrangement; one factor was the diet with and without 2% plasma (from 1 to 7 d of age) and the other factor was the diet with and without mixed mycotoxins. When there were significant differences (5%), between the treatments, the comparison of means was made by Tukey's test. Values with different sides are significantly different according to the probability indicated in the standard table. a>b>c.

TABLE 6 Evaluation of the immune response, through the measurement of IL-2, MIP-3α and M-CSF from male and female chickens 14 days of age fed with 2% plasma and mycotoxins.

Factor		IL-2	MIP-3α	M-CSF
		(pg/mL)		
Plasma (P)				
+ P		4344.1	237	703.0[a]
− P		4056.8	236.7	624.8[b]
Probability **		0.403	0.997	0.005
Mycotoxin (M)				
+ M		3754.0[b]	126.8[b]	666.1
− M		4646.9[a]	346.9[a]	661.7
Probability **		0.013	0.013	0.867
− P	- M	4371.3	347	563.9[c]
− P	+ M	3742.3	126.4	685.8[ab]
+ P	- M	4922.5	346.8	759.6[a]
+ P	+ M	3765.7	127.1	646.5[bc]
Probability **		0.441	0.996	0.001
Mean		4200.4	236.8	663.9
SEM***		183.1	44.38	17.67

P, SDP; M, mycotoxins, +, added, − without. Results of a 2 × 2 factorial arrangement; one factor was the diet with and without 2% plasma (from 1 to 7 d of age) and the other factor was the diet with and without mixed mycotoxins. When there were significant differences (5%), between the treatments, the comparison of means was made by Tukey's test. Values with different sides are significantly different according to the probability indicated in the standard table. a>b>c.

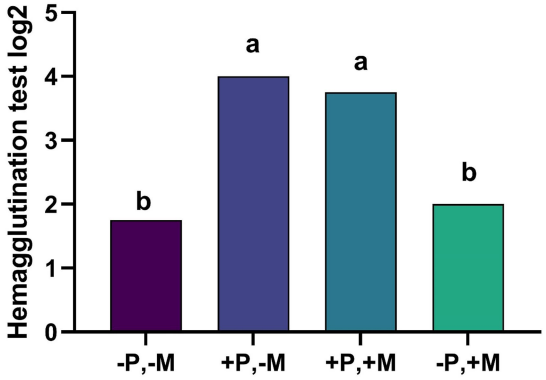

FIGURE 1
Assessment of the hemagglutination inhibition from male and female chickens 14 days of age fed with 2% plasma and mycotoxins. (−P without SDP, +P with SDP, −M without mycotoxins, +M with mycotoxins). Values are means. Labeled means without a common letter differ, $p < 0.05$, a > b.

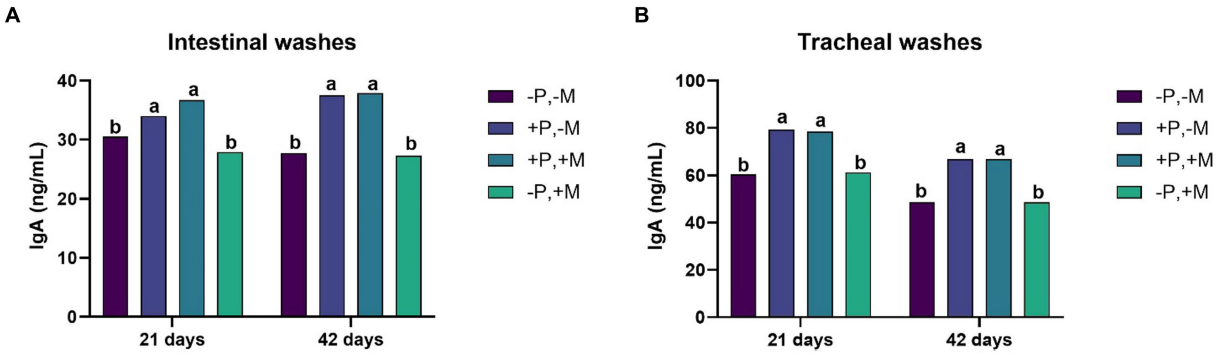

FIGURE 2
Assessment of the IgA concentration from intestinal washes **(A)** and tracheal washes **(B)** from male and female chickens of 21 and 42 days of age fed with 2% plasma and mycotoxins. (−P without SDP, +P with SDP, −M without mycotoxins, +M with mycotoxins). Values are means. Labeled means without a common letter differ, $p < 0.05$, a > b.

