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Early life programming in poultry: Recent insights and interventional approaches

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Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-83251-370-5
DOI 10.3389/978-2-83251-370-5

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Early life programming in poultry: Recent insights and interventional approaches

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Citation

Abdel-Moneim, A.-M. E., Shehata, A. M., Paswan, V. K., eds. (2023). *Early life programming in poultry: Recent insights and interventional approaches*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-370-5

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frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 05 January 2023
DOI 10.3389/fvets.2022.1105653

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 22 November 2022
ACCEPTED 12 December 2022
PUBLISHED 05 January 2023

CITATION
Abdel-Moneim A-ME, Shehata AM and Paswan VK (2023) Editorial: Early life programming in poultry: Recent insights and interventional approaches. *Front. Vet. Sci.* 9:1105653. doi: 10.3389/fvets.2022.1105653

Editorial: Early life programming in poultry: Recent insights and interventional approaches

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KEYWORDS

early-life programming, in-ovo feeding, early nutrition, environmental manipulation, gut health, immune system, antibiotic alternatives, poultry

Editorial on the Research Topic
Early life programming in poultry: Recent insights and interventional approaches

"Early-life programming" describes how exposure to certain conditions during embryonic development or the early post-hatching period can change the normal development process, permanently altering how a bird's body looks and functions (1–4). Early-life programming can affect a bird for the rest of its life, altering its growth performance, tolerance to harsh environmental conditions, resistance to diseases like enteric infection and inflammation, immune function, metabolic disorders, and overall production(2–4). Considerable evidence suggests that early-life programming can alter the phenotype and performance of chicks in significant ways by modifying the expression of specific genes. In both the pre- and post-hatching phases, these treatments alter the environment and the diet of the organisms involved (1, 2).

This Research Topic is aimed at collecting papers suitable for improving our understanding of early life programming and its long-term role in minimizing environmental and health challenges. Its goal is also to share our knowledge on nutritional and environmental factors involved in early-life programming in poultry, such as in-ovo feeding, inadequate conditions, nutrient deficiencies, and sexing methods.

In this special e-book, there are nine papers covering the above-mentioned aspects. Three papers out of nine (33.3%) discuss the role of in-ovo feeding on growth performance, antioxidative capacity, breast development, glycogen reserves, nutrient absorption, immunity, and disease resistance. The excessive use of antibiotics in commercial poultry farms results in multidrug resistance and hinders the efficiency of antibiotics, causing increased threats to human and animal health. Therefore, the use of some natural bioactive molecules that can enhance the immune system, growth performance, and health status can be a promising alternative to antibiotics (5–7). A

study by Bhanja et al. shows how silver nanoparticles alone or in combination with some nutrients play an important role in inducing innate or adaptive immune responses in broilers. They demonstrated that in-ovo supplementation of silver nanoparticles with amino acids, vitamins, and trace elements improved post-hatch growth and immune responses in broilers and in particular that 50 μg/egg silver nanoparticles, in combination with vitamins (B1 and B6) and trace elements (Zn and Se), improved growth performance, and that 50 μg/egg silver nanoparticles with trace elements and amino acids enhanced immune responses in broilers challenged with Newcastle disease virus.

Given the biological link between methionine and/or disaccharide and post-hatching growth and development, Dang et al. investigated the effects of in-ovo feeding with methionine (Met) and/or disaccharide (DS) on the breast muscle and small intestine of the goose, together with the contents of its glycogen stores, the activities of its digestive enzymes, and levels of antioxidants in the jejunum. They found that DS injections enhance glycogen reserves and control muscle growth-related gene expression, increasing breast muscle metrics and hatchling weight. Delivering DS into the embryo improved digestive enzyme activities, nutrient transport enzyme activities, jejunal villus indices, and regulated nutrient transport-related gene expression, increasing post-hatch nutritional absorption. Met treatment in-ovo improved breast muscle metrics by regulating growth-related gene expression. Furthermore, Met treatment temporarily improved digestive enzymes, nutrient transport enzymes, small intestine parameters, and nutrient transport-related gene expression, and constantly improved jejunal villus parameters and jejunal antioxidant capacity status, which supported nutrient absorption post-hatching. As a result, they concluded that in-ovo administration of DS with Met is an effective method for enhancing the goslings' post-hatching nutrient absorption and breast muscle growth.

Heat stress promotes oxidative stress, which alters enzyme functions and immunological responses in broilers. Han et al. examined the effects of L-Leucine in-ovo feeding on broiler growth, organ weight, serum metabolites, antioxidant indices, and gene expression during chronic heat stress. Overall, they concluded that L-Leucine in-ovo feeding mitigates oxidative damage and improves antioxidant capacity in broiler chickens exposed to heat stress.

The burden of pathogens can be decreased by the use of probiotics because they initiate resistance to bacterial colonization and boost mucosal immunity (1, 2, 8–10). Yu et al. investigated how consumption of *Bacillus coagulans* (*B. coagulans*) and *Lactobacillus plantarum* (*L. plantarum*) affected broilers when they were exposed to *Escherichia coli* lipopolysaccharide (LPS). They demonstrated that supplementing broiler chickens with *B. coagulans* and *L. plantarum* enhanced their growth performance, immunity, and antioxidant ability, and reduced the LPS-induced inflammatory response *via* modulating the gut microbiota.

Exogenous emulsifiers improve lipid utilization and enhance nutrient absorption in broilers. The purpose of the research conducted by Li, Abdel-Moneim, Mesalam et al. was to investigate the genes that are crucial to the regulatory impact of lysoforte. They reported that at least 29 genes (including REG4, GJB1, KAT2A, APOA5, SERPINE2, ELOVL1, ABCC2, ANKRD9, CYP4V2, and PISD) and several signaling pathways may be involved in enhancing jejuna morphology in broiler birds. These findings clarified the role of LFT in maintaining the health and integrity of the intestines in broiler chickens.

The effects of hypoxia exposure (HE) on the embryogenesis of chickens are undesirable; however, the mechanism underlying the responses of the heart to HE during embryogenesis in birds is not yet fully understood. The research of Li, Abdel-Moneim, Hu et al. aimed to identify the hub genes as well as the signaling pathways that are associated with chronic hypoxic stress. Numerous genes, including SGCD, DHRS9, HELQ, MCMDC2, and ESCO2, along with multiple signaling pathways (including MAPK, PPAR, insulin, ERI, and adrenergic signaling pathways), were revealed to potentially contribute to the heart's response to HE in chickens.

The research article by Li, Shi et al. identified the key genes involved in LPC regulation in the jejunum of birds. Many genes may be involved in influencing the jejuna morphology of birds, including RSAD2, OASL, EPSTI1, CMPK2, IFIH1, IFIT5, USP18, MX1, and STAT1.

The substantial roles that methionine (Met) plays in the synthesis of proteins and the methylation process of DNA, and its role as a precursor in the production of cysteine, glutathione, and taurine, point to the crucial nature of this amino acid. Liu et al. looked at how a methionine (Met) deficiency during the chicks' early growth (0–6 weeks) affected their performance, egg quality, and serum amino acid metabolism later in life. The growth performance, blood amino acid content, intestinal maturation, and gut microbiota of layer hens were all negatively affected by a lack of Met during the evaluation period (0–6 weeks). Met deficit during this stage led to a serum amino acid imbalance, which impacted growth performance, and had a negative effect on the development and productivity of egg-laying hens throughout the duration of the research (from week 7 to 24).

Because turkey farms often use two distinct lines: a heavy line, which is composed of males, and a laying line, which supplies dams, early sex determination is an extremely important factor in the industry. Pardo et al. demonstrated that combining egg external traits, down feather colors, and two behavioral procedures ("English method" and "slap technique") permit successful

sexing in newly hatched Andalusian turkey poults, especially for the two roan varieties (black roan and bronze roan).

Thus, this Research Topic presents an overview of existing knowledge, highlights new insights on early-life programming in poultry and draws attention to some useful applications during the perinatal period in poultry.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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 in Veterinary Science

ORIGINAL RESEARCH
published: 21 February 2022
doi: 10.3389/fvets.2022.846649

Effects of Probiotics on the Growth Performance, Antioxidant Functions, Immune Responses, and Caecal Microbiota of Broilers Challenged by Lipopolysaccharide

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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: *31 December 2021*
Accepted: *31 January 2022*
Published: *21 February 2022*

Citation:
Yu Y, Li Q, Zeng X, Xu Y, Jin K, Liu J and Cao G (2022) Effects of Probiotics on the Growth Performance, Antioxidant Functions, Immune Responses, and Caecal Microbiota of Broilers Challenged by Lipopolysaccharide. Front. Vet. Sci. 9:846649. doi: 10.3389/fvets.2022.846649

We aimed to study the effects of dietary *Bacillus coagulans* (*B. coagulans*) and *Lactobacillus plantarum* (*L. plantarum*) on broilers challenged by *Escherichia coli* lipopolysaccharide (LPS). One-day-old Cobb 500 chicks (360) were divided randomly into three treatment groups for 47 days: no supplementation (control, CON), *B. coagulans* supplementation (BC), and *L. plantarum* supplementation (LA). Broilers were routinely fed for 42 days and intraperitoneally injected with 500 μg LPS per kg body weight at 43, 45, and 47 days of age, respectively. Samples were collected 3 h after the last injection. At 1–21 days of age, the ADG in the BC and LA groups was higher than that in the CON group, and the feed to gain ratio (F/G) in the BC group was significantly decreased ($P < 0.05$). Compared with that in CON birds, the ADG was increased and the F/G was decreased in the BC and LA birds at 22–42 and 1–42 days of age, respectively ($P < 0.05$). After LPS stimulation, the endotoxin (ET), diamine oxidase (DAO), and D-lactic acid (D-LA) levels in the BC group were lower than those in the CON group ($P < 0.05$). The IgY, IgA, and IgM contents in the BC group and the IgY and IgM contents in the LA group were higher than those in the CON group ($P < 0.05$). The pro-inflammatory factor and interferon-β (IFN-β) contents ($P < 0.05$) decreased, and the anti-inflammatory factor content in the serum ($P < 0.05$) increased in the BC and LA groups. Compared with the CON and LA treatments, the BC treatment increased the concentrations of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT), and decreased that of malondialdehyde (MDA) ($P < 0.05$). In contrast with the CON treatment, the BC and LA treatments increased the abundance of *Ruminococcaceae* and reduced that of *Desulfovibrio* ($P < 0.05$). Moreover, BC increased the abundance of beneficial bacteria. Overall, supplementation with *B. coagulans* and *L. plantarum* promoted the growth of broilers, improved their immunity and antioxidant capacity, and alleviated the LPS-stimulated inflammatory response by regulating the intestinal flora.

Keywords: probiotics, lipopolysaccharide, immunity response, caecal microbiota, broiler

INTRODUCTION

High human demand for poultry meat has led to intensive production, and intensive systems are particularly susceptible to production diseases such as oxidative stress, diarrhea, and enteritis (1, 2). In addition, inflammation associated with the innate immune responses is a common challenge for poultry farms and leads to significant economic losses (3). Such as GSH Px, SOD, and CAT are antioxidant enzymes and MDA is the product of lipid peroxidation. Recent a study found that excess cadmium (Cd) decreased SOD and CAT, increased MDA, and caused common carp liver oxidative stress (4). Another study demonstrated that the down-regulation of GSH-Px, SOD, and CAT, as well as the up-regulation of MDA took part in Cd-induced oxidative stress; meanwhile the up-regulation of TNF-α and the down-regulation of IL-10 took part in Cd-induced immunosuppression in common carp gills (5). Ammonia gas exposure increased IL-1β and IL-6, and caused immunotoxicity in broiler spleens (6). Probiotics and prebiotics initiate resistance to bacterial colonization and enhance the mucosal immunity of the host, thereby minimizing the burden of pathogens (7, 8). Previous studies have demonstrated the safety of *Bacillus coagulans* and its potential for food and medical applications (9, 10). Moreover, Benbara et al. (11) have demonstrated the safety of *Lactobacillus plantarum* (*Lactobacillus plantarum S27*) and its beneficial effects on the performance of chickens.

Lipopolysaccharide (LPS), the main component of the outer membrane of Gram-negative bacteria, leads to the production of inflammatory mediators through toll-like receptors and is an effective activator of the innate immune response. Therefore, it is widely used in the establishment of animal immune stress models (12, 13). Several studies have indicated that probiotics affected the growth performance and immune status of LPS-challenged animals. For example, *yeast* and its derivatives had improved LPS-induced changes in white the counts of blood cells, lymphocytes, and monocytes levels in broilers (14). Probiotics also reduced LPS-induced changes in the body weight of female mice 48 h post-treatment. Moreover, probiotic treatment prevented LPS-induced increases in pro- and anti-inflammatory (IL-1β, TNF-α, IL-6) peripheral cytokines at 8 h following LPS treatment, reduced the mRNA expression of central cytokine in the hypothalamus, hippocampus, and prefrontal cortex (PFC), and prevented LPS-induced changes in the gut microbiota (15). Deng et al. (16) reported that the administration of probiotic strains *Bacillus licheniformis* or *Bacillus subtilis* improved intestinal function, ameliorated the inflammation response, and modulated the microflora after LPS-induced acute inflammation in rats. However, the effects of probiotics on the intestinal microflora of broilers have been less studied in LPS-induced models. Thus, the aim of this study was to elucidate the beneficial effects of probiotics (*B. coagulans* and *L. plantarum*) on LPS-induced broilers by the determination of relevant indices.

MATERIALS AND METHODS

Birds' Management

A total of 360 one-day-old Cobb broilers (half male and half female) were purchased from a local commercial company and randomly divided into the following three groups: (1) birds fed basal diet (CON); (2) birds fed basal diet supplemented with 5×10^9 cfu/kg *Bacillus coagulans* (BC); (3) birds fed basal diet supplemented with 5×10^8 cfu/kg *Lactobacillus. plantarum* (LA). Each group consisted of 6 replicates with 20 broilers per replicate. The experimental period was 47 days, and during this period, birds could feed and drink freely. The initial brooding temperature was 35°C, which was gradually reduced to 26 ± 1°C by 2°C per week until the end of the trial. The death and feed consumption were recorded daily for 42 days. On days 43, 45, and 47, all broilers were intraperitoneally injected with 50 μg/kg of LPS (**Figure 1**). Samples were collected 3 h after the last stimulation. The basic diet composition and nutrition level followed NRC 1994 (**Table 1**). The strains (*Bacillus coagulans* and *Lactobacillus. Plantarum*) and lipopolysaccharide used in this trial are commercially available.

Sample Collection

At day 42, 6 birds per replicate were weighed. After 3 h of stimulation on day 47, euthanasia (Cervical dislocation was performed by an experienced operator) and sampling were conducted. Blood samples were collected from the vein under the wing and centrifuged at 3,000 × *g*, and the supernatant was separated and stored at −80°C. After euthanizing, Jejunal mucosa of broilers was removed and stored at −80°C for immune and antioxidant measuring. Further, the cecal contents were collected and immediately stored at −80°C for bacterial flora 16S ribosomal RNA (rRNA) sequencing. During the sampling process, the sampling tools and sample storage containers were sterile.

Performance Evaluation

At the beginning of experiment, every bird was weighed and recorded for individual error reduction. At the end of experiment, the average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F/G) during 1–21, 22–42, and 1–42 days of age were calculated according to the data recorded every day during the trial.

Immunoglobulin Content

Referring to the specific manual, the contents of immunoglobulin A (IgA), immunoglobulin M (IgM), and immunoglobulin Y (IgY) in the serum and jejunal mucosa were tested with ELISA kits (Cusabio, Wuhan, China).

Inflammatory Factor Level

Tumor necrosis factor-α (TNF-α), interferon-β (IFN-β), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin10 (IL-10) contents in the serum and jejunal mucosa were detected using specific ELISA kits (Nanjing Jiancheng, Nanjing, China) following the manufacturer's instructions.

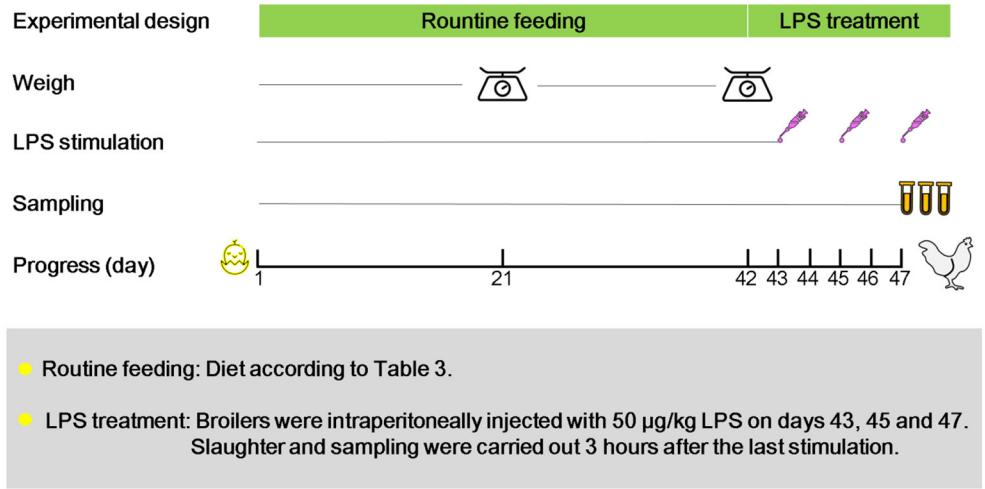

FIGURE 1 | Experimental design. The experiment was divided into 3 groups: birds fed basal diet (CON); birds fed basal diet supplemented with 5×10^9 cfu/kg *Bacillus coagulans* (BC); birds fed basal diet supplemented with 5×10^8 cfu/kg *Lactobacillus plantarum* (LA). All broilers were raised routinely in 1–42 days old, the death and feed intake were recorded daily. Weighing and statistics were conducted on the 21st and 42nd day.

Mucosa Injury Indices

The glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA), antioxidant capacity (AOC), and catalase (CAT) contents in the serum were measured for antioxidation determination. The concentration of endotoxin (ET), diamine oxidase (DAO), and D-lactic acid (D-LA) in the serum was tested for determination intestinal injury. These targets were measured using commercial kits purchased from Nanjing Jiancheng (Nanjing, China) following the manufacturer's instructions.

Cecal Microbial Sequencing

The process was entrusted to Shanghai Mega Biological Co., Ltd. The brief description of the process is as follows: the Illumina MiSeq platform (Illumina Inc., California, USA) was used for 300 paired-end sequencing in this study, wherein two samples of the same group were mixed into one biological sample. Then, the microbial genomic DNA of the cecum was extracted using a specific kit (Qiagen GmbH, Hilden, Germany). The primer used for the V3-V4 hypervariable region was 338F_806R (5′-ACTCCTACGGGAGGCACAG-3′; 5′-GGACTACHVGGGTWTCTAAT-3′). After PCR amplification and product purification, and PCR product quantification and homogenization, a PE library was constructed and Illumina (Illumina, San Diego, CA, USA) sequencing was performed. The Ultrafast sequence analysis (USEARCH) version 7.1 software was used for operational taxonomic unit (OTU) classification to classify the high-quality sequence valid tags obtained from the quality control according to the sequence similarity of 97%. Simpson and Shannon indices were used to analyze the alpha diversity in this study, and Student's *t*-test was used for to test the differences among the groups. Beta diversity analysis was conducted based on the OTU sequence similarity and community structure to compare the differences between different groups of samples, such as principal coordinates analysis (PCoA) and ternary phase diagrams. Unweighted_unifrac algorithm was adopted in PCoA. Microbial multivariate analysis was performed using the ANOVA algorithm to compare the significance of differences between groups.

TABLE 1 | Raw material composition and nutritional level of basic dietary (air-dry basis).

Items	1–21d	22–42d
Ingredients (%)		
Corn	61.80	65.60
Soybean meal	22.50	17.55
Extruded soybean	8.45	10.00
Import fish meal	3.00	3.00
CaHPO4	1.66	1.45
Limestone	1.10	1.00
NaCl	0.32	0.30
DL- methionine	0.16	0.10
L- lysine	0.01	
Premix*	1.00	1.00
Total	100.00	100.00
Nutrition levels		
Metabolizable energy (MJ/kg)	12.45	12.70
Crude protein	21.00	19.20
Lysine	1.15	0.95
Methionine	0.54	0.44
Calcium	0.99	0.89
Available phosphorus	0.53	0.49

**Premix is provided for feed per kg: VA 1,500 IU, VB_1 1.5 mg, VB_6 3.0 mg, VB_{12} 0.01 mg, VD_3 200 IU, VE 10 IU, VK 0.5 mg, Biotin 0.15 mg, D-pantothenic acid 10 mg, Folic acid 0.5 mg, Nicotinic acid 30 mg, Trace elements Cu, Fe, Zn, Mn, Se, I are 8, 80, 40, 60, 0.15, 0.18 mg respectively.*

TABLE 2 | Growth performance.

Item	CON	BC	LA	Pooled-SE	*P*-value
1-21 d					
ADFI (g)	52.97^{b}	55.97^{b}	61.43^{a}	1.057	<0.01
ADG (g)	32.92^{b}	36.14^{a}	36.31^{a}	0.548	0.008
F/G (g/g)	1.618^{a,b}	1.549^{b}	1.692^{a}	0.207	0.008
22–42 d					
ADFI (g)	106.3^{b}	121.3^{a}	126.7^{a}	3.223	0.017
ADG (g)	48.75^{b}	66.86^{a}	69.09^{a}	2.572	<0.01
F/G (g/g)	2.181^{a}	1.814^{b}	1.833^{b}	0.565	0.004
1–42d					
ADFI (g)	159.2^{b}	177.2^{a}	188.1^{a}	3.872	0.002
ADG (g)	81.66^{b}	103.0^{a}	105.4^{a}	2.439	<0.01
F/G (g/g)	1.950^{a}	1.721^{c}	1.785^{b}	0.001	<0.01

CON, broilers were not treated except for the base diet; BC, broilers were supplemented with Bacillus coaglulans; LA, broilers were supplemented with Lactobacillus plantarum; ADFI, the average daily feed intake; ADG, the everage daily gain; F/G, feed to gain ratio. The different alphabet in the same line represent significant difference. N = 6.

Statistical Analysis

One-way ANOVA and Duncan's test in IBM SPSS statistics (version 26.0, SPSS Inc., IIIinois, USA) were used for data analysis, and Graph Pad Prism 8.0 (Graph Pad Prism Inc., California, USA) was used for diagramming, wherein $P < 0.05$ meant significant difference and were marked with "*" in figures, and $P < 0.01$ meant significant difference and were marked with "**" in figures.

RESULTS

Effects of Probiotics on the Growth Performance of Broilers Induced by LPS

As shown in **Table 2**, compared with the CON and BC birds at 1–21 days of age, supplementation with *L. plantarum* significantly increased the ADFI ($P < 0.01$). Adding *B. coagulans* and *L. plantarum* evidently improved the broilers' ADG as compared to that of the CON broilers at 1–21 days of age ($P < 0.05$). The value of the F/G in the BC treatment was lower than that of the LA treatment ($P < 0.05$).

In contrast with that of the CON birds, dietary *B. coagulans* and *L. plantarum* markedly improved the ADFI and ADG of the BC and LA birds at 22–42 days of age ($P < 0.05$). Moreover, the F/G of the BC and LA birds was lower than that of the CON birds at 22–42 days of age ($P < 0.05$).

In contrast with that of the CON birds, the effects of *B. coagulans* and *L. plantarum* on ADFI and ADG at 1–42 days of age were consistent with those of the birds at 22–42 days of age ($P < 0.05$). The F/G of the BC and LA birds decreased significantly compared to that of the CON birds at 1–42 days of age, and the F/G of the BC birds was lower than that of the LA birds ($P < 0.05$).

Effects of Probiotics on Intestinal Injury in Broilers Induced by LPS

To investigate the effect of LPS attack on the intestinal injury of broilers, we detected the ET, DAO, and D-LA contents in the serum. As shown in **Figure 2**, the levels of ET and D-LA in the BC birds were lower than those in the CON and LA birds ($P < 0.05$, **Figures 2A,C**), and the level of DAO in the BC birds was lower than that in the CON birds ($P < 0.05$, **Figure 2B**).

Effects of Probiotics on Immunoglobulin Content in Broilers Induced by LPS

As shown in **Figure 3**, the level of serum IgY in the BC and LA birds was higher than that in the CON birds ($P < 0.05$, **Figure 3A**). Compared with that of the CON birds, the BC group had an increased serum IgA content ($P < 0.05$, **Figure 3B**). The level of serum IgM in the BC and LA treatments was higher than that of the CON treatment. Moreover, the IgM content in the CB group was higher than that of the LA group ($P < 0.05$, **Figure 3C**).

In the jejunal mucosa of the broilers, the IgY, IgA, and IgM contents in the CB treatment was significantly higher than that of the CON ($P < 0.05$, **Figures 4A–C**). In addition, compared with that of the LA broilers, the IgY and IgM levels in the BC group significantly increased ($P < 0.05$, **Figures 4A,C**). The concentration of IgY in the LA group was significantly higher than that of the CON group ($P < 0.05$, **Figure 4A**).

Effects of Probiotics on the Inflammatory Factor Level in the Broilers Induced by LPS

To evaluate the immune effect of *B. coagulans* and *L. plantarum* on broilers challenged by LPS, the inflammatory factors in the serum and jejunal mucosa were individually detected. In contrast with that in the CON treatment, contents of the serum proinflammatory factors (TNF-α, IL-1β, IL-6) in the BC and LA treatments were reduced evidently ($P < 0.05$, **Figures 5A,C,D**), and the TNF-α and IL-1β contents in the BC treatment were lower than those in the LA treatment ($P < 0.05$, **Figures 5A,C**). The serum IFN-β content in the BC treatment was lower than that in the LA treatment ($P < 0.05$, **Figure 5B**). The IL-10 content in the BC and LA treatments was higher than that in the CON treatment ($P < 0.05$, **Figure 5E**). Moreover, in contrast with that of the BC group, the concentration of IL-10 in the LA group was obviously increased ($P < 0.05$, **Figure 5E**).

In the jejunal mucosa, TNF-α and IL-1β levels of the CON group were higher than those of the BC and LA groups ($P < 0.05$, **Figures 6A,C**). Compared with that of the CON birds, the level of IFN-β was decreased significantly in the BC and LA birds ($P < 0.05$, **Figure 6B**). The level of IL-6 in the BC group was lower than that in the CON and LA groups, whereas the level of IL-1β was higher than that in the other two groups ($P < 0.05$, **Figures 6D,E**).

Effects of Probiotics on the Antioxidant Enzyme Activity in the Broilers Induced by LPS

In **Figure 7**, the antioxidant enzyme (GSH-Px, SOD, CAT) activities of the BC group were higher than those of the CON and LA groups in the broilers' serum ($P < 0.05$, **Figures 7A–C**), whereas the level of MDA in the BC group was lower than that in the CON and LA groups ($P < 0.05$, **Figure 7D**).

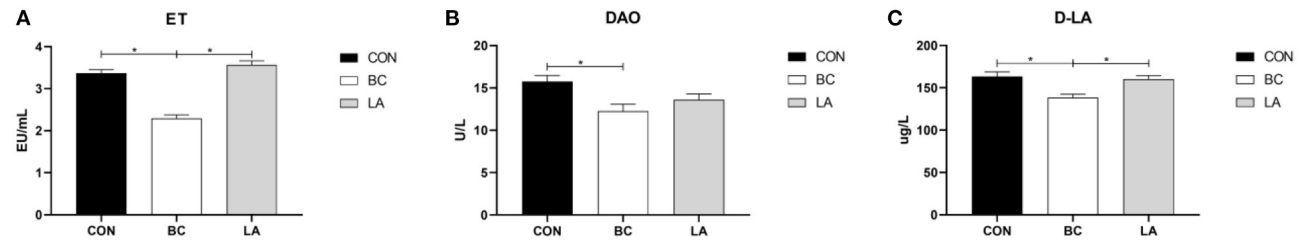

FIGURE 2 | (A–C) Effects of probiotics on intestinal injury induced by lipopolysaccharide in broilers. CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagu-lans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "*" means significantly difference. $N = 6$.

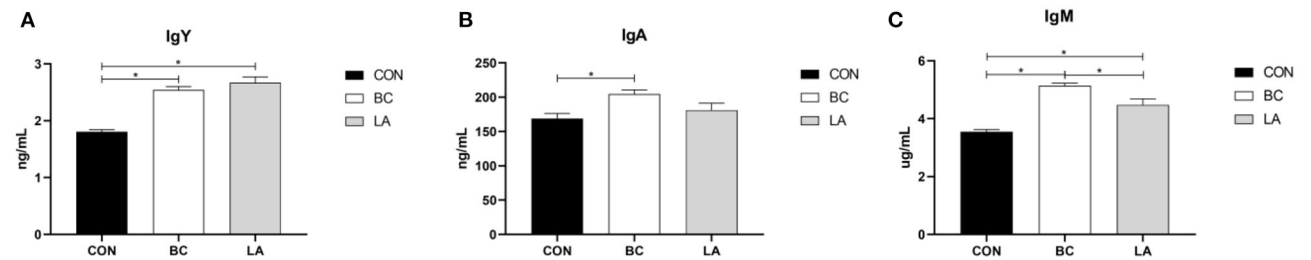

FIGURE 3 | (A–C) Effects of probiotics on serum immunoglubins induced by lipopolysaccharide in broilers. CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagulans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "*" means significantly difference. $N = 6$.

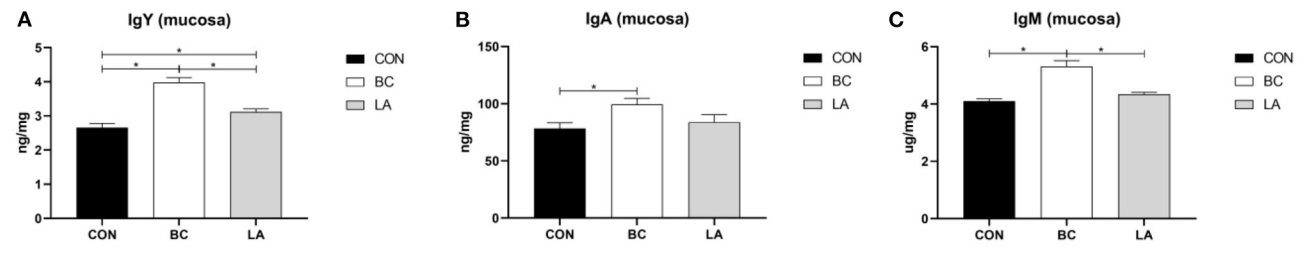

FIGURE 4 | (A–C) Effects of probiotics on jejunal mucosa immunoglubins induced by lipopolysaccharide in broilers. CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagulans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "*" means significantly difference. $N = 6$.

Effects of Probiotics on Gut Microbiota in the Broilers Induced by LPS

After the LPS challenge treatment, the OTUs in the BC and LA treatments were higher than that in the CON treatment (**Figure 8A**). Firmicutes and Bacteroidota were the dominant flora (phylum level), but the proportion of Firmicutes in the CON treatment (48.12%) was lower compared with that in the BC (57.83%) and LA treatments (55.15%) (**Figure 8B**). The Simpson and Shannon indices indicated that the richness and evenness of the bacterial community of the CON group were significantly different from those of the BC and LA groups (**Figures 8C,D**, $P < 0.05$). The distance of the PCoA analysis indicated that the species composition of the BC group was different from that of the CON and LA groups (**Figure 8E**). *Rikenellaceae* were more distributed in the LA and BC groups, and *Ruminococcaceae* were more enriched in the LA group. There was no difference in the distribution of the other levels of microorganisms among the three groups in the ternary phase diagram (**Figure 8F**).

In order to identify the species diversity, we analyzed the species abundance diversity at the genus level, and the intergroup differences were tested for some species (**Figures 9A–C**). The *Lachnoclostridium* abundance in the BC birds was evidently higher than that in the CON birds ($P < 0.05$, **Figure 9A**). Compared with the BC and LA treatments, the *Ruminococcaceae* abundance was significantly decreased in the Con treatment ($P < 0.01$, **Figure 9B**). Moreover, the abundance of *Desulfovibrio* in the CON treatment was significantly reduced compared with that in the BC and LA treatments ($P < 0.05$, **Figure 9C**).

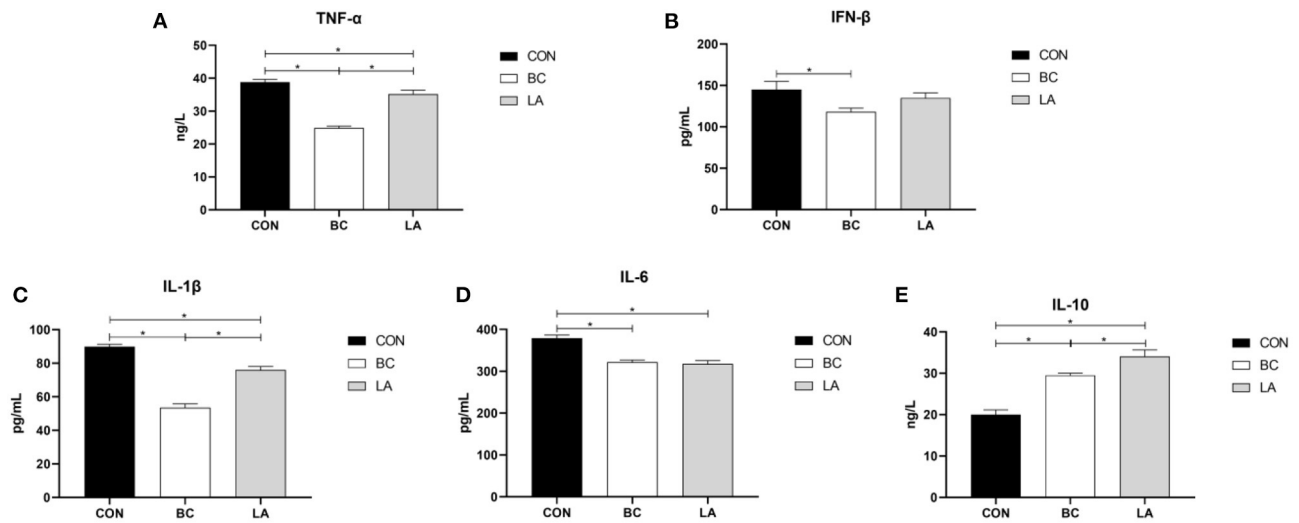

FIGURE 5 | (A–E) Effects of probiotics on serum inflammatory factors induced by lipopolysaccharide in broilers. CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagulans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "·" means significantly difference. $N = 6$.

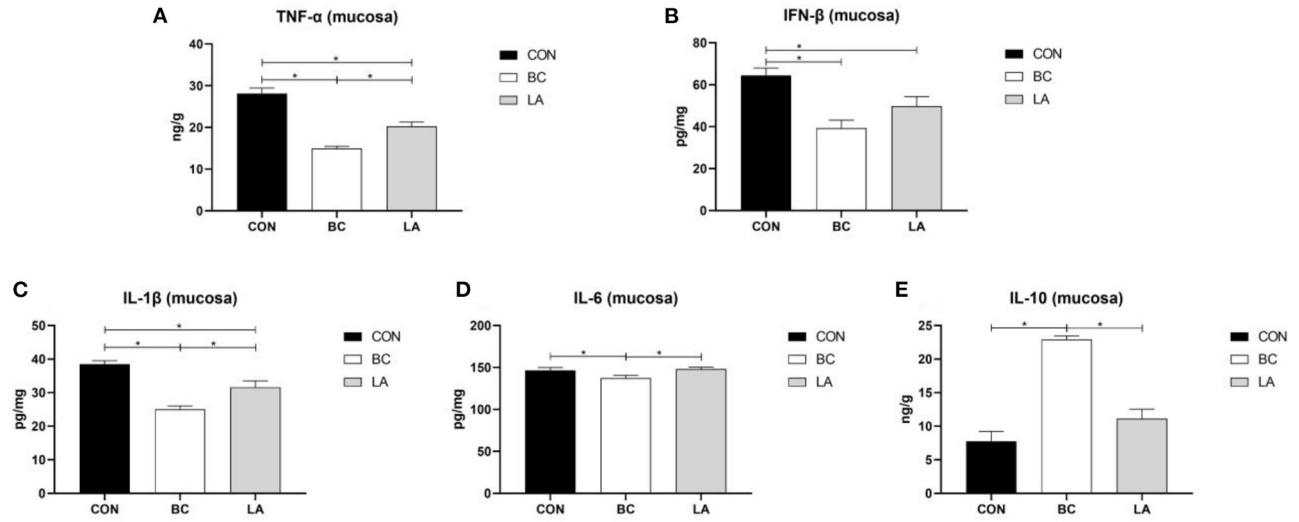

FIGURE 6 | (A–E) Effects of probiotics on jejunal mucosa inflammatory factors induced by lipopolysaccharide in broilers. CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagulans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "·" means significantly difference. $N = 6$.

DISCUSSION

Many studies have shown that probiotics used in animal husbandry can significantly improve the growth performance of livestock and poultry; for example, adding *L. plantarum B1* to the diet improved the weight gain and feed conversion ratio of broilers (17), while feeding *B. licheniformis* could significantly increase body weight and ADG (18). In our study, feeding *B. coagulans* and *L. plantarum* increased the ADG of the early broilers. Moreover, the addition of two probiotics evidently improved ADG and F/G in later- and full-term broilers. Repeated LPS stimulation reduced the body weight of the birds, while the supplementation of diets with *B. amyloliquefaciens* alleviated the LPS-induced reduction in the ADG of the broilers (19, 20).

Although the weight of the broilers after LPS stimulation were not recorded due to experimental errors, it could be inferred from the results of this study and those of previous studies that the addition of *B. coagulans* and *L. plantarum* could improve the growth performance of broilers (17, 18), which may benefit the resistance of broilers to anti-inflammatory consumption.

The D-LA mainly originates from bacterial production in the intestinal tract and is commonly used as a marker of bacterial infection. A higher concentration of intestinal bacteria implies that more D-LA is produced. The DAO activity is associated with

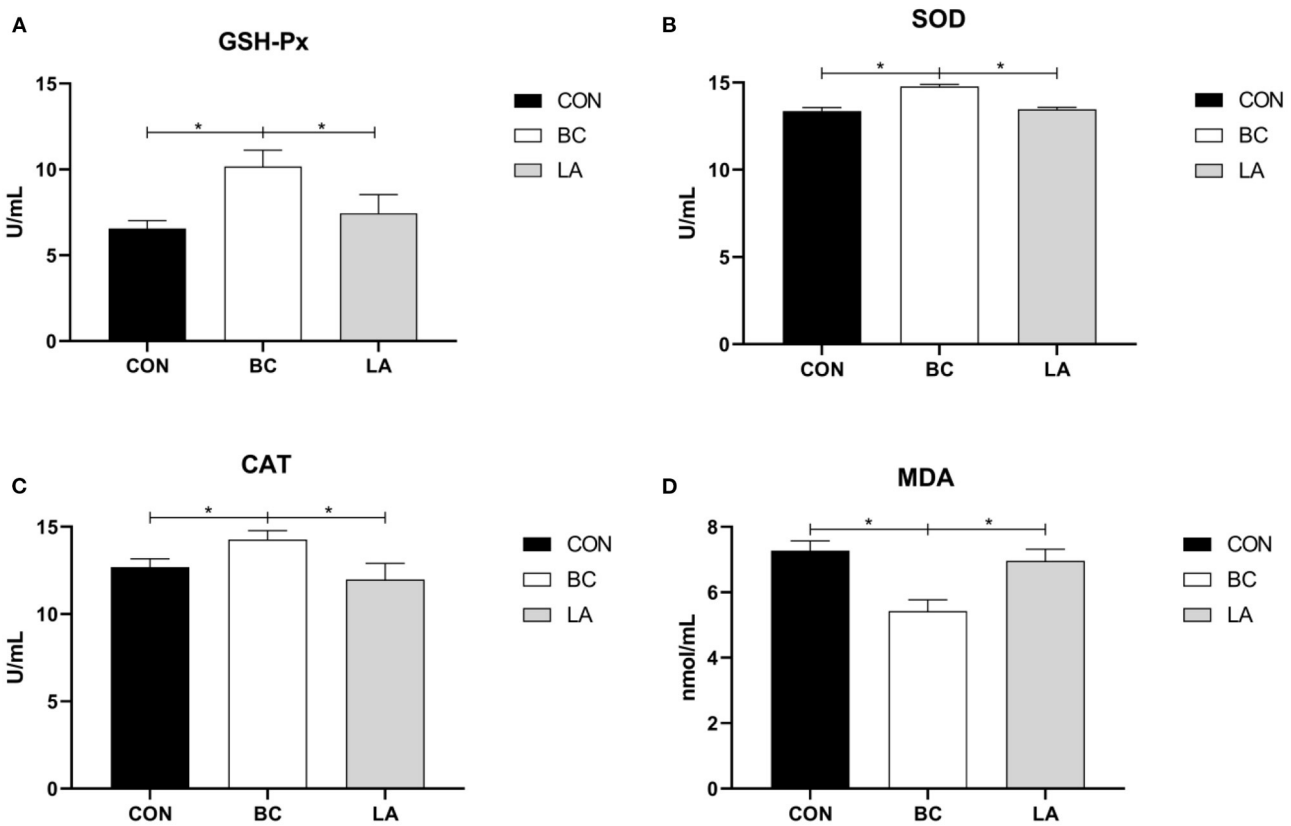

FIGURE 7 | (A–D) Effects of probiotics on serum antioxidant enzyme activity induced by lipopolysaccharide in broilers. CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagulans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "*" means significantly difference. $N = 6$.

the maturation and integrity of the small intestinal mucosa, and DAO activity is a useful biomarker for estimating the severity of intestinal mucosal disorders (21). It has been reported that D-LA level, ET level, DAO activity were increased in intestinal mucosa damage (22). Our study showed that *B. coagulans* evidently reduced the levels of ET, DAO, and D-LA in the serum of broilers challenged by LPS. This is similar to the effect of other probiotics, that is, the triple live agents of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus thermophilus* decreased the DAO activity, and D-LA and ET contents in rats undergoing cardiopulmonary bypass (22). Moreover, supplementation with *L. salivarius* significantly reduced the serum D-LA and DAO contents of LPS-attacked piglets (23). However, *L. plantarum* had no obvious effect in our experiment. The data suggested that dietary *B. coagulans* could reduce LPS-induced intestinal mucosal injury.

The beneficial effects of probiotics *in vivo* have been proven, for example, increased peripheral immunoglobin production stimulated IgA secretion (24). As the biggest producer of immunity *in vivo*, the intestinal tract produces a large amount of IgA by its activated mucosal B cells, which plays the role of the first-line immune defense (25). In this study, the contents of IgA, IgY, IgM were increased in the broilers' serum and jejunal mucosa after *B. coagulans* supplementation. These indicated that the immunity of broiler chickens fed with *B. coagulans* was enhanced by regulating the caecal microbiota, wherein *B. coagulans* helped to resist the infection stress. Supplementation of *L. plantarum* increased the levels of IgY and IgM in the serum and jejunal mucosa, suggesting that dietary *L. plantarum* had a positive effect on the immunity of broilers, but the expression of different immunoglobulins may vary. The contents of IgY and IgM (including serum and jejunal mucosa) in the BC birds were higher than that in the LA birds, which indicated that *B. coagulans* was more beneficial than *L. plantarum* in improving the immunity of broilers in our study.

When animals received repeated LPS stimulation, the peripheral blood immune organs such as spleen and thymus significantly proliferated, produced inflammation and pro-inflammatory cytokines (TNF-α) (26, 27). Mazkour et al. (28) demonstrated that the combination of *B. coagulans* and *B. subtilis* significantly reduced the level of the serum inflammatory cytokine TNF-α, which was induced by Salmonella. In addition, *B. coagulans* downregulated the expression of the pro-inflammatory cytokine IFN-γ, and it is believed that *B. coagulans* had potential to suppress intestinal inflammation in broilers challenged by *S. enteritidis* (29). In the present study, dietary *B. coagulans* significantly decreased the contents of the pro-inflammatory factors TNF-α, IL-1β, IL-6, and IFN-β in the serum and jejunum mucosa of the broilers. Supplementation

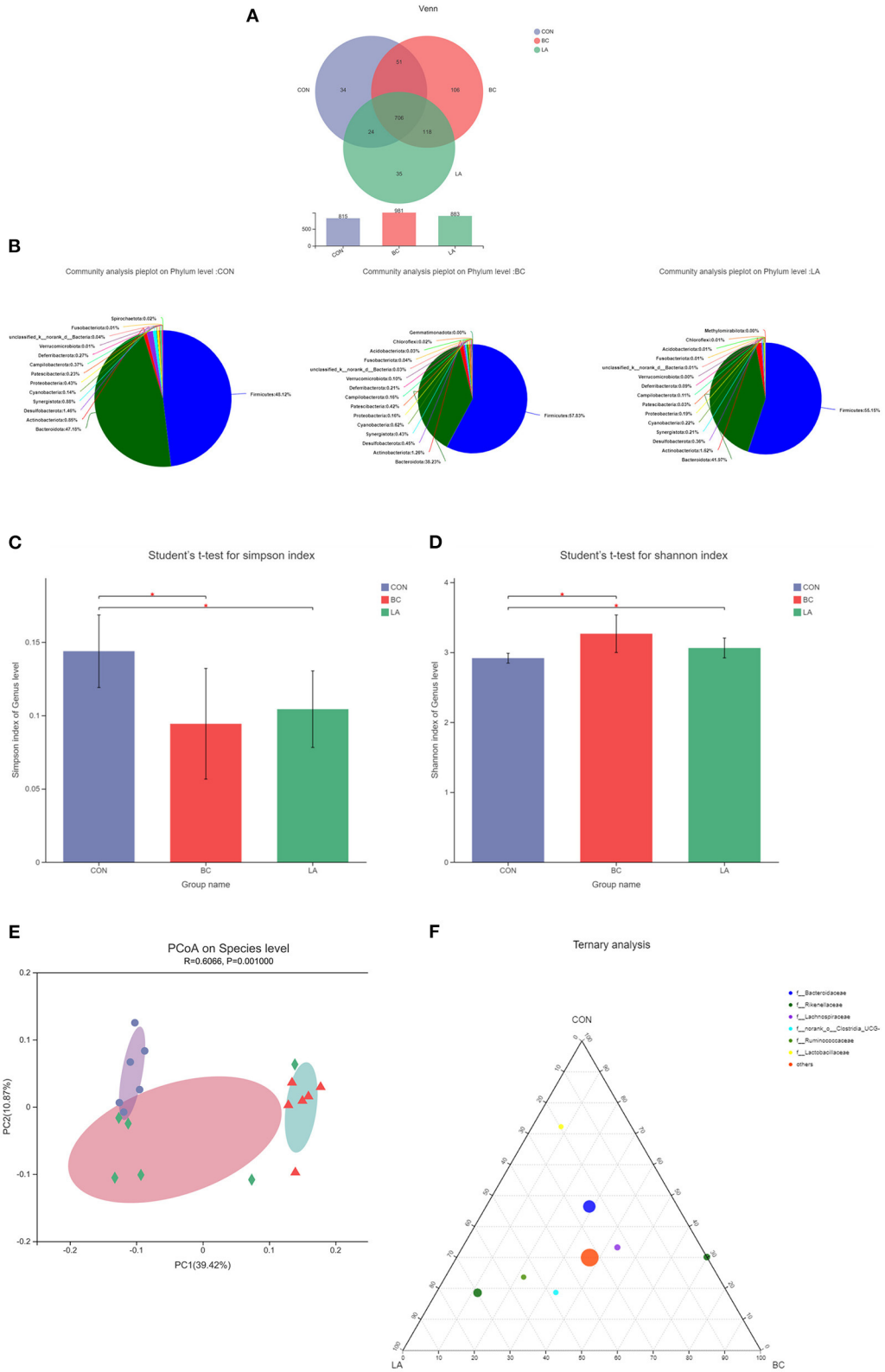

FIGURE 8 | Effects of probiotics on cecal microbiota composition of LPS-attacked broilers. **(A)** OUT, **(B)** community composition, **(C)** simpson index, **(D)** shonnon index, **(E)** PCoA analysis, **(F)** ternary analysis. CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagulans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "*" means significantly difference. $N = 6$.