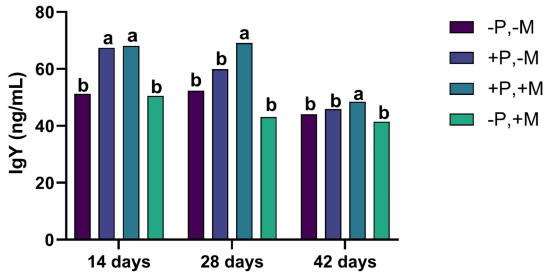

FIGURE 3
Assessment of the IgY from male and female chickens of 14, 28 and 42 days of age fed with 2% plasma and mycotoxins. (−P without SDP, +P with SDP, −M without mycotoxins, +M with mycotoxins). Values are means. Labeled means without a common letter differ, $p < 0.05$, a > b.

4 Discussion

The first diet provided to chicks is critical for their growth and development, and this primary diet also contributes to the establishment of their microbiota (3) and plays an important role in the development of their immune response (34). Our results showed that feeding with SDP during the first 7 days of life promotes weight gain and improves the feed conversion rate, even when there are mycotoxins in the diet, which is of great importance given that the rate of feed conversion indicates the efficiency by which animal bodies use food to achieve optimal weight gain (35). The benefit of plasma in the primary diet was reflected in a greater weight of the chickens and a lower rate of conversion at sacrifice.

Mycotoxins are the secondary fungal metabolites of certain toxigenic fungal species which are proved to be detrimental for the health conditions of humans, animals, and birds. To date, approximately 400 different chemically diverse mycotoxins have been identified, but ochratoxins and aflatoxins are thought to be the most lethal in the poultry industry (36, 37) and these two mutagens have been reported as major carcinogens by International Agency for Research on cancer (38). The presence of mycotoxins in poultry diets has been associated with an increase in mortality rates, as well as a decrease in productive efficiency (39). Our results did not show a clear effect of mycotoxin on mortality during the first week of the study, so it is possible that a higher concentration is required to observe these differences. It has been reported that administering plasma in the pre started diet of chicks had no effect on carcass performance under normal conditions; however, when the chicks were raised in a stressful

situation created by using the litter from the previous experiment, the presence of SDP in the diet increased meat yield (40). Therefore, the findings of this study could imply that mycotoxin contamination in the diet did not create a stressful situation sufficient to observe an effect of SDP addition on meat quality.

However, chickens fed mycotoxins had a higher consumption at 42 days, which resulted in a higher conversion rate at 42 days. ($p < 0.001$), demonstrating that the presence of mycotoxins, as previously stated, the mycotoxins are capable of impair animal productivity by affecting weight gain and reducing feed conversion.

The results obtained in the present study indicate that the use of 2% SDP in the first 7 days of life may be a promising strategy to ensure the development of broilers regardless of the presence of mycotoxins, provided that the mycotoxin level in cereal-based feed does not exceed the maximum level that can be biologically tolerated by birds. Similar results have been obtained in pigs (23, 41) in which piglets diets where contaminated with different levels of mycotoxins and observed that the presence of SDP mitigated the negative effects of the added mycotoxins on the growth of the animals.