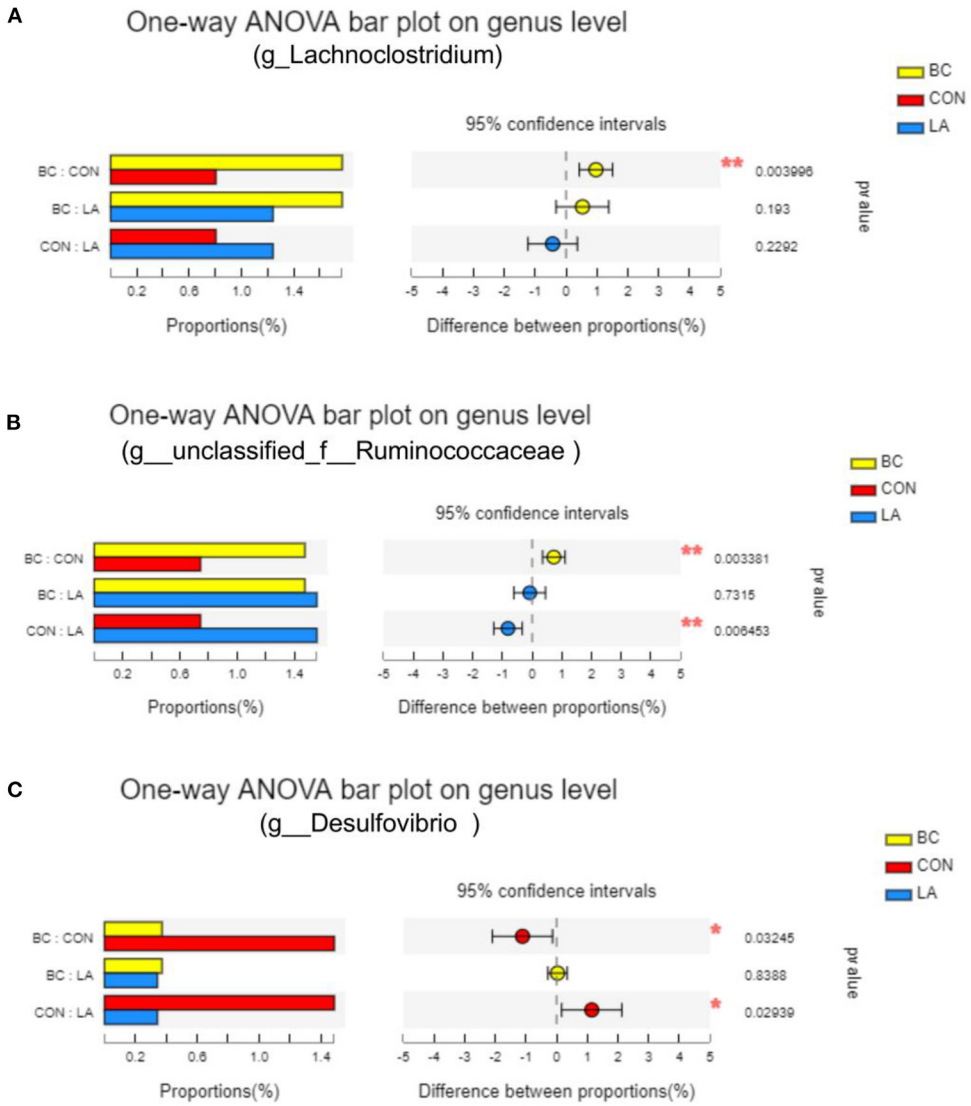

FIGURE 9 | Effects of probiotics on cecal species abundance of LPS-attacked broilers. **(A–C)** Were the test results of the significance of intergroup differences of *Lachnoclostridium*, *Ruminococcaceae* and *Desulfovibrio*, respectively, inspection methods: one-way ANOVA, correct ways: False discovery rate; CON, broilers were not treated except for the base diet; BC, broilers were supplemented with *Bacillus coagulans*; LA, broilers were supplemented with *Lactobacillus plantarum*; "*" means significantly difference. "·" means extremely distinct difference. $N = 6$.

with *L. plantarum* reduced the levels of the pro-inflammatory factors TNF-α and IL-1β in the serum and jejunal mucosa. Dietary supplementation of the two probiotics increased the contents of anti-inflammatory factors (IL-10) in the serum. Moreover, *B. coagulans* increased the IL-10 content of the broilers (in serum and jejunum mucosa) and was superior to *L. plantarum* in terms of immunogenicity. The results showed that adding probiotics into the broilers' diet could activate the immune system of the broiler, which could help them resist LPS stimulation.

Lipopolysaccharide stimulation not only easily led to intestinal inflammation, but also often caused acute liver injury (30, 31). Probiotics are an important factor affecting the oxidative status of the gut by exhibiting direct antioxidant properties and inducing the intrinsic organisms signaling antioxidant defense (32). Chorawala et al. (33) showed that probiotics could resist LPS-induced oxidative stress by reducing the MDA content and restoring the glutathione content in the colon. Besides, our previous study proved that *B. coagulans* elevated the serum CAT, SOD, and GSH-Px activity levels and decreased the serum MDA content in conventionally grown broilers (34). *B. coagulans* increased the levels of GSH-Px, SOD, and CAT, decreased the levels of MDA, and it performed better than *L. plantarum* in this study. These results were consistent with previous studies, indicating that *B. coagulans* could reduce LPS-induced oxidative stress injury, while the antioxidation of *L. plantarum* was not ideal.

At the phylum level, Bacteroidetes and Firmicutes were the dominant bacterial groups, which was consistent with previous studies in which Bacteroidetes and Firmicutes constituted most of the microbial communities in chickens at the phylum level, and these bacteria are known to play a role in energy production and metabolism (35–37). The ternary phase diagram helped to prove this point. The Simpson and Shannon indices of the probiotics treatment indicated that the colonization of probiotics had no adverse effect on the intestinal microflora of broilers and increased the community richness of intestinal flora.

In this study, the enrichment of the genus *Lachnoclostridium* was significantly increased by the addition of *B. coagulans*. Many species belonging to the genus are producers of butyrate (38), which is associated with the gut barrier, inflammation, and endotoxin levels (39). Combing with the above indices of mucosal injury, it could be concluded that *B. coagulans* attenuated inflammation, and resisted the intestinal barrier injury by increasing the *Lachnoclostridium* abundance in the LPS-challenged broilers' intestinal tract. The content of ET, DAO, D-LA in the LA group were not significantly different from those in the CON group, correspondingly. There was no significant difference in the *Lachnoclostridium* richness between the two groups (CON and LA). On the other hand, supplementation with *L. plantarum* improved some of immune and antioxidant properties of the broilers attacked by LPS. It's suggested that the way through which *L. plantarum* activated host immunity in this trial may not be through regulating the *Lachnoclostridium* abundance.

Bacillus. coagulans increased the abundance of *Ruminococcaceae* in LPS-challenged broilers. Interestingly, Ma et al. (40) reported that the increased abundance of *Ruminococcaceae* due to *B. subtilis* addition was associated with increased ADG and body weight. This could explain the significant improvement in the ADG and F/G of the BC and LA groups. Moreover, *Desulfovibrio* is an inflammatory-promoting taxon of bacteria associated with anxiety and depression (41). One recent study showed that probiotics (*L. rhamnosus LS-8* and *L. crustorum MN047*) manipulated the gut microbiota by decreasing the abundance of *Desulfovibrio* and increasing *Lactobacillus* and *Bifidobacterium*, thereby reducing the circulating LPS levels (42). In this study, both *B. coagulans* and *L. plantarum* supplementation evidently reduced the abundance of *Desulfovibrio*.

CONCLUSION

In conclusion, diets supplemented with *B. coagulans* and *L. plantarum* improved the growth performance of broilers under LPS stimulation and alleviated the mucosal injury, inflammatory response, and oxidative stress, which may be related to changes in the intestinal flora caused by the addition of probiotics. Findings from our study demonstrate the potential applications of *B. coagulans* and *L. plantarum* in poultry, specifically its beneficial effects in the performance of chickens, which is of great significance because of the increasing demand for poultry meat. The specific mechanism needs further in-depth study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was conducted according to the guidelines of the Animal Management Rules of the Ministry of Health of the People's Republic of China, and approved by the Ethics Committee of Zhejiang Agricultural and Forestry University, Hangzhou, China.

AUTHOR CONTRIBUTIONS

YY: conceptualization and writing—original draft preparation. QL: methodology and data curation. XZ: software and visualization. YX: validation and formal analysis. KJ: investigation. JL: resources and funding acquisition. GC: project administration. YY and GC: writing—review and editing. YX and KJ: supervision. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was supported by Zhejiang Leading Innovation and Entrepreneurship Team (No. 2020R01015); the National Natural Science Foundation of China (No. 32002195); Zhejiang Key Agricultural Enterprise Research Institute (No. 2021Y30004); and the Nature Science Foundation of Zhejiang Province (No. LQ20C170003).

ACKNOWLEDGMENTS

Thanks to the members of the Scientific Research Project of Zhejiang Education Department (No. YZ20200002) and some of the experimental equipment provided by the project.

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Conflict of Interest: XZ and JL was employed by Zhejiang Vegamax Biotechnology Co., Ltd.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 18 March 2022
doi: 10.3389/fvets.2022.862572

In ovo Feeding of L-Leucine Improves Antioxidative Capacity and Spleen Weight and Changes Amino Acid Concentrations in Broilers After Chronic Thermal Stress

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OPEN ACCESS

Edited by:
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Subrat Kumar Bhanja, Indian Council of Agricultural Research (ICAR), India

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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: *26 January 2022*
Accepted: *21 February 2022*
Published: *18 March 2022*

Citation:
Han G, Cui Y, Shen D, Li M, Ren Y, Bungo T, Chowdhury VS, Li Y and Li C (2022) In ovo Feeding of L-Leucine Improves Antioxidative Capacity and Spleen Weight and Changes Amino Acid Concentrations in Broilers After Chronic Thermal Stress. Front. Vet. Sci. 9:862572. doi: 10.3389/fvets.2022.862572

L-Leucine (L-Leu) was demonstrated to confer thermotolerance by *in ovo* feeding in broiler chicks and chickens in our previous studies. However, the L-Leu-mediated roles in recovering from the detrimental effects of heat stress in broilers are still unknown. This study aimed to investigate the effects of L-Leu *in ovo* feeding on the growth performance, relative weight of organs, serum metabolites and antioxidant parameters, and gene expression profiles in broiler chickens after chronic heat stress. Fertilized broiler eggs (Ross 308) were subjected to *in ovo* feeding of sterile water (0.5 mL/egg) or L-Leu (69 μmol/0.5 mL/egg) on embryonic day 7. After hatching, the male chicks were separated and used for the current study. All chickens were subjected to thermal stress exposure from 21 to 39 days of age and 1 week of recovery from 40 to 46 days of age. The results showed that *in ovo* feeding of L-Leu did not affect the body weight gain or relative weight of organs under chronic heat stress; however, the serum glutathione peroxidase was significantly increased and serum malondialdehyde was significantly decreased by L-Leu at 39 days of age. After 1 week of recovery, *in ovo* feeding of L-Leu significantly improved the relative spleen weight at 46 days of age. Subsequent RNA-seq analysis in the spleen showed that a total of 77 significant differentially expressed genes (DEGs) were identified, including 62 upregulated DEGs and 15 downregulated DEGs. Aspartic-type endopeptidase and peptidase activities were upregulated after recovery in the L-Leu group. The expression of genes related to B cell homeostatic proliferation and vestibular receptor cell differentiation, morphogenesis and development was downregulated in the L-Leu group. Moreover, the concentrations of serum catalase, total antioxidative capacity, isoleucine and ammonia were significantly decreased by L-Leu *in ovo* feeding after recovery. These results suggested that L-Leu *in ovo* feeding promoted the recovery of antioxidative status after chronic heat stress in broiler chickens.

Keywords: amino acid, *in ovo* feeding, heat stress, spleen, chicken

INTRODUCTION

Commercial broilers have the characteristics of fast growth and a high feed conversion rate after decades of intensive selection (1) and serve as one of the most widely consumed meats worldwide. However, chickens are very sensitive to heat stress (2, 3), as they lack sweat glands and have high metabolic rates (4). Heat stress is a critical problem that causes negative effects on the efficient and healthy production of broiler chickens (5). To mitigate the heat stress-induced negative impacts on poultry production, many studies have been conducted to propose suitable and efficient strategies to overcome this problem in broiler chickens (6, 7). The technology of *in ovo* administration was first proposed for vaccination against Marek's disease and well-developed with definition of deposition sites and injection days in 1980s (8). The technique of delivering various nutrients, supplements, immunostimulants, vaccines, and drugs via the *in ovo* route is gaining wide attention among researchers for boosting the production performance and immunity and for safeguarding the health of poultry.

In our previous studies, it was demonstrated that L-leucine (L-Leu) *in ovo* feeding afforded thermotolerance, reduced food intake and improved lipid metabolism under acute heat stress in broiler chicks (9–12). Some essential amino acids, including Leu and isoleucine (Ile), were significantly increased in the liver and decreased in the plasma by L-Leu *in ovo* administration under acute heat stress, which was considered a contributor to L-Leu-mediated thermotolerance (13). Interestingly, the daily body weight (BW) gain was also significantly higher in L-Leu *in ovo*-treated broilers than in control broilers under chronic heat stress (13). Chronic heat stress alters hypothalamic integrity and increases serum albumin, cholesterol and triglyceride (TG) levels (14, 15). After chronic heat stress, the total protein (TP), total globulin and glucose (GLU) concentrations were found to be elevated in Japanese quail (16). Partial recovery was also observed in TP, TG, blood pH, rectal temperature and respiration rate after chronic heat stress in slow-growing chicks (17). However, whether *in ovo* L-Leu-mediated thermotolerance affects the recovery process of broilers after chronic heat stress is still unclear. Thus, the first objective of the current study was to investigate the effects of L-Leu *in ovo* feeding on the growth, relative weight of organs, plasma metabolites and amino acid changes after chronic heat stress in broiler chickens.

Heat stress causes oxidative stress by producing reactive oxygen species, which damage enzyme functions and impair the immune response in broilers (5, 18). Chronic heat stress also reduces the relative weight of lymphoid organs (thymus, bursa, and spleen) in broiler chickens (19). The spleen, as the largest immune organ, plays an important role in regulating cellular and humoral immunity in poultry. Different responses were observed in the physiological and immunological parameters under chronic heat stress between different breeds of broilers, which indicated that heat tolerance was correlated with the immunological parameters under thermal stress (20). Therefore, the second aim of this study was to investigate the *in ovo* L-Leu-mediated effects on serum antioxidative parameters and the transcriptome profiles in the spleen after chronic heat stress in broiler chickens.

MATERIALS AND METHODS

Experimental Design

A total of 320 fertilized broiler eggs (Ross 308 strain; 48-week-old parent stock) were purchased from a local hatchery in Nanjing, China. Eggs were individually weighed, numbered in pencil, and then separated into two groups (control and L-Leu; $n = 160$/group) based on egg weight to form groups as uniform as possible. The average egg weights of the control and L-Leu groups were 67.3 ± 0.3 g and 66.8 ± 0.3 g, respectively. All eggs were placed into an incubator (Hongde 2112 type incubator, Hongde Comp., Shandong, China). The incubation temperature was 37.6°C with 60–70% relative humidity and autoturning every 1.5 h. On embryonic day (ED) 7, all eggs were candled, and 23 unfertilized eggs (12 eggs from the control group and 11 eggs from the L-Leu group) were detected and discarded properly. The remaining eggs were subjected to *in ovo* injection of L-Leu solution (69 μmol/0.5 mL sterile water/egg; Beijing Solarbio Science & Technology Co., Ltd., China) or sterile water (0.5 mL/egg) for the corresponding group, as described elsewhere (9). In brief, a small hole was made at the blunt end of the egg after sterilization. L-Leu solution or sterile water was injected to a depth of 25 mm, and the small holes were immediately sealed with a glue gun (Deli Group Co., Ltd., China). After injection, the eggs were returned to the incubator. The eggs were shifted to hatching trays at the end of ED 18 as a preparation of hatching.

After hatching, chicks were housed in groups in metal cages under a controlled thermoneutral temperature (CT) following management guidelines. The room temperature was controlled with air conditions. The chicks were provided with free access to feed with commercial standard diets. All birds were under a light cycle of 23 h light plus 1 h dark (23:00–00:00) from 1 to 7 days of age and 18 h light plus 6 h dark (22:00–04:00) from 8 to 46 days of age. At 2 days of age, male chicks were selected by feather identification, as it was confirmed that *in ovo* feeding of L-Leu afforded thermotolerance in male but not female broiler chicks (11, 13). At 15 days of age, 24 chicks with similar body weights were selected from each group and equally assigned to separate cages (length × width × height: 0.9 m × 0.6 m × 0.5 m) as 8 replicates with 3 male chicks each. From 21 to 39 days of age, the birds were subjected to natural summer heat waves. The room temperature and relative humidity were recorded by a digital thermometer and hygrometer (RC-4HC type, Elitech Technology Inc., Jiangsu, China), and the room temperature results are shown in **Figure 1**. Food and water were provided *ad libitum* throughout the experiment. The behavior of panting was confirmed daily under heat stress. At 39 days of age, 8 birds (one bird per replicate) were randomly selected from each group for sampling after completing the heat stress exposure. The chickens were properly anesthetized with dry ice (Jiangsu Yongtai Dry Ice Co., Ltd, Taixing, China) before being killed for sample collection. Body weight was measured after anesthetization. To collect serum, blood was immediately collected from the jugular

vein into ice-cold tubes and centrifuged at 10,000 × *g* at 4°C for 4 min. The heart, liver, spleen and bursa were collected and weighed by the same person without knowing the grouping information. The relative organ weight (%) of the heart, liver, spleen and bursa was expressed as the ratio of organ weight (g) to body weight (g). Spleen and liver were immediately collected following weighing and snap frozen using liquid nitrogen. All the collected samples were stored at −80°C until further analysis. After chronic heat stress, the birds were kept under CT conditions for recovery from 40 to 46 days of age. At 46 days of age, 8 birds (one bird per replicate) were randomly selected from each group, and the measurement, sample processing and storage were the same as those at 39 days of age.

This study was performed according to the Guidelines for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University [permit number SYXK (Su) 2011-0036].

RNA Isolation, Library Construction, and Sequencing

To study the transcriptome profiles in the spleen after 1 week recovery from chronic thermal stress, spleen samples of 46-day old broilers (n = 3/group) were applied transcriptome analysis. Total RNA was extracted using TRIzol (Invitrogen, CA, USA) following the manufacturer's instructions. The RNA purity and integrity were checked by a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and an RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Then, the concentration of RNA was determined with a Qubit® RNA Assay Kit in a Qubit®2.0 Flurometer (Life Technologies, CA, USA). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. PCR products were purified (AMPure XP system, Beckman Coulter, CA, USA), and library quality was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform, and 125/150 bp paired-end reads were generated.

Quality Control, Transcriptome Assembly and Bioinformatics Analyses

Raw data (raw reads) in fastq format were first processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads from the raw data. At the same time, the Q20, Q30, and GC contents of the clean data were calculated. All downstream analyses were based on clean data with high quality. Reference genome and gene model annotation files were downloaded from the genome website directly. The mapped reads of each sample were assembled by StringTie (v1.3.3b) in a reference-based approach. FeatureCounts v1.5.0-p3 was used to count the read numbers mapped to each gene.

Differential expression analysis was performed using the DESeq2 R package (1.16.1), based on the same RNA samples of spleen of 46-day old broilers. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. GO terms with corrected $P < 0.05$ were considered significantly enriched by differentially expressed genes. KEGG is a database resource for understanding the high-level functions and utilities of biological systems, such as cells, organisms and ecosystems, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used the clusterProfiler R package to test the statistical enrichment of differentially expressed genes in KEGG pathways.

Analysis of Serum Free Amino Acids, Metabolites and Antioxidative Parameters

The serum free amino acid concentrations were analyzed using a fully automatic amino acid analyzer (L-8080 type, Hitachi, Japan) according to the method described elsewhere (21). The serum was well mixed with a 5% sulfonic acid solution for deproteinization. After 30 min, the serum samples were centrifuged at 4°C and 20,000 × *g* for 20 min. The supernatant was collected and filtered using a 0.22-μm filter (Biosharp, Guangzhou Saiguo Biotech Co., Ltd., Guangzhou, China). The filtrate and standard solution were incorporated into the amino acid analyzer. The amino acid concentrations were expressed as pmol/μL in the serum. Since the system used here could not separate the L- and D-forms of the amino acids, only the names of the amino acids are used in the results of the determined amino acids.

Serum concentrations of GLU, TP, uric acid (UA), total cholesterol (T-CHO), non-esterified fatty acid (NEFA) and TG were measured by a biochemical automatic analyzer (Hitachi 7020, Hitachi, Tokyo, Japan) as described elsewhere (22).

The serum concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) activity and total antioxidant capacity (T-AOC) were analyzed by the corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer as described elsewhere (23).

Statistical Analysis

The body weight, organ indices, serum metabolites, antioxidative parameters and amino acid levels were statistically analyzed using a Student's t-test. A $P < 0.05$ was used to denote significant differences. Statistical analysis was conducted using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). The results are expressed as the mean ± standard error of the mean (SEM). The number of chickens used for statistical analysis in each group is shown in the figure legends and table notes.

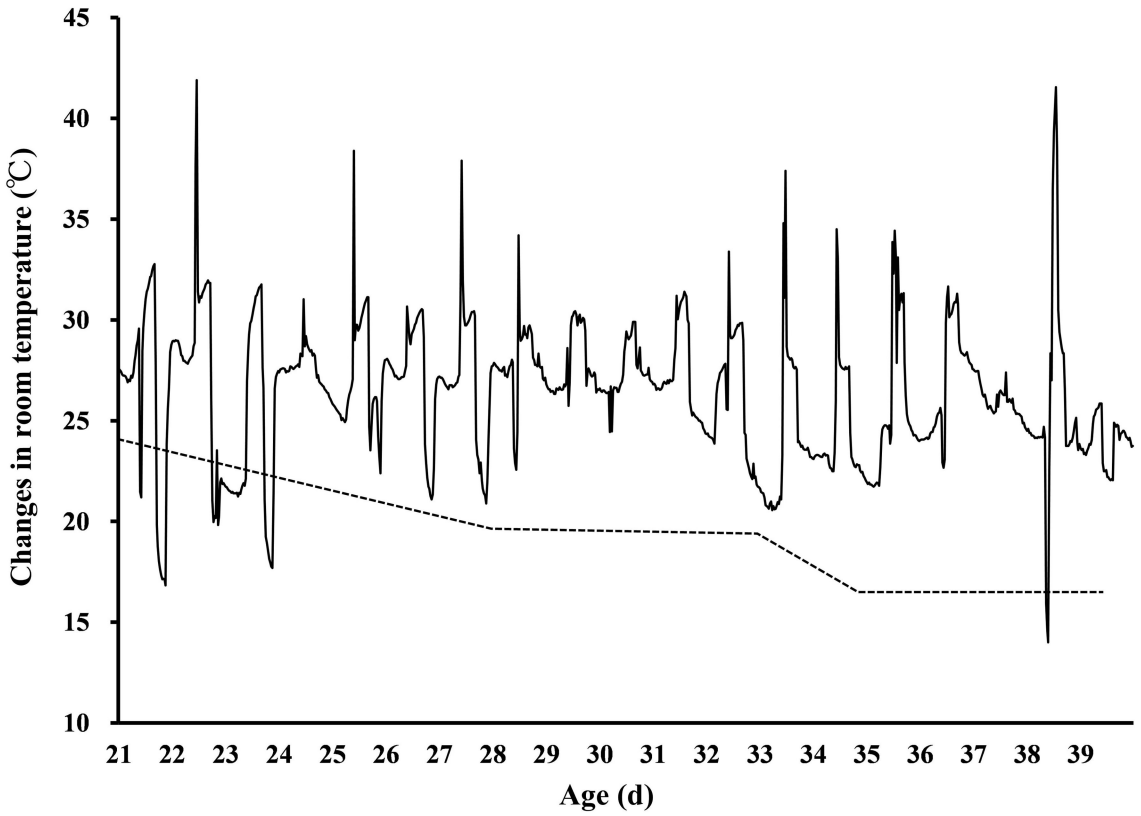

FIGURE 1 | Changes in the ambient temperature of the poultry room during chronic heat stress. The dotted line indicates the standard control temperature for broiler rearing.

RESULTS

Changes in Body Weight and Organ Indices After Chronic Heat Stress

After chronic heat stress, the body weight and organ indices were not affected by L-Leu treatment at 39 days of age. After 1 week of recovery, L-Leu *in ovo* feeding did not improve the body weight; similarly, the weight and relative weights of the liver, heart and bursa were also not affected by L-Leu treatment. Interestingly, the relative spleen weight was significantly ($P < 0.05$) increased by L-Leu *in ovo* feeding after chronic heat stress at 46 days of age (**Table 1**).

Transcriptomic Analysis Overview

Six cDNA libraries were constructed, and 131.76 and 143.48 million raw sequence reads were generated from the control and L-Leu libraries, respectively. After removing the low-quality reads, 122.58 and 132.21 million clean reads were retained with 94.64–95.27% Q30 bases and 46.28–47.49% GC content, respectively. The correlation of gene expression levels between samples is an important indicator to test the reliability of the experiment and the accuracy of the sample selection. The correlation analysis shows that the R^2 between the biological duplicates in this study was higher than 0.95, as shown in **Supplementary Figure 1**.

A total of 21,527 unigenes were detected in the current study. Among these unigenes, 77 were identified as differentially expressed genes (DEGs) with padj < 0.05 and Ilog2foldchangeI > 1, including 62 upregulated and 15 downregulated genes in the L-Leu group vs. the control group. This information is presented in a hierarchical clustering and a volcano plot (**Figures 2A,B**).

Functional Classification of Identified DEGs

As a significant difference was found in the relative spleen weight between the control and L-Leu groups after recovery from heat stress, enrichment GO terms were analyzed for the DEGs to evaluate the effects of L-Leu *in ovo* treatment on spleen recovery. After 1 week of recovery, the upregulated DEGs were associated with molecular function (MF), aspartic-type endopeptidase activity and aspartic-type peptidase activity. In contrast, downregulated DEGs were associated with biological process (BP), including B cell homeostatic proliferation, vestibular receptor cell differentiation, vestibular receptor cell morphogenesis and vestibular receptor cell development (**Figure 2C**). Subsequently, KEGG analysis was applied to reflect the transcriptional changes in metabolic pathways. A total of 23 pathways were enriched, including cytokine–cytokine receptor interaction and caffeine metabolism (*P*

TABLE 1 | The changes in body weight and (relative) organ weight after chronic heat stress (39-day old) and 1 week recovery (46-day old) in broiler chickens.

	BW	Liver	Heart	Spleen	Bursa
39-day old					
Control	1,693 ± 95	43.23 ± 1.71	10.03 ± 0.74	2.23 ± 0.29	4.18 ± 0.30
L-Leu	1,699 ± 55	44.84 ± 1.01	9.51 ± 0.39	2.61 ± 0.02	4.09 ± 0.39
P-value	NS	NS	NS	NS	NS
46-day old					
Control	2,646 ± 167	57.27 ± 3.55	12.63 ± 0.75	3.32 ± 0.20	6.14 ± 0.47
L-Leu	2,388 ± 135	53.78 ± 2.29	10.71 ± 0.60	4.55 ± 0.42	5.59 ± 0.63
P-value	NS	NS	NS	NS	NS
Relative weight (%)					
39-day old					
Control		2.61 ± 0.19	0.59 ± 0.04	0.14 ± 0.02	0.25 ± 0.02
L-Leu		2.65 ± 0.08	0.56 ± 0.01	0.16 ± 0.02	0.24 ± 0.03
P-value		NS	NS	NS	NS
46-day old					
Control		2.18 ± 0.09	0.48 ± 0.02	0.13 ± 0.01 ^{b}	0.24 ± 0.02
L-Leu		2.27 ± 0.09	0.45 ± 0.01	0.20 ± 0.03 ^{a}	0.23 ± 0.03
P-value		NS	NS	P<0.05	NS

The number of replicates used in each group was as n = 7–8. The unit for body weight and organ weight is g. Different superscripts in the same row indicate significant differences (P < 0.05) between treatments. Values are means ± SEM; L-Leu, L-leucine; BW, body weight; NS, not significant.

< 0.05). However, no significantly enriched pathway [false discovery rate (FDR) <0.05] was identified between the two groups (**Figure 2D**).

Changes in Serum Antioxidative Parameters, Free Amino Acids and Metabolites After Chronic Heat Stress

The serum concentrations of TP, GLU, UA, T-CHO, TG, and NEFA were not affected by L-Leu *in ovo* treatment after chronic heat stress or 1 week of recovery (**Supplementary Table 1**). However, L-Leu *in ovo* feeding caused a significant decrease in serum MDA and an increase in serum GPx at 39 days of age (**Figures 3A,B**). After 1 week of recovery, serum CAT and T-AOC concentrations were significantly decreased by L-Leu treatment at 46 days of age (**Figures 3C,D**). Moreover, L-Leu *in ovo* feeding caused a significant reduction in serum Ile and ammonia concentrations and a decreasing trend in serum threonine ($P = 0.087$) at 46 days of age (**Figure 4**).

DISCUSSION

Chronic heat stress, cyclic or continuous, reduces food intake, increases food conversion ratios, and retards growth in broiler chickens (6). Recently, we reported that L-Leu *in ovo* feeding improved growth performance under chronic heat stress, especially during its cyclic period (13). However, body weight was not affected by L-Leu *in ovo* feeding after chronic heat stress in the current study. Our previous studies demonstrated that *in ovo* injection of L-Leu afforded thermotolerance in broiler chicks and chickens, as the body temperature increase was suppressed by L-Leu under acute heat stress (9, 11, 13). However, the difference in body temperature between control and L-Leu-treated birds narrowed after 3 h of thermal exposure and disappeared after 6 h under continued high ambient temperature in neonatal broiler chicks (*unpublished data*). The L-Leu-mediated improvement in body weight gain was also diminished under continuous chronic heat stress in broiler chickens from 30 to 44 days of age (13). A dietary supply of some antioxidant minerals was reported to decrease the food conversion ratio without improving growth under chronic heat stress (24). However, the final body mass can be significantly improved when supplied with a high dose of antioxidant minerals under chronic heat stress (25). Therefore, the current results indicated that L-Leu-mediated thermotolerance was limited for promoting growth under chronic heat stress, especially a continuous pattern of heat stress.

It was demonstrated that heat stress causes impairment of the immune system, and chronic heat stress is considered to increase heat-related maladies in poultry (26). The spleen is an important peripheral lymphoid tissue (27), and the relative weight of the spleen is reduced under chronic heat stress (28). In the current study, the relative spleen weight was not affected by L-Leu treatment after chronic heat exposure. It was expected that chronic heat stress would cause a reduction in the relative spleen weight in both groups. However, the relative spleen weight in the L-Leu group was significantly higher than that in the control group after 1 week of recovery in the current study. Supplementation with additives, including zinc, ascorbic acid and chromium, was reported to increase spleen weight and improve the immune status of broilers reared under heat stress (18, 29). The significantly increased relative spleen weight indicated that the heat stress-related

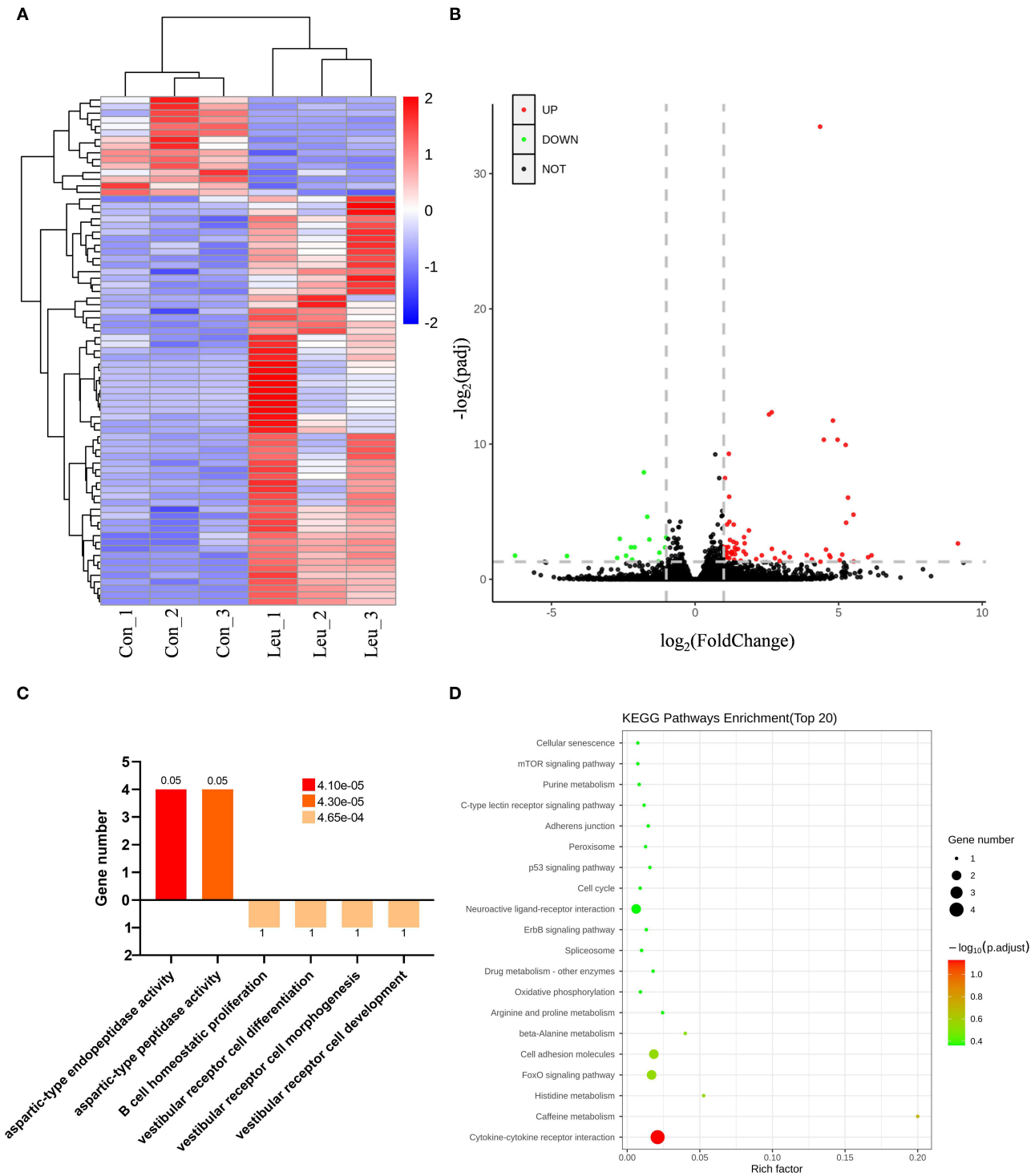

FIGURE 2 | Transcriptomic analysis of the spleens of the control (Con) and L-leucine (L-Leu) groups. **(A)** Heatmap showing the significant differentially expressed genes (DEGs); **(B)** Volcano plots showing the DEGs between the Con and L-Leu groups; **(C,D)** Functional classification of identified DEGs by GO enrichment analysis **(C)** and KEGG enrichment analysis **(D)** between the Con and L-Leu groups.

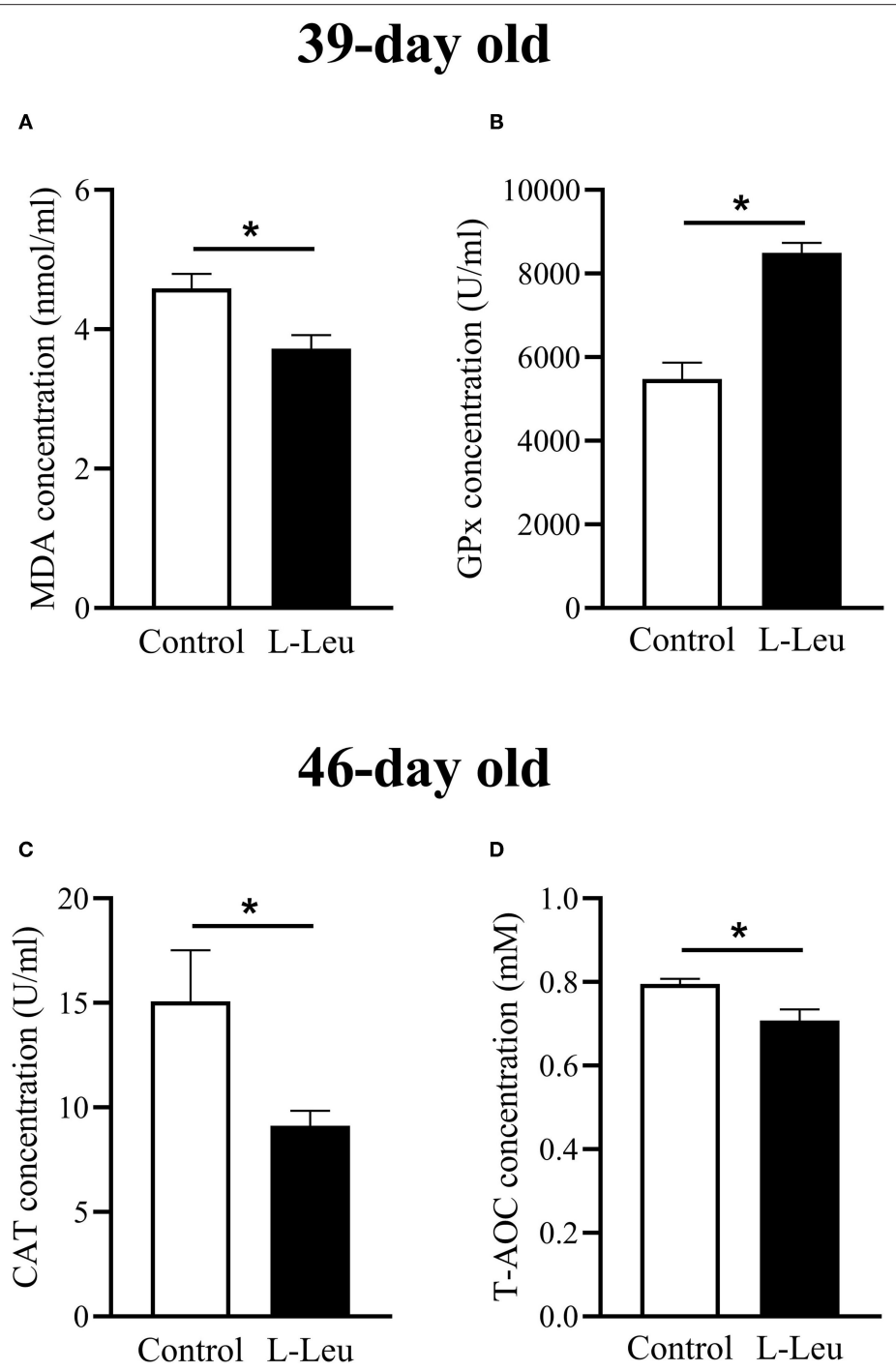

FIGURE 3 | Changes in serum antioxidative parameters in the control and L-leucine (L-Leu) groups. **(A,B)** Serum concentrations of malondialdehyde (MDA) and glutathione peroxidase (GPx) after chronic heat stress at 39 days of age; **(C,D)** serum concentrations of catalase (CAT) activity and total antioxidant capacity (T-AOC) after 1 week of recovery at 46 days of age. The number of chickens in each group was $n = 6$. $^*P < 0.05$.

depression in immunocompetence was reduced by L-Leu *in ovo* treatment in the current study. Combined with the results of body weight changes, it could be suggested that L-Leu-mediated thermotolerance was not enough to support immunity during chronic heat stress, at least for supporting peripheral lymphoid tissues, in broiler chickens. However, the recovery of immune function could be expected to be promoted by L-Leu *in ovo* feeding after ending the heat exposure. Future studies will clarify

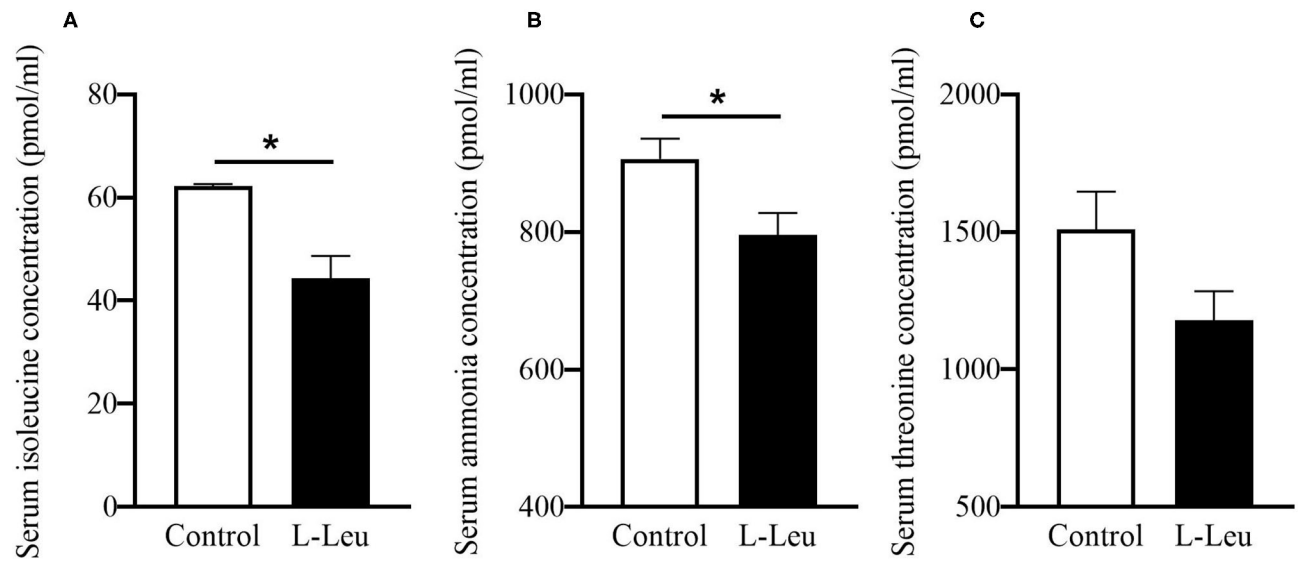

FIGURE 4 | Effects of L-leucine (L-Leu) *in ovo* feeding on serum isoleucine **(A)**, ammonia **(B)**, and threonine **(C)** in male broiler chickens after 1 week of recovery at 46 days of age. The number of chickens in each group was $n = 6$. $^*P < 0.05$.

this matter with an investigation of immune parameters in serum and spleen tissue under chronic heat stress.

To determine the possible mechanism for spleen recovery after chronic heat stress, splenic transcriptome profiles were conducted in this study. Overall, the aspartic-type endopeptidase and peptidase activities were enhanced in the L-Leu *in ovo* group. Endopeptidase and peptidase are known to break peptide bonds between amino acids and catalyze the hydrolysis of a peptide bond. Previous studies found that amino acid metabolism was modified by L-Leu *in ovo* treatment under acute or chronic heat stress (10, 13). B cell homeostatic proliferation and vestibular receptor cell development in the spleen were downregulated by L-Leu treatment, which might be feedback to the improved splenic cell proliferation during recovery. In birds, B cells mature in the bursa and emigrate to the spleen after hatching (30). However, the bursa weight was not affected in the current study. Moreover, serum immunoglobulin A and G were also not affected by L-Leu treatment after recovery (*data not shown*). In the current study, quantitative real-time PCR was not conducted to confirm the DEGs, and future studies will clarify the mechanisms of changing splenic weight after chronic heat stress.

Thermal stress causes an increase in body temperature and accelerates metabolism (31). The energy requirement for maintain homeostasis is increased, which stimulates glucose metabolism and suppresses lipid metabolism under heat stress (2, 32). Previous study supported that L-Leu *in ovo* feeding causes to activate lipid metabolism and affords thermotolerance in broiler chicks under acute heat stress (11). However, the serum metabolites, includes glucose, TG, UA, etc., were not affected by L-Leu *in ovo* feeding after chronic heat stress in this study. It is agreed with our previous results under chronic heat stress (13), as the strategies for coping with heat stress might also be different between short- and long-term patterns in broilers (33). Heat stress is a significant cause of economic losses in poultry production through a decrease in growth, and impacts on the physiological status of poultry by inducing oxidative stress in the body. Heat stress has been confirmed to induce oxidative stress *in vivo* or *in vitro* (34, 35). Reactive oxygen species (ROS) are byproducts of oxygen metabolism and are continuously produced in all aerobic organisms. Enhanced ROS disturbs mitochondrial homeostasis and induces lipid peroxidation. GPx is a ubiquitous intracellular enzyme that breaks down lipid hydroperoxide by utilizing lipid peroxide as a substrate. It has been reported that a decrease in GPx activity is associated with an imbalance between oxidative stress and antioxidants (36). The serum GPx concentration was significantly increased by L-Leu *in ovo* feeding under chronic heat stress, which suggested that heat stress-induced lipid peroxidation might be attenuated in L-Leu-treated broilers. MDA is the principal product of polyunsaturated fatty acid peroxidation, and heat stress results in higher mitochondrial and plasma levels of MDA (37). L-Leu *in ovo* administration has been shown to reduce serum MDA levels after chronic heat stress, which indicated that heat stress-induced oxidative damage is reduced in L-Leu-treated birds, as plasma MDA is considered a biomarker of oxidative stress (38).