In birds, the consumption of feed, as well as the absorption of nutrients, is highly regulated by the conditions of the gastrointestinal tract (42). Moreover, the prime responsibility of intestinal mucosa is the digestion and/or absorption of nutrients ultimately and a healthy mucosa ensures the optimum growth of the organisms (43). The inclusion of plasma improved the surface capacity of the contact area in the duodenum and ileum at 21 days meanwhile the presence of mycotoxins caused a detrimental effect in the surface capacity of the contact area in the duodenum, jejunum, and ileum. The contact surface capacity develops according to the growth of the chicks, and in our study, it is interesting to note that the absorption capacity of the distal segments (jejunum and ileum) increased with time and with the addition of SDP, which indicates that the use of plasma in the first days of life may be a promising strategy to improve intestinal function; however, more studies are needed to test this hypothesis. In general, the improvement in intestinal absorption capacity due to the presence of SDP positively correlated with length of the microvilli, especially at the ileum level. A study (44) in pigs demonstrated that the presence of SDP increased the surface area of intestinal microvilli due to an increase in their length instead of an increase in crypts. The greater presence of mycotoxins in the diet during the first 7 days was correlated with a lower absorption surface capacity in the duodenum, jejunum, and ileum, especially at 21 days, which could explain the lower growth observed in these birds during the study. The observed increase in the concentration of IgY can be associated with the greater relative weight (%) of the thymus as previously reported (45).

As previously mentioned, the presence of mycotoxins in grains destined for animal consumption can have detrimental effects on health due to the complex immune response that they provoke to these compounds (46). In the present study, we evaluated the response of different cytokines to the presence of mycotoxins and plasma. The presence of mycotoxins in the diet decreased ($p < 0.004$) the concentration of IgY, which is the main isotype that occurs in chickens (47), from 14 days of age, while the animals fed SDP presented a higher ($p < 0.001$) concentration of this immunoglobulin at 14 days of age. The observed increase in the concentration of IgY can be associated with the greater relative weight (%) of the thymus in the birds that were consuming SDP in the preliminary feed the first 7 days of life, which would probably also explain the higher titer of antibodies reported by the hemagglutination inhibition method at 14 days of age against Newcastle disease virus in birds that received SDP in diets with and without mycotoxins. This finding is interesting due to a possible stimulating effect on this primary lymphoid organ, and with it some immunostimulatory effects on the release of interleukins and chemokines that regulate and modulate the immune response (48). In chicks challenged with *Salmonella sofia* that the presence of plasma had no effect on the differences in the weights of the organs related to the immune system such as the liver, spleen and thymus, while the weight of the bursa of Fabricius was increased (25). In our study, an increase due to supplementation with SDP was observed in the thymus ($p < 0.033$), although numerically, the bursa of Fabricius also increased.

Regarding interleukins and cytokines, lower concentrations of IL-2 and MIP-3α were found in the serum at 14 days of age in the animals that consumed the diet with mycotoxin supplementation. These results are interesting since IL-2 is described as a growth factor of T lymphocytes, which induces all types of lymphocyte subpopulations and activates the proliferation of B lymphocytes (49) and therefore its effective action. In the literature, MIP-3α is described to be produced in the mucosa and skin by activated epithelial cells that attract activated B cells and memory T cells (50). Therefore, this decrease in birds that consume mycotoxins could be interpreted as an immunosuppressive effect at the immune level (51).

In contrast, chickens that consumed plasma had increased M-CSF levels, which is considered a macrophage colony-stimulating factor secreted by hematopoietic stem cells to differentiate into macrophages or other related cell types (52). In general, the observed interleukin and cytokine results indicate the potential of plasma to modulate the immune response and the importance of supplying it in the first days of life.

It is interesting to observe that at 25 days of age, the supplementation of SDP was associated with an increase in the H/L indices that could indicate an increase in innate immunity, while the presence of mycotoxins reduced it. It was observed in pigs fed diets contaminated with mycotoxins that the presence of these mycotoxins reduced the number of leukocytes and monocytes (53), as our results seem to indicate.