Heat stress is associated with modified CAT activity, which is one of the key antioxidant enzymes. An *in vitro* study demonstrated that heat stress caused oxidative damage with a decreased CAT activity, and betaine treatment attenuated the heat stress-mediated oxidative damage with enhanced CAT activity (35). Moreover, serum T-AOC is considered an important parameter for assessing oxidative status (39), as T-AOC considers the cumulative effect of all antioxidants in blood

and body fluids. In the current study, the decreased CAT and T-AOC concentrations in L-Leu-treated birds indicated that the oxidative status was, at least, ameliorated in comparison with the control group after 1 week of recovery. Thus, it could suggest that L-Leu *in ovo* feeding promoted broiler chickens to recover after chronic heat exposure. Similarly, acute heat stress (35°C for 3 h) induced a significant increase in ROS and antioxidative enzymes (SOD, CAT, GPx), and the above parameters gradually approached preheat levels after 12 h of recovery (40, 41). Chronic heat stress causes heavy damage and takes a long time for recovery. It was reported that 3 days of recovery was not enough for quails to fully recover from a prior 9 days of heat stress exposure (16). However, it was not clear whether the L-Leu-treated broiler chickens fully recovered after 1 week of recovery. Future studies will clarify this matter with prolonged measurements compared with one non-stresses group.

Amino acids serve as building blocks of protein. Recent studies have demonstrated that some amino acids also play important roles in the regulation of body temperature and food intake. Previous studies investigated the amino acid profiles following L-Leu *in ovo* injection in embryos and heat-exposed chicks or chickens (10, 13, 21). This is the first study to clarify the amino acid changes in L-Leu-injected chickens after recovery from heat stress. L-Leu *in ovo* injection was shown to cause a significant increase in hepatic Ile and a decrease in plasma Ile after chronic heat stress (13). Dietary Ile supplementation was reported to improve the immune response and alleviate rotavirus infection in piglets (42). After 1 week of recovery, the decreased serum Ile in the L-Leu group indicated that the immune response was defused, as L-Leu-treated birds were expected to recover quickly compared with the control chickens. The lower serum ammonia in the L-Leu-treated group suggested that ammonia might be utilized to synthesize certain biomolecules (43). In previous reports, L-Leu *in ovo* administration was shown to decrease plasma ammonia during embryogenesis, which may cause a prenatal imprinting on ammonia-related metabolism (11). Interestingly, the serum Leu concentration was not affected by *in ovo* feeding of L-Leu after 1 week of recovery. Similar results showed that the blood Leu concentrations in embryos and heat-exposed chicks were also not affected by L-Leu feeding *in ovo* (10, 21). These results suggested that L-Leu is a trigger rather than a long-term regulator of L-Leu-mediated thermotolerance, as our trial experiments showed that central or oral administration of L-Leu had no effects on body temperature regulation in chicks.

CONCLUSIONS

In summary, L-Leu *in ovo* feeding reduced oxidative damage and improved antioxidative ability and relative spleen weight after chronic heat stress, which suggested that L-Leu promoted the recovery process in heat-exposed broiler chickens. The amino acid Ile was expected to be one of the contributors to the L-Leu-mediated benefit during recovery. Future studies will confirm the effects of dietary Ile on heat-exposed broilers.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Nanjing Agricultural University.

AUTHOR CONTRIBUTIONS

GH and CL designed this research. GH, YC, DS, and ML conducted the animal experiment. GH, YC, YR, and YL performed the sample analysis and statistical analysis. GH and VC wrote the manuscript. TB and CL reviewed and edited the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was partly supported by grants from the Jiangsu Agricultural Industry Technology System (grant no. JATS [2021] 480) and the National Nature Science Foundation of China (grant no. 32072781) to CL.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.862572/full#supplementary-material

Supplementary Figure 1 | The results of the correlation analysis among the samples.

Supplementary Table 1 | The changes in serum metabolites after chronic heat stress (39-day old) and 1 week recovery (46-day old) in broiler chickens.

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ORIGINAL RESEARCH
published: 25 April 2022
doi: 10.3389/fvets.2022.878107

Effect of Methionine Deficiency on the Growth Performance, Serum Amino Acids Concentrations, Gut Microbiota and Subsequent Laying Performance of Layer Chicks

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OPEN ACCESS

Edited by:
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Reviewed by:
Ahmed Kewan, Kobe University, Japan
Samiullah Khan, University of Adelaide, Australia

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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: *17 February 2022*
Accepted: *31 March 2022*
Published: *25 April 2022*

Citation:
Liu Y, Wang D, Zhao L, Zhang J, Huang S and Ma Q (2022) Effect of Methionine Deficiency on the Growth Performance, Serum Amino Acids Concentrations, Gut Microbiota and Subsequent Laying Performance of Layer Chicks. Front. Vet. Sci. 9:878107. doi: 10.3389/fvets.2022.878107

This study was conducted to investigate the effect of methionine (Met) deficiency in the rearing period on the growth performance, amino acids metabolism, intestinal development and gut microbiome of egg-laying chicks and the continuous effects on the performance, egg quality, and serum amino acids metabolism of the subsequent development process. Three hundred sixty one-day-old chicks were randomly divided into two groups and fed on a basal diet (NC group, Met 0.46%) and Met deficiency diet (Met- group, Met 0.27%). Each group included six replicates with 30 chicks per replicate. The trial lasted 6 weeks (0–6 weeks), both groups were fed the same basal diet which met the needs of Met during the observation period (7–24 weeks). Results showed that Met deficiency significantly decreased ($P < 0.05$) body weight (BW), average daily weight gain (ADG), average daily feed intake (ADFI) and tibia length (TL) compared to the NC group during the trial period (0–6 weeks). Also, Met deficiency dramatically increased ($P < 0.05$) feed conversion ratio (FCR) during the trial and observation period (7–24 weeks). In addition, during the observation period, the BW and ADG were decreased ($P < 0.05$) in the Met- group. Moreover, Met- group decreased ($P < 0.05$) villi height and villi height/crypt depth ratio in jejunum at 6th weeks. In addition, the concentrations of serum main free amino acids (FAA) in the Met- group were significantly increased ($P < 0.05$) at 6th weeks, while were decreased at 16th weeks. Based on the α-diversity and PCoA analysis in β-diversity, there were no significant differences in the cecal microbial composition between NC and Met- groups. However, the LEfSe analysis revealed that differential genera were enriched in the NC or Met- groups. The Haugh unit, shell thickness and egg production in the Met- group were significantly lower ($P < 0.05$) than in the NC group. In conclusion, these results revealed that dietary supplementation of appropriate Met could substantially improve the growth performance, host amino acid metabolism and intestinal development and continuously improve the laying performance and thus boost the health of growing hens.

Keywords: methionine deficiency, egg-laying chicks, growth performance, amino acid, intestinal development

INTRODUCTION

The importance of Methionine (Met) is indicated by its significant functions in protein synthesis, methylation reaction of DNA, and as a precursor in the synthesis of cysteine, glutathione, and taurine (1–3). Met is an essential amino acid that can't be synthesized by animals, particularly in poultry. In layers, Met is classified as the first limiting amino acid. Dietary Met levels directly affect production performance and egg quality (4, 5). Met is essential for the growth, production, development of feathers and immune responses enhancement of poultry (6–8). For laying hens, an optimal dose of Met supplementation in diets can improve the efficiency of protein utilization and affect the egg weight, albumen and yolk ratio (9). However, excessive supplementation with Met impaired growth and had no significant beneficial effect on laying performance (10, 11). Previous studies showed that Met deficiency in poultry reduced growth, feed intake (FI), body weight gain (BWG), egg size, production and increased feed conversion ratio (FCR) for layers and breeders (12–14). Additionally, low methionine diets decrease laying performance and egg quality (15, 16). Moreover, a deficiency of Met in the diet of laying hens impairs the whole-body protein metabolism, significantly reducing protein synthesis (17). In addition, Met has been generally recognized as a critical molecule in gut microbial metabolism. Met deficiency also alters the gut microbial structure (18). Met deficiency induces the small intestinal villus atrophy, decreases goblet cell number and diminishes small intestinal redox capacity affecting intestinal development (19). The small intestine is one of the most important organs responsible for the digestion and absorption of nutrients required for growth and development; meanwhile, as the primary media of feed digestion and absorption, the activities of digestive enzymes are inextricably linked with the digestion and utilization of nutrients (20).

The rearing period is critical for the growth and development of poultry. During the rearing period, the nutritional requirements for layer chicks should be ensured so that the chicks can grow rapidly and healthy and provide a solid foundation for later growth, development, and production. However, no research has been conducted on the investigation of methionine deficiency in the serum amino acids concentration, gut microbiota, and intestinal development of egg-laying chicks. Meanwhile, the impact of Met deficiency during the rearing period on the subsequent development of egg-laying chicks has not been reported yet. Therefore, the objective of the present experiment was to determine the effects of Met deficiency in the rearing period diets on growth performance, intestinal development, gut microbiota, the content of serum free amino acids of egg-laying chicks and evaluate its continuous impacts on the subsequent development index and production performance.

MATERIALS AND METHODS

Birds, Diets and Management

All experimental procedures of the present study were permitted by the China Agricultural University Animal Care and Use Committee (AW13301202-1-11, Beijing, China).

A total of 360 one-day-old Peking Pink strain egg-laying chicks (Yukou Poultry Co., Ltd. of Beijing, China) were randomly divided into two groups. The dietary groups were as follows: NC group (NC), fed basal diet, Met 0.46%; Met- group (Met-), fed Met deficiency diet, Met- 0.26%. Each group included six replicates with 30 chicks per replicate. The trial lasted 6 weeks. Subsequently, the same diet was fed to carry out a continuous observation of the test chicks until the laying period (7–24 weeks). Birds were housed in stainless steel cages (W 65 × L 62 × H 37 cm). Room temperature and artificial light were controlled, and the vaccination programs were implemented according to the management guide of Peking Pink hens. Birds had *ad libitum* access to water throughout the experimental period. Chicks were fed *ad libitum* commercial corn- and soybean meal-based diet containing the nutritional requirements for layer chicks (National Research Council, 1994) (**Table 1**).

Growth Performance

The body weight and feed intake of chicks in each replicate were measured weekly. Average daily weight gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**) were calculated at different stages (0–2 weeks, 3–4 weeks, 5–6 weeks, 0–6 weeks, 7–16 weeks, and 17–24 weeks). The ADFI and FCR were corrected for mortality. Body weight (**BW**) and tibial length (**TL**) were observed until the end of the 24th week. During the feeding trial, the data was measured every two weeks to observe the influence of the rearing period on growth performance.

Laying Performance and Egg Quality

During the observation period, eggs, broken and shell-less eggs, were collected daily; eggs were weighed and recorded daily by replicate. The see egg age, which represents the age of the first egg appearance, was recorded. Egg production and daily egg mass were calculated on a per replicate basis. The age of the first production day was calculated according to the period when the daily egg production rate reached 50% by replicate.

At weeks 21 and 24, egg quality parameters were measured based on five eggs collected at random from each replicate. The eggshell strength was measured using the egg force reader (F0241, Orka Technology Ltd) and the digital egg tester (ESTG-01, Orka Technology Ltd) was used to measure the eggshell thickness. Haugh unit and yolks color were measured using a multifunctional egg quality tester (EA-01, Orka Technology Ltd). The eggshell was weighed, yolks were separated using a separator. They were weighed on digital technical balance with a precision of ± 0.1 g to determine the relative albumen and yolk proportion.

Blood Collection and Analysis

At the end of the 6th, 16th, and 24th weeks of the experiment, two birds were selected randomly from each replicate (12 birds per group) to collect 6 mL of blood from the wing vein. The blood was centrifuged at 3,000 rpm/min for 15 min and stored at −20 °C. After that, 0.5 mL serum was collected by pipette into tubes, added 1.5 mL 4% sulfosalicylic acid, shook at high speed for 1 min, put the samples in an ice-bath for 20 min; then added 175 μL LiOH solution (2 mol/L). After thoroughly mixed, samples were centrifuged at 12,000 rpm/min for 40 min;

TABLE 1 | Ingredients and nutrient content of the diets (% DM).

Ingredients (%)	0–6 weeks		7–12 weeks	13–16 weeks	17–20 weeks	21–24 weeks
	NC	Met-				
Corn	68.20	68.20	67.18	67.26	66.47	65.05
Dehulled soybean meal	25.70	25.70	23.50	19.00	22.80	24.20
Zeolite powder	0.00	0.19	–	–	–	-
Wheat bran	–	–	5.60	9.50	3.60	0.00
Calcium monohydrogen phosphate	2.00	2.00	1.50	1.80	1.70	1.70
Limestone	1.30	1.30	1.20	1.40	4.60	8.20
NaCl (salt)	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin premix[a]	0.04	0.04	0.04	0.03	0.03	0.03
Mineral premix[b]	0.30	0.30	0.30	0.30	0.30	0.30
50%Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine-HCl (78.5%)	0.15	0.15	0.07	0.05	0.00	0.00
DL-Methionine	0.19	0.00	0.16	0.16	0.10	0.12
Threonine	0.07	0.07	–	–	–	-
Tryptophan	0.02	0.02	–	–	–	-
Isoleucine	0.08	0.08	–	–	–	-
Alanine	1.55	1.55	–	–	–	-
Total	100	100	100	100	100	100
Nutrient (%)[c]						
Crude protein	19.06	19.06	16.51	15.05	15.52	16.04
AME (MJ/kg)	2.85	2.85	2.80	2.74	2.70	2.69
Ca	1.02	1.02	0.89	1.01	2.20	3.60
Total P	0.67	0.67	0.64	0.69	0.66	0.65
Non-phytate phosphorus	0.45	0.45	0.39	0.44	0.39	0.39
Methionine	0.46	0.27	0.40	0.40	0.35	0.38
TSAA	0.74	0.55	0.66	0.67	0.62	0.65
Lys	1.01	1.01	0.85	0.75	0.75	0.78
Trp	0.22	0.22	0.17	0.17	0.15	0.16
Thr	0.72	0.72	0.66	0.63	0.57	0.59
Ile	0.76	0.76	–	–	–	-

[a]Vitamin premix supplied (per kg of diet): vitamin A, 11,700 IU(0–6 weeks); vitamin A, 8,000 IU;(7–24 weeks);vitamin D3, 3,600 IU; vitamin E, 21 IU; vitamin K3, 4.2 mg; vitamin B1, 3 mg; vitamin B2, 10.2 mg; folic acid, 0.9 mg; calcium pantothenate, 15 mg; niacin 45 mg; vitamin B6, 5.4 mg; vitamin B12, 24 μg; and biotin: 0.15 mg. 7–24 weeks: vitamin A, 8,000 IU; vitamin D3, 3,600 IU; vitamin E, 21 IU; vitamin K3, 4.2 mg; vitamin B1, 3 mg; vitamin B2, 10.2 mg; folic acid, 0.9 mg; calcium pantothenate, 15 mg; niacin 45 mg; vitamin B6, 5.4 mg; vitamin B12, 24 μg; and biotin: 0.15 mg. [b]Mineral premix provided (per kg of diet): 0–6 weeks: Cu, 6.8 mg; Fe, 66 mg; Zn, 83 mg; Mn, 80 mg; I, 1 mg. [c]The nutrient levels were calculated values.

the supernatant was used across the 0.22 μm membrane and adjusted to the appropriate concentration. The L-8,900 amino acid analyzer (Hitachi, Japan) was used to determine the content of 17 kinds of free amino acids in the serum.

Digestive Enzyme Activity Assay

At the end of the trial, two birds per replicate (12 birds per group) were randomly selected for euthanasia with cervical dislocation. The samples in duodenal chyme were collected and stored at −80 °C. The levels of amylase, lipase, chymotrypsin and trypsin activities in duodenum samples were detected with the corresponding kits provided by Nanjing Jiancheng Institute of Bioengineering following the manufacturer's instructions.

Small Intestinal Morphology

Duodenum, jejunum, and ileum segments were fixed in 4% paraformaldehyde (pH 7.2) solution for 24 h, dehydrated and embedded in paraffin. Each selected sample was sectioned into six μm thickness with a microtome. The sections of the intestine were stained with hematoxylin and eosin. Using an image processing system composed of CMOS (OLYMPUS, Japan) and its special software (Imaging Technology, USA), the computer obtains the villus height, crypt depth, and ratios. Villus height and crypt depth were measured at × 40 magnification using a microscope (BA400Digital, Mike Audi Industrial Group Co., Ltd. Xiamen, China). The images were evaluated by using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axiocam HR digital camera.

DNA Extraction, Amplification, and Sequencing of Gut Microbiota

Cecal samples were collected and stored at −80°C. Total DNA of the cecum contents was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To assess the quantity and purity of the DNA, the extracted DNA was determined by a NanoDrop 2000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The V3-V4 region of the 16S rRNA gene was amplified with primer pairs 338F (5′ACTCCTACGGGAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Paired-end reads were generated with Illumina MiSeq PE250 (Shanghai MajorBio Biopharma Technology Co., Ltd., China), and the reads were filtered out with default parameters.

Data Statistics and Analysis

Tabulated results were expressed as means with SEM. The comparison of NC group with Met- group values were performed with two-tailed Student's test for unpaired data. All analyses were performed with SPSS 21 software (SPSS, Inc., Chicago, IL, USA). Statistical significance was assigned at $P < 0.05$.

The raw paired-end reads were assembled into longer sequences, and the PANDAseq (version 2.9) was used to filter the quality and remove the low-quality reads. The high-quality sequences were clustered into operational taxonomic units (OTUs) based on a 97% sequence similarity using UPARSE (version 7.0) in QIIME (version 1.17) and using UCHIME to remove the chimeric sequences, and taxonomy was assigned to OTUs using the RDP classifier (21). UPARSE (version 7.0) was used to cluster the subsequent clean reads and annotated with the SILVA 16S rRNA gene database using the MOTHUR program (version v.1.30.1). Alpha-diversity (the Sob index, Chao index, Shannon index, and Ace index) was calculated based on the profiles of OTU by the MOTHUR program. Bar plots and heat maps were generated with the "vegan" package in R (version 3.3.1). A Venn diagram was generated to visualize the shared and unique OTUs among groups using the R package "Venn Diagram" based on the occurrence of OTUs across groups regardless of their relative abundance. principal coordinate analysis (PCoA) was performed based on the Bray-Curtis distance using QIIME (version 1.17). An analysis of similarities (ANOSIM) was performed to compare the similarity of bacterial communities between groups using the "vegan" package of R (version 3.3.1). The LDA effect size (LEfSe) analysis was performed to identify the bacterial taxa differentially enriched in different bacterial communities.

RESULTS

Effects of Met Deficiency on Growth Performance

As shown in **Table 2**, Met deficiency (only 6 weeks for laying chicks) significantly decreased the BW and TL on weeks 2, 4 and 6 ($P < 0.05$). Subsequently, during the observation period (from week seven to week 24), BW was significantly reduced ($P < 0.05$) at 16 weeks. Compared with the NC group, ADFI and ADG of Met- group were decreased on the trial and observation period ($P < 0.05$). On weeks of 7–16, ADFI of Met- group was significantly increased, but ADG of the Met- group was significantly decreased ($P < 0.05$). FCR was significantly higher in Met- group ($P < 0.05$) of the whole test period and the 7–16 weeks of the observation period.

Effects of Met Deficiency on Small Intestinal Digestive Enzyme Activity

Met deficiency had no significant effects ($P > 0.05$) on the activity of amylase, lipase, trypsin, and chymotrypsin (**Table 3**).

Effects of Met Deficiency on Laying Performance and Egg Quality

As shown in **Table 4**, Met deficiency significantly affected the laying performance of hens. For example, main parameters, such as egg production, average egg weight and egg mass in the Met- group were significantly lower ($P < 0.05$) than that in the NC group in 18–24 weeks. Also, the age of the first production day was significantly postponed ($P < 0.05$) in the Met- group. Met deficiency especially increased the unqualified egg rate ($P < 0.05$) in 18–24 weeks.

As shown in **Table 5**, the Haugh unit in the Met- group was significantly lower ($P < 0.05$) than that in the NC group at 21 weeks. Meanwhile, the eggshell percentage and thickness in the Met- group were significantly lower ($P < 0.05$) than that in the NC group in 24 weeks.

Effects of Met Deficiency on Small Intestinal Morphology

Chicks treated with Met deficiency diet significantly decreased the height of jejunal villi (**Figures 1A,B**) and the V/C value ($P < 0.05$) (**Figure 1D**) and significantly increased the depth of ileum crypts ($P < 0.05$) (**Figure 1C**).

Effects of Met Deficiency on Content of Serum Free Amino Acids

Met deficiency significantly affected the content of amino acids in the serum of egg-laying chicks. The content of Thr, Ser, Cys, Val, Ile, Leu, Pro, Asp, and Lys in the Met- group on the 6th week was higher ($P < 0.05$) than that in the NC group (**Figures 2A–D**). Moreover, the content of Ala in the NC group was higher ($P < 0.05$) than that in the Met- group on the 16th week (**Figure 2E**). However, there were no significant differences ($P > 0.05$) between the two groups in the content of serum free amino acids on the 24th week (**Figures 2I–L**).

Effects of Met Deficiency on the Cecal Microbiota

16S rDNA sequencing was performed to investigate how Met deficiency impacted the gut microbiota composition of egg-laying chicks. Based on the Venn diagram illustrating the overlap of bacterial OTUs, the NC group had a total of 2,395 OTUs, and the Met- group, had 3,108 OTUs. A total of 2,081 common

TABLE 2 | Effects of Met deficiency in rearing period diets on growth performance of egg-laying chicks.

Period	Group[1]				
	NC	Met-	Difference[c]	SEM[2]	*P value*
			BW (g)		
2 wk	121.02[a]	114.18[b]	−6.83	1.06	< 0.001
4 wk	250.01[a]	224.73[b]	−25.28	3.89	< 0.001
6 wk	419.45[a]	360.78[b]	−58.67	8.97	< 0.001
16 wk	1,209.75[a]	1,177.41[b]	−32.34	6.91	0.022
18 wk	1,302.05	1,271.41	−30.64	7.59	0.052
24 wk	1,533.05	1,524.89	−9.08	8.26	0.611
			TL (mm)		
2 wk	45.26[a]	44.53[b]	−0.72	0.16	0.013
4 wk	58.24[a]	56.68[b]	−1.56	0.30	0.003
6 wk	72.38[a]	69.12[b]	−3.26	0.57	< 0.001
16 wk	100.01	100.01	−0.01	0.30	0.994
18 wk	99.24	99.82	0.58	0.41	0.510
24 wk	99.77	99.99	0.23	0.59	0.858
			ADFI (g/bird/d)		
0–2 wk	11.80	11.87	0.07	0.05	0.506
3–4 wk	21.25[a]	20.27[b]	−0.98	0.17	< 0.001
5–6 wk	30.95[a]	28.14[b]	−2.81	0.47	< 0.001
0–6 wk	21.58[a]	20.30[b]	−1.28	0.21	< 0.001
7–16 wk	54.06[b]	54.52[a]	0.45	0.11	0.030
17–24 wk	85.39	85.44	0.05	0.17	0.889
			ADG (g/bird/d)		
0–2 wk	6.19[a]	5.67[b]	−0.53	0.08	< 0.001
3–4 wk	9.92[a]	8.50[b]	−1.42	0.23	< 0.001
5–6 wk	12.10[a]	9.72[b]	−2.39	0.38	< 0.001
0–6 wk	9.48[a]	8.01[b]	−1.47	0.22	< 0.001
7–16 wk	12.31[a]	11.82[b]	−0.49	0.11	0.022
17–24 wk	5.80[b]	6.21[a]	0.42	0.11	0.048
			FCR		
0–2 wk	1.91[b]	2.10[a]	0.19	0.03	< 0.001
3–4 wk	2.14[b]	2.38[a]	0.24	0.04	< 0.001
5–6 wk	2.56[b]	2.90[a]	0.34	0.05	< 0.001
0–6 wk	2.28[b]	2.54[a]	0.26	0.04	< 0.001
7–16 wk	4.40[b]	4.61[a]	0.22	0.04	0.003
17–24 wk	14.78	13.78	−1.00	0.26	0.052

[a,b]Means with different superscripts within a row differ significantly ($P < 0.05$).
[c]Means difference of mean value between NC and Met- group.
[1]NC, fed basal diet, Met 0.46%; Met-, fed Met deficiency diet, Met- 0.26%.
[2]SEM: standard error of the means.
TL, tibial length.

OTUs were shared between the two groups. The NC group exhibited the number of unique sequences (314 OTUs) and the total number of unique sequences in the Met- group was 1,027 OTUs (**Figure 3A**).

The alpha-indices (Sobs index, Ace index, Chao index and Shannon index) were used to describe the degree of cecal microbial diversity. As shown in **Supplementary Figure 1**, it was found that no difference was observed in the Shannon index between the two groups ($P > 0.05$). Meanwhile, there was no significant difference in other indices between the group ($P > 0.05$). The relative abundance at phylum and genus levels was studied. Principal coordinate analysis (PCoA), a multivariate statistical analysis method suitable for high-dimensional data was performed (**Figure 3B**). At the phylum level, Bacteroidetes and Firmicutes were the two major bacteria in the cecum of chicks, accounting for more than 90% (NC group, 98.62%; Met- group, 97.03%) of the cecum bacterial community (**Figure 3C**). Additionally, the Met- group had lower Firmicute/ Bacteroidetes ratios and higher Bacteroidetes than the NC group ($P > 0.05$). At the genus level, the most

TABLE 3 | Effects of Met deficiency in rearing period diets on digestive enzymes activities in duodenum of egg-laying chicks at 6 weeks.

Item	Group[1]		Difference[a]	SEM[2]	*P* value
	NC	Met-			
Typsin activity (U/mg prot)	591.03	388.59	−202.44	150.86	0.563
Chymotrypsin activity (U/mg prot)	2.15	2.93	0.79	0.58	0.557
Lipase activity (U/mg prot)	4.69	4.05	−0.64	1.01	0.787
Amylase activity (U/mg prot)	0.13	0.12	−0.01	0.01	0.588

[a]*Means difference of mean value between NC and Met- group.*
[1]*NC, fed basal diet, Met 0.46%; Met-, fed Met deficiency diet, Met- 0.26%.*
[2]*SEM: standard error of the means.*

TABLE 4 | Effects of Met deficiency in rearing period diets on Subsequent laying performance of laying hens.

Item	Group[1]		Difference[c]	SEM[2]	*P* value
	NC	Met-			
See egg age (d)	130.00	134.50	4.50	1.367	0.100
The age of the first production day (d)	142.83[b]	144.83[a]	2.00	0.405	0.006
	18–20 weeks				
Egg production (%)	10.78[a]	6.71[b]	−4.07	0.009	0.006
Average egg weight (g)	42.64[a]	41.26[b]	−1.37	0.265	0.006
Egg mass (g/bird/d)	4.61[a]	2.78[b]	−1.83	0.366	0.005
	21–22 weeks				
Egg production (%)	75.92[a]	62.30[b]	−13.62	0.024	< 0.001
Average egg weight (g)	48.24	46.86	−1.38	0.368	0.057
Egg mass (g/bird/d)	36.96[a]	29.96[b]	−7.00	1.170	< 0.001
	23–24 weeks				
Egg production (%)	97.25	96.64	−0.61	0.003	0.380
Average egg weight (g)	53.33	52.69	−0.64	0.212	0.139
Egg mass (g/bird/d)	52.08	51.02	−1.07	0.325	0.102
	18–24weeks				
Egg production (%)	61.32[a]	55.21[b]	−6.10	0.011	0.001
Average egg weight (g)	48.07[a]	46.94[b]	−1.13	0.227	0.005
Egg mass (g/bird/d)	31.21[a]	27.92[b]	−3.30	0.551	< 0.001
Unqualified egg rate (%)	0.09	0.26	0.16	0.000	0.072

[a,b]*Means with different superscripts within a row differ significantly (P < 0.05).*
[c]*Means difference of mean value between NC and Met- group.*
[1]*NC, fed basal diet, Met 0.46%; Met-, fed Met deficiency diet, Met- 0.26%.*
[2]*SEM: standard error of the means.*

common genera in the NC and Met- groups were accounted by *Bacteroides*, norank_f_norank_o_*Clostridia*_UCG014, norank_f_norank_o_RF39, *Lactobacillus*, *Faecalibacterium* and Unclassified_ f_ *lachnospiraceae* (**Figure 3D**). The level of predominant genera such as *Bacteroides*, *Lactobacillus* and *Faecalibacterium* in the Met- group was higher than the NC group. The relative abundance of *Bacteroides* in the NC group was 14.85%, and that in the Met- group was 19.62%. The data showed the composition of the gut microbiota in the Met- group was not significantly altered. To further determine which bacterial taxa contributed to the differences both statistically and biologically, we utilized linear discriminant analysis (LDA) effect size (LEfSe) analysis (**Figure 3E**). As shown in **Figure 3E**, a variety of genera were significantly enriched in the Met- group compared to the NC group, including *Odoribacter*, *Butyricicoccus*, norank_o__*Gastranaerophilales*, *Bifidobacterium*, *Bosea* and *Eubacterium*.

DISCUSSION

As Met is usually the first or second limiting amino acid (22), its adequate supply of Met is essential in farm animal diets. Met deficiency had different effects on animals, such as reduced

TABLE 5 | Effects of Met deficiency in rearing period diets on egg quality of 21 and 24 weeks laying hens.

Item	Group[1]		Difference[c]	SEM[2]	*P* value
	NC	Met-			
21 weeks					
Egg shape index	1.31	1.30	−0.01	0.006	0.291
Shell percentage (%)	9.52	9.38	−0.14	0.097	0.485
Albumen percentage (%)	68.85	68.79	−0.06	0.268	0.914
Yolk percentage (%)	21.63	21.83	0.20	0.249	0.695
Shell color	57.28	54.69	−2.59	0.903	0.155
Yolk color	4.17	4.17	0.00	0.102	1.000
Shell strength (kg/cm^2)	4.32	4.07	−0.25	0.125	0.333
Shell thickness (mm)	0.401	0.394	−0.01	0.004	0.413
Haugh unit	82.94[a]	73.96[b]	−8.99	2.135	0.033
24 weeks					
Egg shape index	1.30	1.30	0.00	0.005	0.866
Shell percentage (%)	11.10[a]	10.48[b]	−0.62	0.123	0.009
Albumen percentage (%)[3]	64.61	65.29	0.67	0.299	0.266
Yolk percentage (%)[3]	24.29	24.24	−0.06	0.273	0.921
Shell color	60.20	58.58	−1.62	1.156	0.491
Yolk color	4.22	4.39	0.17	0.104	0.431
Shell strength (kg/cm^2)	4.17	3.89	−0.28	0.078	0.074
Shell thickness (mm)	0.408[a]	0.392[b]	−0.02	0.004	0.024
Haugh unit	83.92	82.76	−1.16	1.274	0.657

[a,b]*Means with different superscripts within a row differ significantly ($P < 0.05$).*
[c]*Means difference of mean value between NC and Met- group.*
[1]*NC, fed basal diet, Met 0.46%; Met-, fed Met deficiency diet, Met- 0.26%.*
[2]*SEM: standard error of the means.*
[3]*The albumen and yolk percentage were calculated based on the whole egg weight.*

protein synthesis and inhibited growth development (23, 24). For poultry, Met deficiency decreased the egg quality and laying performance, such as lowering shell thickness and egg weight (13, 15, 25). Our results demonstrated that the methionine-restricted diet containing 0.26% Met significantly affected BW and TL of egg-laying chicks during the trial period (0–6 weeks). Compared with the NC group (Met 0.46%), the ADFI and ADG of Met- group (Met 0.26%) were significantly decreased during the observation period (from week seven to week 24). The result agrees with the previous studies that found that Met deficiency had significantly decreased the growth performance, such as decreased BW, FI, and increased FCR (26–28). These results showed that Met deficiency (Met 0.26%) affected the growth performance of egg-laying chicks during the rearing period and had the subsequent effect on the growth performance: BW and ADG of the Met- group were significantly decreased, but FCR was increased.

Egg quality is a significant concern for consumers and egg producers alike. The adverse effects of Met deficiency during the laying period on egg quality, such as the decreased Haugh unit and the lower shell thickness, had been widely reported (29, 30). Surprisingly, the present study showed chicks who fed Met deficient diet during the rearing period (0–6weeks) significantly reduced egg quality; even normal diets of 18 weeks could not alleviate the adverse effects of Met deficiency during the rearing period. It indicated that the effect of dietary methionine deficiency during the rearing period on chicks is sustainable. As we know that the life span of commercial laying hens is close to 80 weeks. Although we used the observation period (7–24 weeks) to study whether Met deficiency during the rearing period will have a lasting impact on growth performance and egg quality of laying hens, but the further work is needed to evaluate the duration of these effects, because the observation period of this study is relatively short for the life span of commercial laying hens.

It is plausible that dietary deficiency of an essential amino acid could affect digestibility, transport and absorption of nutrients. But our results showed that Met deficiency did not have significant effect on the digestive enzyme activity. These results agree with study by Nitsan et al., who stated that the enzymic activities (trypsin, chymotrypsin, and amylase) were scarcely affected by the level of Met of chicks (31).

The serum concentration of AA results from the amount and form of ingested AAs, which reflects AA absorption (32). Met takes part in many critical metabolic pathways, including protein synthesis, cysteine and carnitine metabolism and one-carbon metabolism (33). To a certain extent, the concentration of free amino acids in serum can reflect the metabolism of amino acids. In this study, the higher levels of Ser and Cys in the Met- group were observed at week 6. Met and Cys are the key factors of one-carbon metabolism, and Ser and Gly are the main sources of

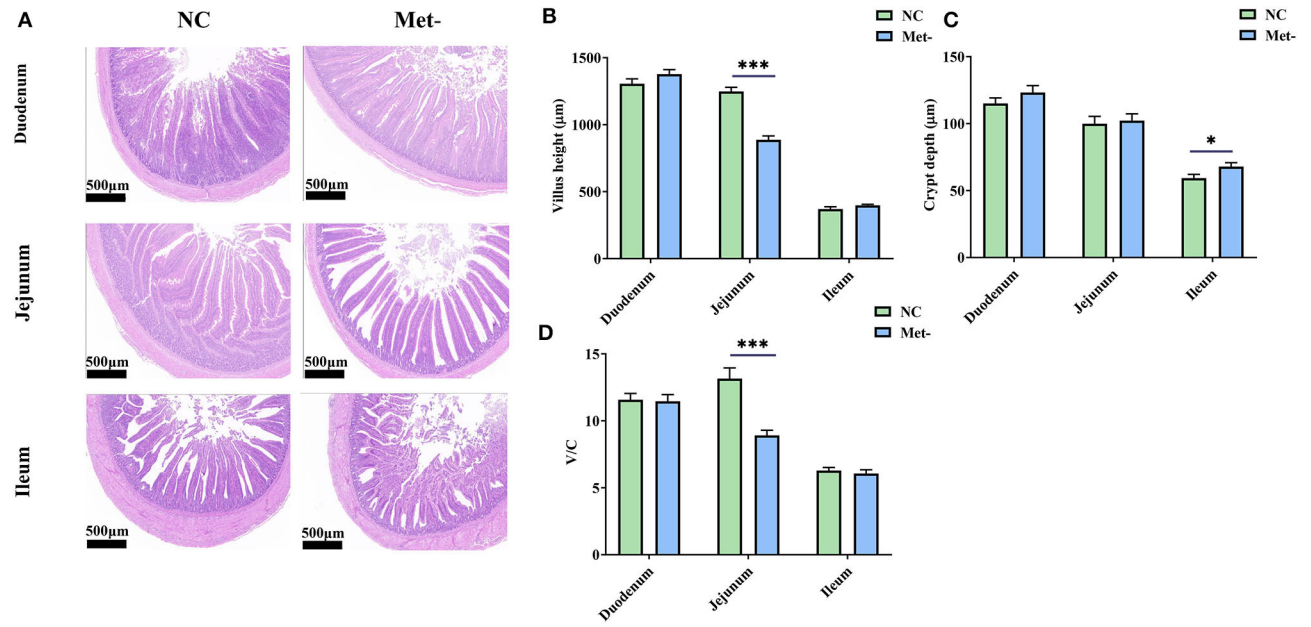

FIGURE 1 | Effects of Met deficiency in rearing period diets on small intestine morphologic structure of egg-laying chicks. **(A)** Representative samples from histological stained sections showed Met- group had lower jejunal villus height and deeper ileal crypt depth. **(B)** Villus height of duodenum, jejunum, and ileum. **(C)** Crypt depth of duodenum, jejunum, and ileum. **(D)** Villus height-to-crypt depth ratio (V/C) of duodenum, jejunum, and ileum. Data were presented as means ± SEM. Significant differences were tested by student's *t*-test. *Indicates significant difference, $P < 0.05$; ***, $P < 0.001$.

one-carbon groups (34, 35). The requirement for Ser is reduced when both Met and Cys are supplied adequately in broilers. When the Met was supplied insufficiently, the levels of Ser and Cys may be altered (36). At week 16, the lower content of Ala in Met- group was observed, which was consistent with the study by Wan et al. (37). They found that plasma Ala concentration was linearly increased as DL-Met supplementation level increased. In this study, these results clearly demonstrated that Met deficiency had a significant influence on the content of amino acids in serum of chicks. However, how the Met deficiency affects the serum AA concentration remains to be elucidated.

Met is critical for a rapidly growing animal (38). Met plays a vital role in intestinal development and maintains the integrity of the intestinal mucosa and barrier function (39). Notably, when comparing the EAA that is being metabolized in the gastrointestinal tract, on average, the utilization of Met tends to be greater than other EAA. Therefore, there appears to be a specific functional need for Met in the gastrointestinal tract of animals. In this study, the current study revealed the villi height of jejunal was decreased in the Met- group. Conversely, the crypts depth of the ileum was increased. Zhang et al. found that dietary Met supplementation increased villus height of the ileal, which improved the small intestinal morphology of Pekin ducks (40). Shen et al. (41) found that the supplementation of Met could increase the villus height and decrease the crypts depth of young chickens. On the contrary, Met deficiency caused the decrease of villus height and the increase of crypts depth. The amino acid imbalance caused by the lack of methionine during the rearing period may also be one of the reasons that hinder intestinal development besides.

Gut microbial communities play critical roles in animal health and productivity. There is increasing evidence that microbes in the gastrointestinal tract may play an important role in AA metabolism and host protein (42). Notably, Dai et al. discovered that AA could regulate the composition of the intestinal bacterial population from the small intestine in pigs (43). In this study, we found that there were no significant differences in alpha diversity indices between NC (Met 0.46%) and Met- group (Met 0.26%), which was similar to the previous study (44). Wu et al. found that D-methionine supplementation had no effects on alpha diversity indices in male Wistar rats (176–200 g, 6 weeks old), who uncovered the Met diet does not alter gut microbiota structure (45). Meanwhile, Wallis et al. found that when sexes were combined, there were no differences in the composition of the intestinal microflora between the control and Met restriction groups (18). Interestingly, *Firmicutes* and *Bacteroidetes* were the major phyla in the cecal digesta of chickens. Numerous studies have consistently demonstrated that the *Firmicutes*/*Bacteroidetes* (F/B) proportion is increased in obese people compared to lean people and tend to decrease with weight loss (46). Koliada et al. (47) found that the content of *Firmicutes* was gradually increased while the relative abundance of *Bacteroidetes* was decreased with increasing body mass index in Ukrainian population. In the present study, at the phylum level, the Met- group (Met 0.26%) had lower *Firmicute*/*Bacteroidetes* ratios and higher *Bacteroidetes* than the NC group (Met 0.46%), which may support our results

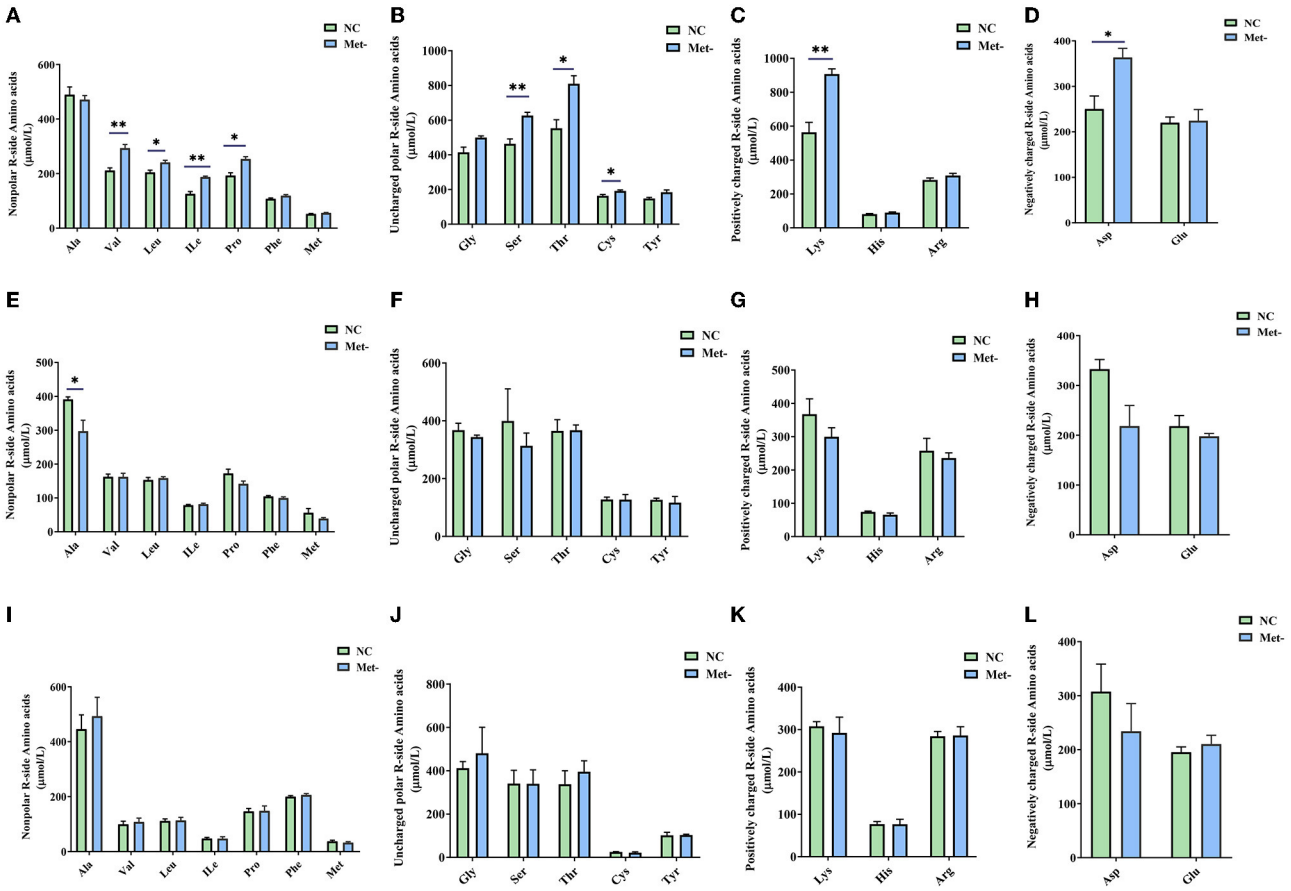

FIGURE 2 | Effects of Met deficiency in rearing period diets on serum concentration of amino acids (AA) of laying hens. **(A–D)** serum concentration of amino acids (AA) of laying hens at 6th weeks, **(A)** Nonpolar R-side Amino acids. **(B)** Uncharged polar R-side Amino acids. **(C)** Positively charged R-side Amino acids. **(D)** Negatively charged R-side Amino acids. **(E–H)** serum concentration of amino acids (AA) of laying hens at 16th weeks. **(E)** Nonpolar R-side Amino acids. **(F)** Uncharged polar R-side Amino acids. **(G)** Positively charged R-side Amino acids. **(H)** Negatively charged R-side Amino acids. **(I–L)** serum concentration of amino acids (AA) of laying hens at 24th weeks. **(I)** Nonpolar R-side Amino acids. **(J)** Uncharged polar R-side Amino acids. **(K)** Positively charged R-side Amino acids. **(L)** Negatively charged R-side Amino acids. Data were presented as means ± SEM. Significant differences were tested by student's *t*-test. *Indicates significant difference, $P < 0.05$; **, $P < 0.01$.

that the BW in the Met- group was decreased. *Eubacterium* is linked with amino acid fermentation (48). These results showed that the genera of *Eubacterium* were decreased in the Met-group compared to the NC group.

Lactobacillus has been extensively studied and identified as the predominant amino acid-fermenting bacteria (49, 50). In this study, the results showed that the genera of *Lactobacillus* were higher in the Met-group compared to the NC group. The alteration of *Eubacterium* and *Lactobacillus* was associated with the Amino acid metabolism pathway. Recent research has demonstrated that Met can regulate animal's metabolic processes and digestive functioning (51). Meanwhile, Met is the main source of butyrate (48). However, the interactions between the gut microbiota and Met deficiencies are still unclear, and more research studies are needed.

CONCLUSIONS

According to the results in this study, Met deficiency during the trial period (0–6 weeks) reduced growth performance, changed the concentration of serum amino acids, hindered intestinal development, affected the gut microbial community and structure of layer chicks. Met deficiency in the rearing period induced the imbalance of serum amino acids and affected the current growth performance and had a continuously adverse impact on the growth and production performance of egg-laying chicks during the observation period (from week 7–24). Therefore, it's critical to meet the needs of Met in the rearing period for subsequent growth development and production performance of egg-laying chicks.