The results of the IgA concentration analysis in the intestinal and tracheal washes at 21 and 42 days of age indicate that the inclusion of SDP in the preliminary diet increased the titer of this immunoglobulin isotype, just the opposite effect to that observed with the inclusion of mycotoxins in feed that decreased IgA titers in both intestine and trachea. This information is important given that IgA is the isotype of immunoglobulin found in the mucosa, protecting at this level against antigens and toxins (54), and suggests that chicks receiving SDP in the diet have a greater immune capacity, as well as probably a stronger integrity of the gut barrier, as observed when chicks are subjected to systemic inflammation (55).

The current study adds to our understanding of the advantages of SDP in the feeding of various animal species. It is important to take notice that mycotoxin feed contamination is highly variable and therefore to gain more knowledge about the advantages of SDP we recommend the use of different levels of mycotoxin and different mixture of them. Further research, particularly in poultry, is required to assess its effect on intestinal health in poultry and its relationship with other types of diets.

5 Conclusion

The presence of mycotoxins in feed during the life cycle had negative effect on the growth of chicks probably due to poor intestinal development at this stage of their life and an alteration of the immune capacity that persists over time.

The inclusion of 2% SDP in preliminary corn-soy diets in broilers improves performance, intestinal health and immune response and reduces the negative effects associated with the presence of mycotoxins in the feed. The mitigating effects of SDP on mycotoxins are probably associated with the known effect of SDP to support and maintain a more efficient response of the immune system to inflammatory stress.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author, upon reasonable request.

Ethics statement

The animal study was approved by Institutional Committee for the Care and Use of Animals of the School of Veterinary Med-icine of the National Autonomous University of Mexico (CI-CUAE-FMVZ-UNAM MC-2017/1-14). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GG-V: Methodology, Writing – original draft. JA-M: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – original draft. CL-C: Methodology, Supervision, Validation, Writing – original draft. EA-G: Investigation, Methodology, Supervision, Visualization, Writing – original draft. CM-M: Writing – review & editing. JP: Conceptualization, Funding acquisition, Investigation, Project administration, Validation, Writing – review & editing. LR: Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by APC Proteins Avda. Sant Julià 246-258. P.I. El Congost. 08403. Granollers. Barcelona. Spain.

Conflict of interest

JP and LR are employed by APC LLC, Ankeny, IA, United States, a company that manufacture and sell spray-dried plasma. However, they declared that the company did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Authors ensure the objectivity and accuracy in the collection and presentation and interpretation of the results.

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

Publisher's note

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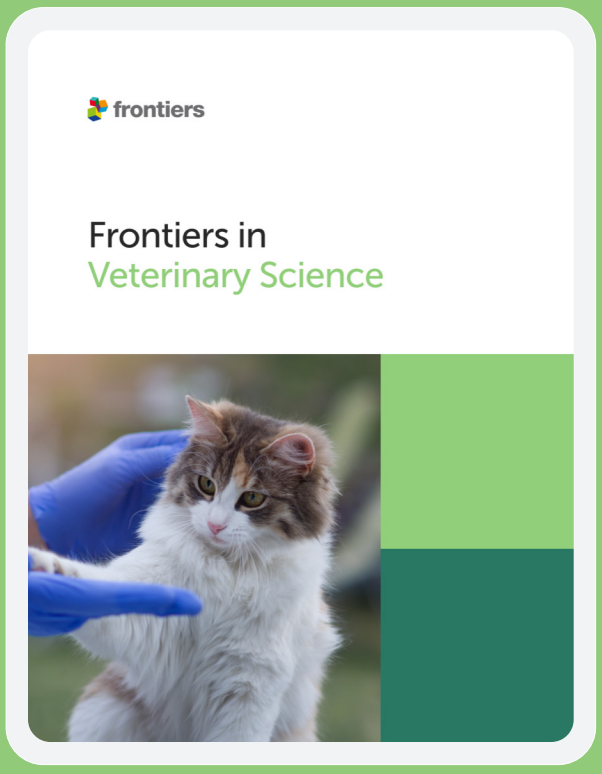

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