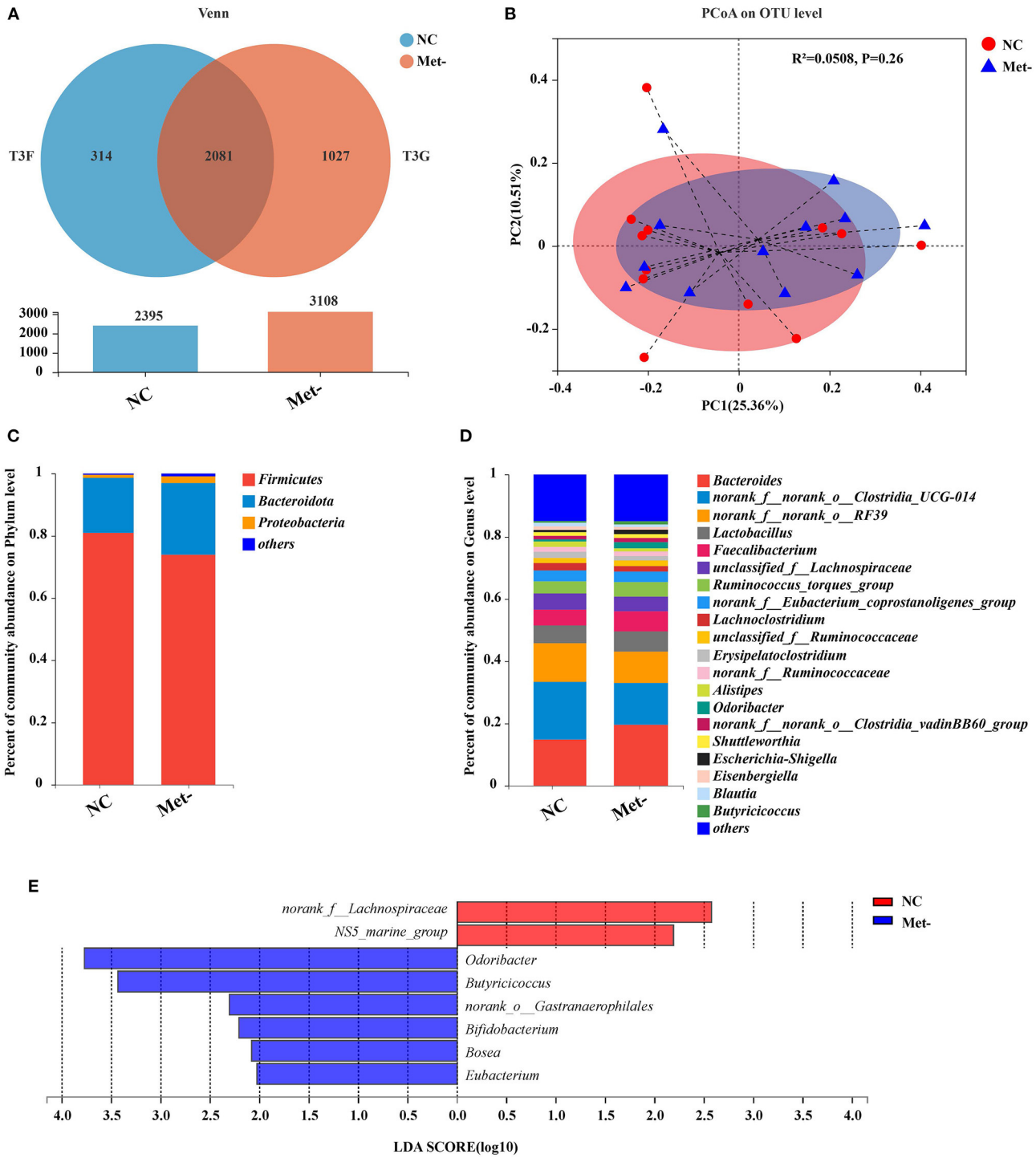

FIGURE 3 | Effects of Met deficiency in rearing period diets on the gut microbiota of egg-laying chicks at 6 weeks. **(A)** Venn diagram of the shared and total operational taxonomic units (OTUs) in the NC and Met- groups. **(B)** The principal coordinate analysis (PCoA) (Bray-Curtis distance) plot of the gut microbial community structure between the NC and Met- group. **(C)** Relative abundance of gut microbiota at the phylum level. **(D)** Relative abundance of gut microbiota at the genus level. **(E)** Histograms of the linear discriminate analysis (LDA) score (threshold ≥ 2) in Met- and NC groups are plotted. Linear discriminate analysis effect size (LEfSe) was performed to determine the difference in abundance.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA791195.

ETHICS STATEMENT

The animal study was reviewed and approved by China Agricultural University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

DW and QM designed the study. DW conducted the experiments. DW and YL collected and analyzed the samples. YL, DW, and SH guided to analyze the experimental data. YL drafted the manuscript. SH, LZ, JZ, and QM polished the manuscript and finished the submission. YL, DW, SH, LZ, JZ, and QM helped with revisiting and reviewing the manuscript. QM obtained funds and managed project. All authors read and approved the final manuscript.

FUNDING

This study was supported by a special fund for China Agricultural Research System program (Grant No. CARS-40-K08).

ACKNOWLEDGMENTS

We thank all technicians in the experimental animal facility of China Agricultural University for providing daily care of layer chicks.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.878107/full#supplementary-material

Supplementary Figure 1 | Effects of Met deficiency in rearing period diets on the alpha diversity of the gut microbiota in egg-laying chicks at 6 weeks. **(A)** Sobs index of the community diversity. **(B)** Chao index of the community richness. **(C)** Shannon index of the community diversity. **(D)** Ace index of the community richness.

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frontiers | Frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 20 June 2022
doi: 10.3389/fvets.2022.911496

Effects of Lysophosphatidylcholine on Jejuna Morphology and Its Potential Mechanism

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Lysophosphatidylcholine (LPC) plays a vital role in promoting jejuna morphology in broilers. However, the potential mechanism behind LPC improving the chicken jejuna morphology is unclear. Therefore, the present study was designed to reveal the important genes associated with LPC regulation in birds' jejuna. Thus, GSE94622, the gene expression microarray, was obtained from Gene Expression Omnibus (GEO). GSE94622 consists of 15 broiler jejuna samples from two LPC-treated (LPC500 and LPC1000) and the control groups. Totally 98 to 217 DEGs were identified by comparing LPC500 vs. control, LPC1000 vs. control, and LPC1000 vs. LPC500. Gene ontology (GO) analysis suggested that those DEGs were mainly involved in the one-carbon metabolic process, carbon dioxide transport, endodermal cell differentiation, the positive regulation of dipeptide transmembrane transport, cellular pH reduction, and synaptic transmission. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated the DEGs were enriched in NOD-like receptor (NLR), RIG-I-like receptor (RILR), Toll-like receptor (TLR), and necroptosis signaling pathway. Moreover, many genes, such as *RSAD2, OASL, EPSTI1, CMPK2, IFIH1, IFIT5, USP18, MX1, and STAT1* might be involved in promoting the jejuna morphology of broilers. In conclusion, this study enhances our understanding of LPC regulation in jejuna morphology.

Keywords: gene, signaling pathway, lysophosphatidylcholine, jejuna, broiler

OPEN ACCESS

Edited by:
Abdelrazeq M. Shehata, Al-Azhar University, Egypt

Reviewed by:
Alzahraa M. Abdelatty, Cairo University, Egypt
Qiugang Ma, China Agricultural University, China
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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: *02 April 2022*
Accepted: *10 May 2022*
Published: *20 June 2022*

Citation:
Li X, Abdel-Moneim A-ME, Mesalam NM and Yang B (2022) Effects of Lysophosphatidylcholine on Jejuna Morphology and Its Potential Mechanism. Front. Vet. Sci. 9:911496. doi: 10.3389/fvets.2022.911496

INTRODUCTION

Lysophosphatidylcholine (LPC), a kind of bioactive lipid, has a robust antimicrobial and immunomodulatory potentials in broilers (1–5). The potent antimicrobial activity of LPC has been documented (1–3). In the *in vitro* study of Yadav et al. (1), the combination of LPC and polymyxin B inhibited the growth of Salmonella and some Gram-negative. In addition, LPC was substantially more effective to induce membrane permeability in Gram-positive bacteria than in Gram-negative counterparts. The bactericidal effect of LPC also enhanced gentamicin sensitivity in resistant Methicillin-resistant *Staphylococcus aureus* strains and markedly alleviated the burden from intracellular bacteria in the liver and spleen (2, 3). Furthermore, LPC improved phagosome maturation to control *Salmonella Typhimurium* (*S. Typhimurium*) growth by activating the NFκB pathway induced by active oxygen species (ROS) (3). In LPC-treated cells, the expression of phagosome maturation markers (including *LAMP1* and *EEA1*) and cleaved cathepsin D, and ROS production were remarkably improved during *S. Typhimurium* infection (3). LPC also enhanced the production of gamma interferon by engaging naive T cells and induced the release of chemokines and IL-8 (4).

Parra-Millan et al. reported that LPC could trigger innate and specific humoral-mediated immunity as LPC administration regulated the immune response in the prognosis of ceftazidime-resistant *Pseudomonas*-induced infections (6). Moreover, in monocyte-derived dendritic cells, LPC could upregulate the *CD86*, *HLA-DR,* and *CD40* genes expression (6). LPC increased the relative weights of bursa and thymus, and improved antibody production titers against Newcastle disease virus and sheep red blood cells in broilers (7).

Importantly, previous investigations also revealed that LPC had the ability to improve intestinal morphology, nutrients digestion and absorption, and growth performance in broilers (6–10). Nutautaite et al. documented significant improvement in intestinal villus height, average daily gain (ADG), and the contents of isovaleric and butyric acid in broilers treated with LPC (8). LPC addition reduced the crypt depth, increased the jejunal villi height, and improved the ratio of villi height to crypt depth in the jejuna and duodena of chicken. LPL promoted the growth performance and nutrient utilization (9). Zhang et al. reported that dietary LPC increased body weight gain and the digestibility of C16:0, C18:1n7, C18:2, C18:3n3, and C18:1n9 (10). Furthermore, LPC decreased the average daily feed intake, increased ADG and feed conversion ratio (7). LPC improves broilers' performance by upregulating the expression of amino acids and cholesterol transporter genes in enterocytes and increasing the fat digestibility and the intake of cholesterol and amino acids (6).

Nevertheless, the mechanism by which LPC improves the jejuna morphology in broilers is unclear. Therefore, we obtained the microarray data of broiler chickens' jejuna treated with or without LPC from the Gene Expression Omnibus dataset (GEO; https://www.ncbi.nlm.nih.gov/geo/) and identified differentially expressed genes (DEGs) in birds' jejuna, aiming to explore the potential mechanism behind the regulation of LPC on the jejuna morphology in broilers.

MATERIALS AND METHODS

Ethics Statement

This study protocol was approved by Anhui Science and Technology University (Bengbu, China) Institutional Animal Care and Use Committee (ECASTU-2015-P08).

Animals, Feed, and Tissue Collection

Seventy-five newly hatched Cobb 500 male broilers were divided into three groups, including the control, LPC500, and LPC1000 groups, with five replications of five chicks each (11). The control was provided with the basal diet, and the LPC500 and LPC1000 groups were provided with the basal diet adding 500 g/T and 1,000 g/T LPC, respectively (11). The experimental time lasted for 4 weeks. Ingredients and nutrient levels of the basal diet were shown in **Supplementary Table 1**. All birds were individually weighed weekly and placed in a room with adjoining floor pens (11). On the 10th day of the experiment, five chicks were randomly chosen from each group and killed via cervical dislocation. Pieces with approximately 10 cm in length were collected from the middle of jejuna (11).

RNA Extraction and Microarray Analysis

Based on the effects of LPC on chicken jejuna morphology (**Supplementary Table 2**), Approximately 50 mg of jejunal mucosa was homogenized using Tri Reagent (11). Total RNA was extracted using Directzol RNA columns, and RNA integrity, quality, and purity were assessed (11). Samples with RNA integrity number (RIN) $>$ 8.7 were used for the subsequent analysis. Microarray analysis was performed with the chicken genome 1.0 array (11). The data of jejuna gene expression were deposited in GEO (accession number: GSE94622) (11).

Microarray Data

The gene expression microarray, GSE94622, was downloaded from the GEO dataset. GSE94622 consisted of LPC1000-treated (n = 5; GSM2479496, GSM2479497, GSM2479513, GSM2479530, and GSM2479531), LPC500-treated (n = 5; GSM2479493, GSM2479510, GSM2479511, GSM2479526, and GSM2479527) and the control (n = 5; GSM2479490, GSM2479491, GSM2479506, GSM2479507, and GSM2479523) chicken jejuna samples obtained at the 10^{th}day of experiment.

Data Processing

To identify the DEGs in the jejuna samples between the LPC-treated groups and the control, GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) software was used the data from GSE94622. Genes with$|\log_2$Fold Change (FC)$|$ $>$ 1and $P < 0.05$ were considered as the DEGs. The probe sets without Entrez gene annotation were deleted.

Analysis of KEGG and Genetic Ontology for DEGs

KOBAS 3.0(http://kobas.cbi.pku.edu.cn/kobas3/genelist/) was used to analyze the signaling pathway for DEGs. As to the Genetic ontology (GO) analysis, DEGs were analyzed with DAVID (https://david.ncifcrf.gov/).

Protein Classification and Reactome Analysis for DEGs

Protein class and Reactome analysis for DEGs were performed with PANTHER classification system (http://pantherdb.org/) and KOBAS 3.0, respectively.

Protein-Protein Interaction Network

Protein-Protein Interaction (PPI) network was analyzed with the STRING database (https://string-db.org/) and further visualized with Cytoscape 3.8.0 (http://www.cytoscape.org/).

Hub Genes and Their Functions

CytoHubba software (http://apps.cytoscape.org/apps/cytohubba) was employed to reveal hub genes from the PPI network, then the functions of hub genes were summarized using GeneCards (https://www.genecards.org/), NCBI database (https://www.ncbi.nlm.nih.-gov/), and previous literature.

RESULTS

Outline of Transcripts and Genes in Broilers Jejuna

Totally 38,535 transcripts and 14,086 genes were identified in the jejuna treated with or without LPC. UMAP and transcripts expression density are shown in **Figures 1A,B**. **Figures 1C–E** represents the volcano plots for DEGs in the comparisons of LPC500 and LPC1000 vs. control and LPC1000 vs. LPC500, respectively. **Figure 1F** represents the Venn diagram of the three comparisons.

As shown in **Supplementary Table 3**, a total of 147 to 306 differentially expressed transcripts (DETs), 98 to 217 DEGs were identified by three ways of comparisons (LPC500 vs. control, LPC1000 vs. control, and LPC1000 vs. LPC500). Compared with the control, 236 transcripts and 179 genes were upregulated, whereas 70 transcripts and 38 genes were downregulated in LPC1000-treated jejuna (**Supplementary Files 1**, **2**); 145 transcripts and 99 genes were upregulated, whereas 70 transcripts and 44 genes were downregulated in LPC500-treated jejuna (**Supplementary Files 3**, **4**). Forty of the 45 common DEGs of the two comparisons (LPC1000 vs. control and LPC500vs.control) were shown in **Supplementary Table 4**. The top 20 up-and downregulated genes in three comparisons (LPC500 and LPC1000 vs. control and LPC1000 vs. LPC500) were revealed in **Supplementary Tables 5**–**10**, respectively.

GO Analysis for DEGs

To reveal the biological processes involved in the regulation of LPC on broiler jejuna heath, GO functional enrichment of DEG sin the three comparisons, including LPC1000 andLPC500 vs. control, and LPC1000 vs. LPC500were illustrated in **Figures 2A–C** and **Supplementary Files 5**–**7**, respectively.

Forty-five common DEGs of two comparisons (LPC1000 and LPC500 vs. control) in the chicken jejuna participated in defense response to viruses, the positive regulation of tumor necrosis factor production, inflammatory response, interleukin-6 production, and innate immune response; neutrophil activation; type I interferon; the negative regulation of viral genome replication, chemokine, interferon-alpha, interferon-beta production (**Figure 2D** and **Supplementary File 8**). In addition, **Figures 2E–O** represents the heatmaps for DEGs in innate immune response; the positive regulation of chemokine production; the positive regulation of G1/S transition of the mitotic cell cycle, the positive regulation of interleukin-6 production, cell adhesion, cell proliferation, endodermal cell differentiation, germinal center B cell differentiation, inflammatory response; the negative regulation of endopeptidase activity; and the negative regulation of NFκB transcription factor activity, respectively.

KEGG Enrichment for DEGs

To discover the signaling pathways related to the regulation of LPC on broiler jejuna heath, KEGG enrichment for DEGs in the three comparisons, including LPC500 vs. control, LPC1000 vs. control, and LPC1000 vs. LPC500, is shown in **Figures 3A–C** and **Supplementary Files 9**, **10**, respectively. Moreover, the common DEGs of two comparisons (LPC500 vs. control and LPC1000 vs. control) mainly participated in TLR, NLR, RILR, pyrimidine metabolism, AGE-RAGE, and necroptosis signaling pathways (**Figure 3D** and **Supplementary File 12**). In addition, **Figures 3E–I** reveals the expression outline for DEGs in metabolic pathways, cellular senescence, necroptosis, PPAR, and TLR signaling pathway, respectively.

Reactome Enrichment for DEGs

To further reveal the pathways related to the regulation of LPC on broiler jejuna heath, Reactome enrichment for DEGs in the three comparisons, including LPC500 vs. control, LPC1000 vs. control, and LPC1000 vs. LPC500, are shown in **Figures 4A–C**, respectively. In addition, the common DEGs of two comparisons (LPC500 and LPC1000 vs. control) mainly taken part in the innate immune system; cytokine signaling in the immune system; metabolism of RNA, hemostasis; caspase-8 and−10 mediated induction of NF-Kb; interleukin-6 signaling; negative regulation of MDA5 signaling; TRAF mediated activation of IRF; and interleukin-20 family signaling (**Figure 4D**).

Protein Classification of DEGs

DEGs between LPC1000 and the control groups might play an important role in extracellular matrix protein, extracellular matrix structural protein, metabolite interconversion enzyme, protein class, peroxidase, protein-binding activity modulator, acyltransferase, actin or actin-binding cytoskeletal protein, protease inhibitor, reductase, oxidoreductase, metalloprotease, deaminase, RNA metabolism protein, hydrolase, serine protease, cadherin, protease, oxidase and glycosidase (**Figure 5A**). DEGs between LPC500 and the control groups might play an important role in RNA helicase, primary active transporter, non-receptor serine/threonine-protein kinase, ATP-binding cassette, transporter (**Figure 5B**). DEGs between LPC1000 and LPC500 groups might play an important role in extracellular matrix structural protein, metabolite interconversion enzyme, oxidoreductase, deaminase, reductase, oxygenase, dehydratase, membrane-bound signaling molecule, hydrolase, lyase, and serine protease (**Figure 5C**).

PPI Network

To further explore key genes, PPI networks of DEGs in the three comparisons, including LPC500 andLPC1000 vs. control, and LPC1000 vs. LPC500, are shown in **Figures 6A–C**, respectively.

Hub Genes and Their Function

As shown in **Figure 7A**, the top 20 hub genes from DEGs between LPC1000 and control groups included *MX1*, *IFIH1*, *RSAD2*, *OASL*, *IFIT5*, *EPSTI1*, *CMPK2*, *SAMD9L*, *STAT1*, *USP18*, *TLR3*, *ZNFX1*, *IL8L2*, *IL8L1*, *MYD88*, *FN1*, *SPARC*, *COL1A2*, *COL6A1*, and *COL5A1*. The top 20 hub genes from DEGs between LPC500 and control groups included *DHX58*, *RSAD2*, *DDX60*, *OASL*, *EPSTI1*, *CMPK2*, *IFIH1*, *IFIT5*, *PARP9*, *USP18*, *MX1*, *STAT1*, *EIF2AK2*, *SAMD9L*, *ZNFX1*, *PARP12*, *TLR3*, *MOV10*, *ZC3HAV1*, and *RNF213* (**Figure 7B**). The top nine hub genes from DEGs between LPC1000 and LPC500 groups included

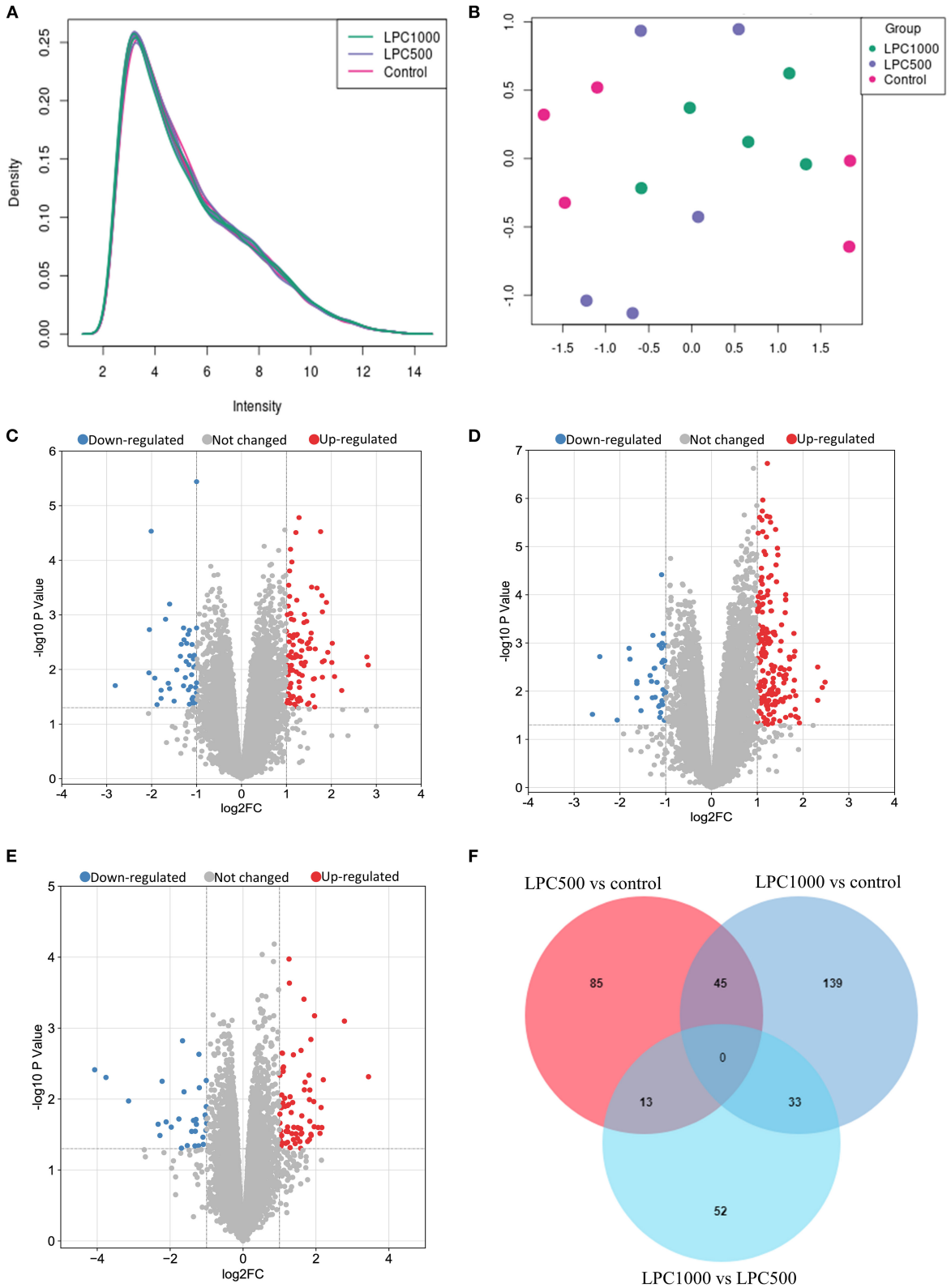

FIGURE 1 | Profile of Transcripts and Genes in Broilers Jejuna Treated with and without LPC. **(A)** Transcripts expression density; **(B)** UMAP; **(C–E)** volcano plot of DEGs identified by three comparisons (LPC500 and LPC1000 vs. control and LPC1000 vs. LPC500, respectively). The red gray, and blue spots represent the upregulated, unchanged and downregulated genes, respectively. **(F)** Venn diagrams for the DEGs identified in the three ways of comparisons mentioned above.

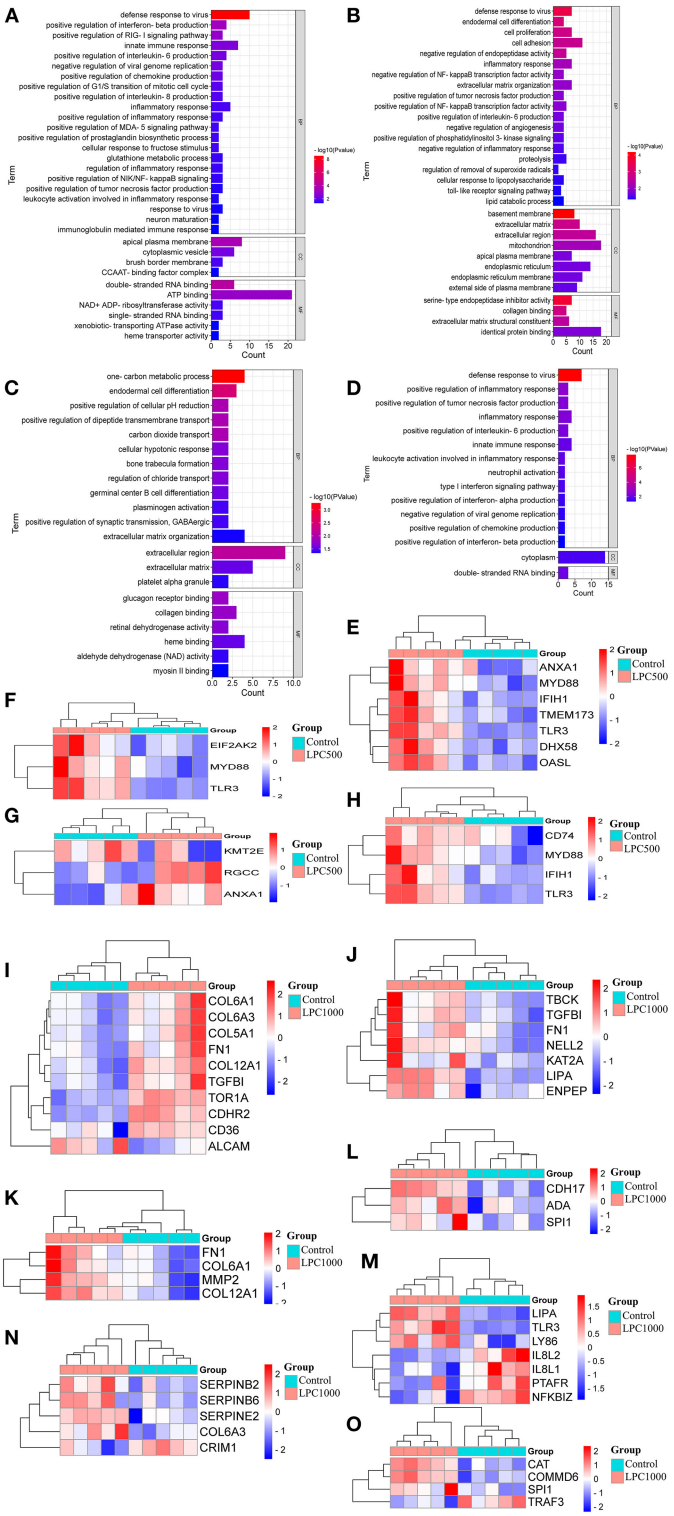

FIGURE 2 | GO Enrichment for DEGs in Broilers Jejuna Treated with and without LPC. **(A–C)** GO enrichment for DEGs identified in the three comparisons (LPC500 and LPC1000 vs. control and LPC1000 vs. LPC500, respectively); **(D)** GO enrichment for the common DEGs in the two comparisons (LPC500 and LPC1000 vs. control);The heatmaps for DEGs in innate immune response **(E)**, the positive regulation of chemokine production **(F)**, the mitotic cell cycle G1/S transition **(G)**, IL-6 production **(H)**,cell adhesion **(I)**, cell proliferation **(J)**, endodermal cell differentiation **(K)**, germinal center B cell differentiation **(L)**, inflammatory response **(M)**, the negative regulation of endopeptidase activity **(N)**, and the negative regulation of NFκB transcription factor activity **(O)**, respectively.

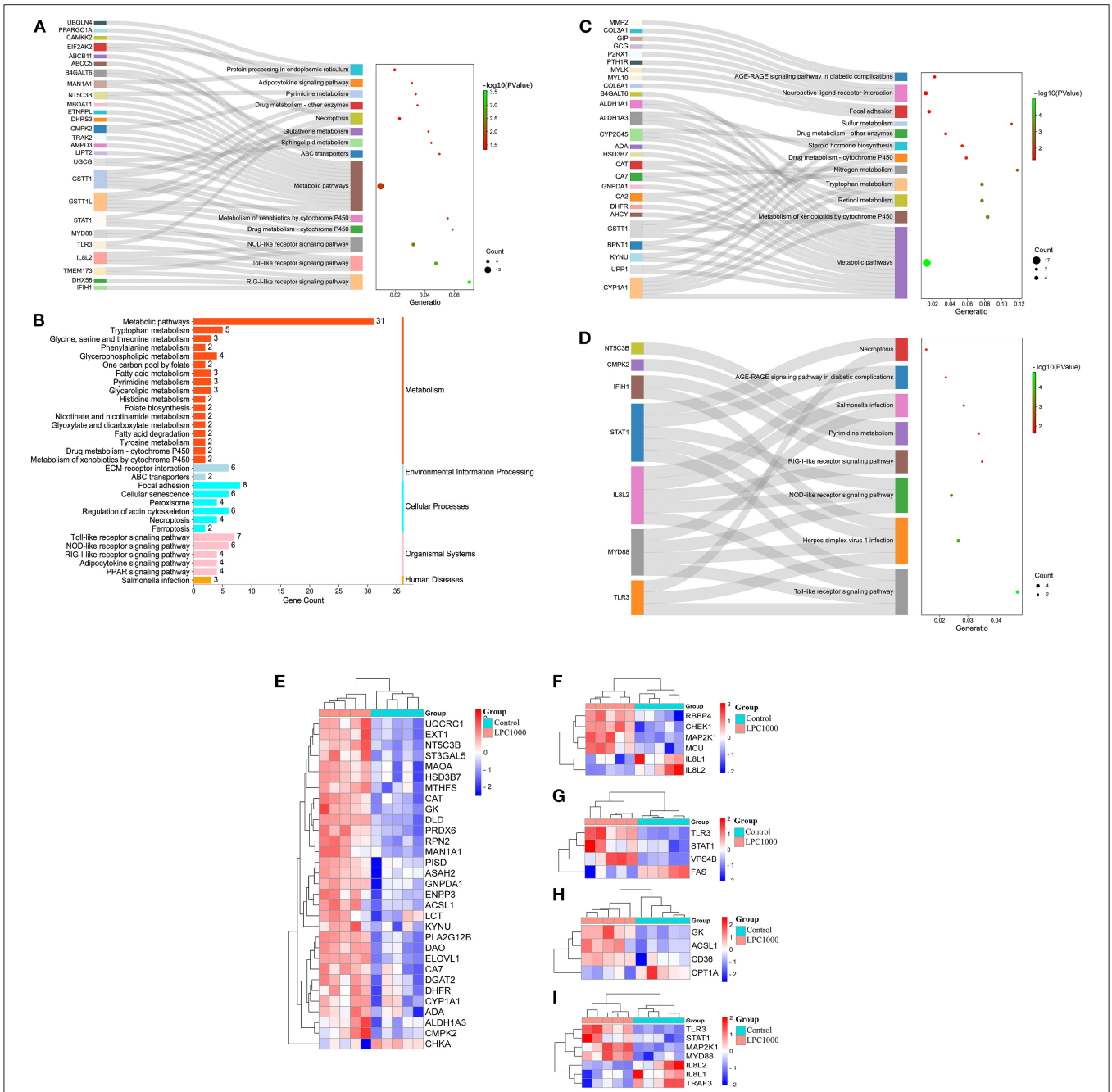

FIGURE 3 | KEGG Enrichment for DEGs in Broilers Jejuna Treated with and without LPC. **(A–C)** KEGG enrichment for DEGs identified by three comparisons (LPC500 and LPC1000 vs. control and LPC1000 vs. LPC500, respectively); **(D)** KEGG enrichment for the common DEGs identified by two comparisons (LPC500 and LPC1000 vs. control); Heatmaps for DEGs in metabolic pathways **(E)**, cellular senescence **(F)**, necroptosis **(G)**, PPAR **(H)**, and TLR signaling pathway **(I)**, respectively.

CYP1A1, *CYP1A4*, *ALDH1A1*, *ALDH1A3*, *CYP2C45*, *MMP2*, *DCN*, *IYD*, and *COL6A1* (**Figure 7C**).

GO enrichment for the top 20 hub genes from DEGs between LPC1000, and the control groups suggested that these genes participated in leukocyte activation involved in TLR; cell adhesion; neutrophil activation; wound healing; type I interferon; inflammatory response; extracellular matrix organization; NFκB inflammatory response; endodermal cell differentiation, chemokine-mediated; neutrophil chemotaxis, the response to virus; and the positive regulation of inflammatory response, and the production of IL-6, IL-8, chemokine and interferon-beta (**Figure 7D**). GO enrichment for the top 20 hub genes from DEGs between LPC500 and control groups suggested that these genes participated in the positive regulation of RIG-I,

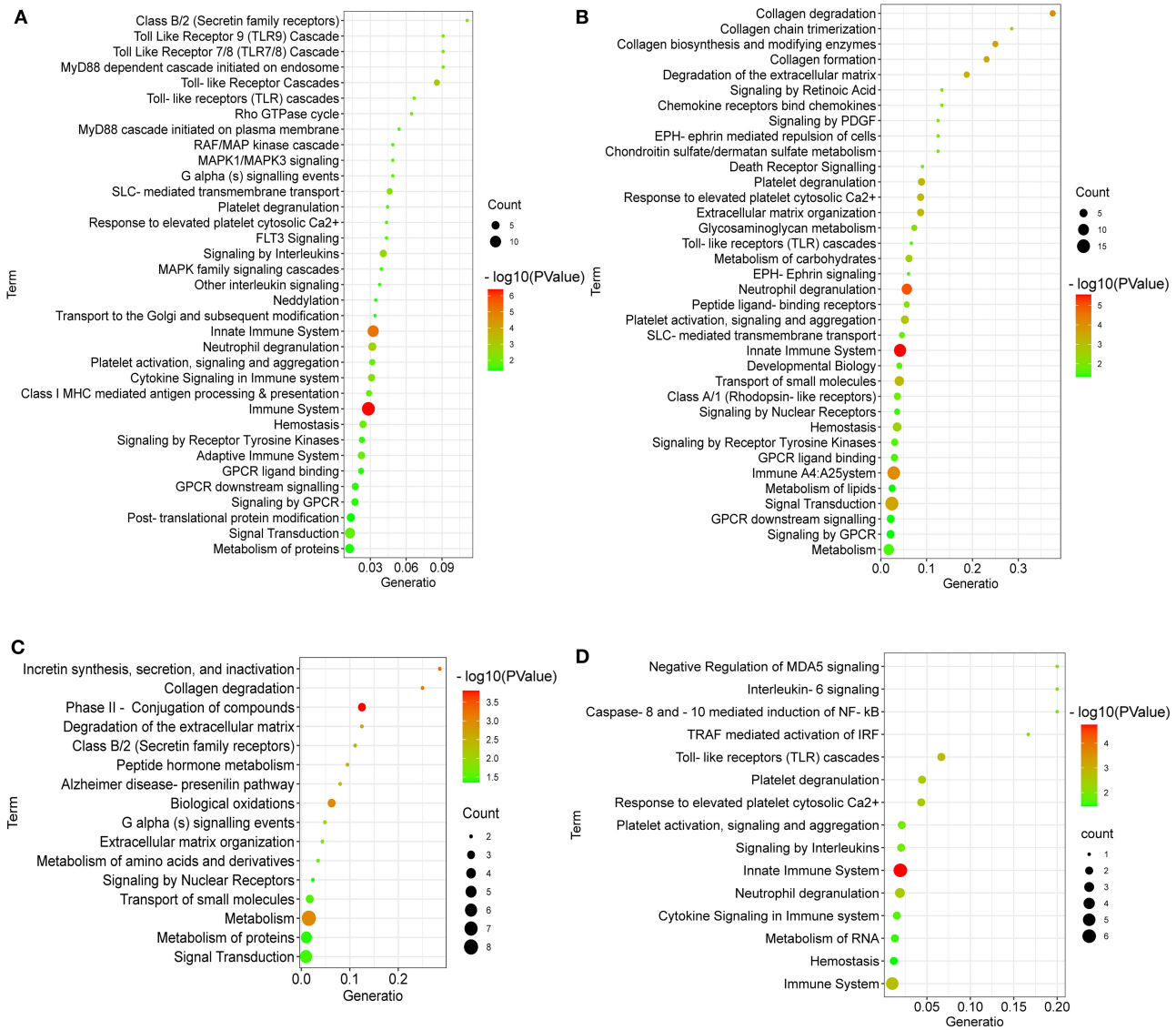

FIGURE 4 | Reactome Enrichment for DEGs in Broilers Jejuna treated with and without LPC. **(A–C)** Reactome enrichment for DEGs identified by three comparisons (LPC500 and LPC1000 vs. control and LPC1000 vs. LPC500, respectively); **(D)** Reactome enrichment for the common DEGs in two comparisons (LPC500 and LPC1000 vs. control).

MDA-5, NIK/NFκB signaling and the productions of chemokine, interferon-alpha, tumor necrosis factor, et al. (**Figure 7E**).

KEGG enrichment suggested the top 20 hub genes from DEGs between LPC1000 and control were involved in multiple signaling pathways, including TLR; necroptosis; cellular senescence; AGE-RAGE; focal adhesion; NLR; and RILR (**Figure 7F**). KEGG enrichment indicated that the top 20 hub genes from DEGs between LPC500 and control groups participated in many signaling pathways, including TLR; necroptosis; pyrimidine metabolism; AGE-RAGE; RILR; C-type lectin receptor; NLR; protein processing in the endoplasmic reticulum; and metabolic pathways (**Figure 7G**).

DISCUSSION

Hub Genes by Which LPC Increased Jejuna Morphology in Broilers

In the present study, many hub genes, such as *RSAD2*, *OASL*, *EPSTI1*, *CMPK2*, *IFIH1*, *IFIT5*, *USP18*, *MX1*, and *STAT1*, might be concentrated in the regulation of LPC on the chicken jejuna morphology. *RSAD2* (Radical s-adenosyl methionine domain containing 2) encoded a vital enzyme for innate immune responses as it was expressed in multiple kinds of cells in response to inflammatory stimuli (including viral infection). For instance, Wiedemann et al. reported that *RSAD2* participated in the NK cells' adaptive behavior after

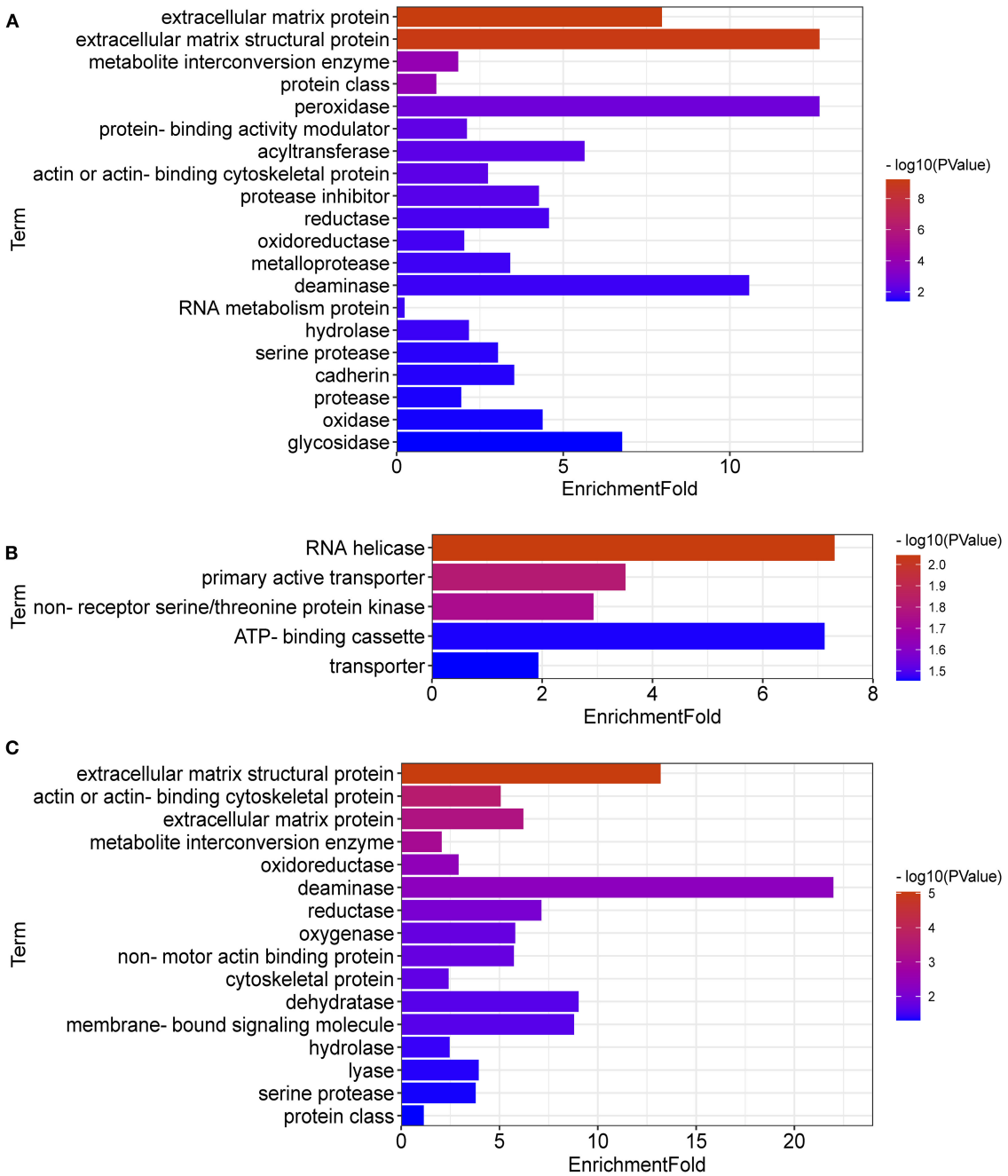

FIGURE 5 | Protein Classification for DEGs in Broilers Jejuna Treated with and without LPC. **(A–C)** Protein classification for DEGs identified by three comparisons (LPC1000 and LPC500 vs. control, and LPC1000 vs. LPC500, respectively).

viral infection (12). In infected 293T cells, *RSAD2* could restrict the measles virus (MV) infection at the virus release stage, However, in SR-B2 cells, the transduction with *RSAD2* expression *in vitro* or *in vivo* impaired the MV release (13). *RSAD2* also catalyzed the transformation of cytidine triphosphate (CTP) to its analog ddhCTP, which inhibited the activity of NAD^+-dependent enzymes, including *GAPDH* (14). Therefore, *RSAD2* might control the cell response to inflammatory stimuli, including viral infection, by regulating cell metabolism (15).

OASL (2′-5′-Oligoadenylate Synthetase Like) exerts various effects on RNA and DNA viruses by improving RIG-I-induced IFN induction and suppressing cGAS-induced IFN production, respectively (16). In $CD4^+$ T cells, *OASL* gene upregulation could increase the expressions of TET1, CD40L, and CD70, the hydroxymethylation levels, and the aberrant cell activation via

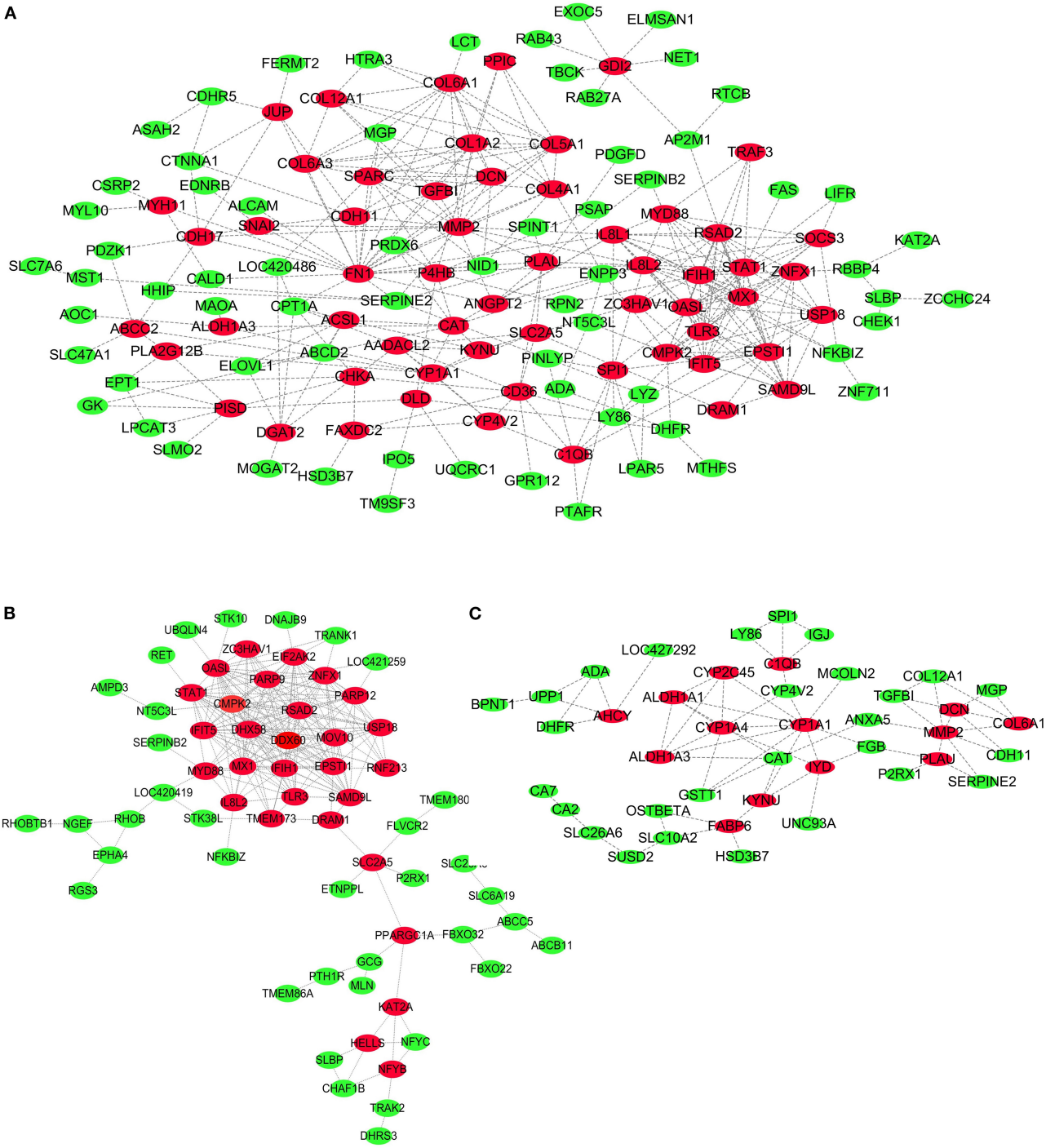

FIGURE 6 | PPI Network for DEGs in Broilers Jejuna Treated with and without LPC. **(A–C)** PPI network for DEGs identified by three comparisons (LPC1000 and LPC500 vs. control, and LPC1000 vs. LPC500, respectively).

IRF1 signaling. Moreover, *IRF1* could regulate *TET1* expression by binding to its promoter (17).

EPSTI1, named epithelial-stromal interaction 1, was widely expressed in immune cell types and had a vital role in immune privilege and function. In our study, *EPSTI1* was significantly up-regulated in chicken jejuna from LPC500 and LPC1000 groups compared to the control which was consistent with Kim et al. who reported that *EPSTI1* was highly expressed in macrophages activated by the exposure to lipopolysaccharide (LPS) and IFNγ (18). Macrophage polarization was important

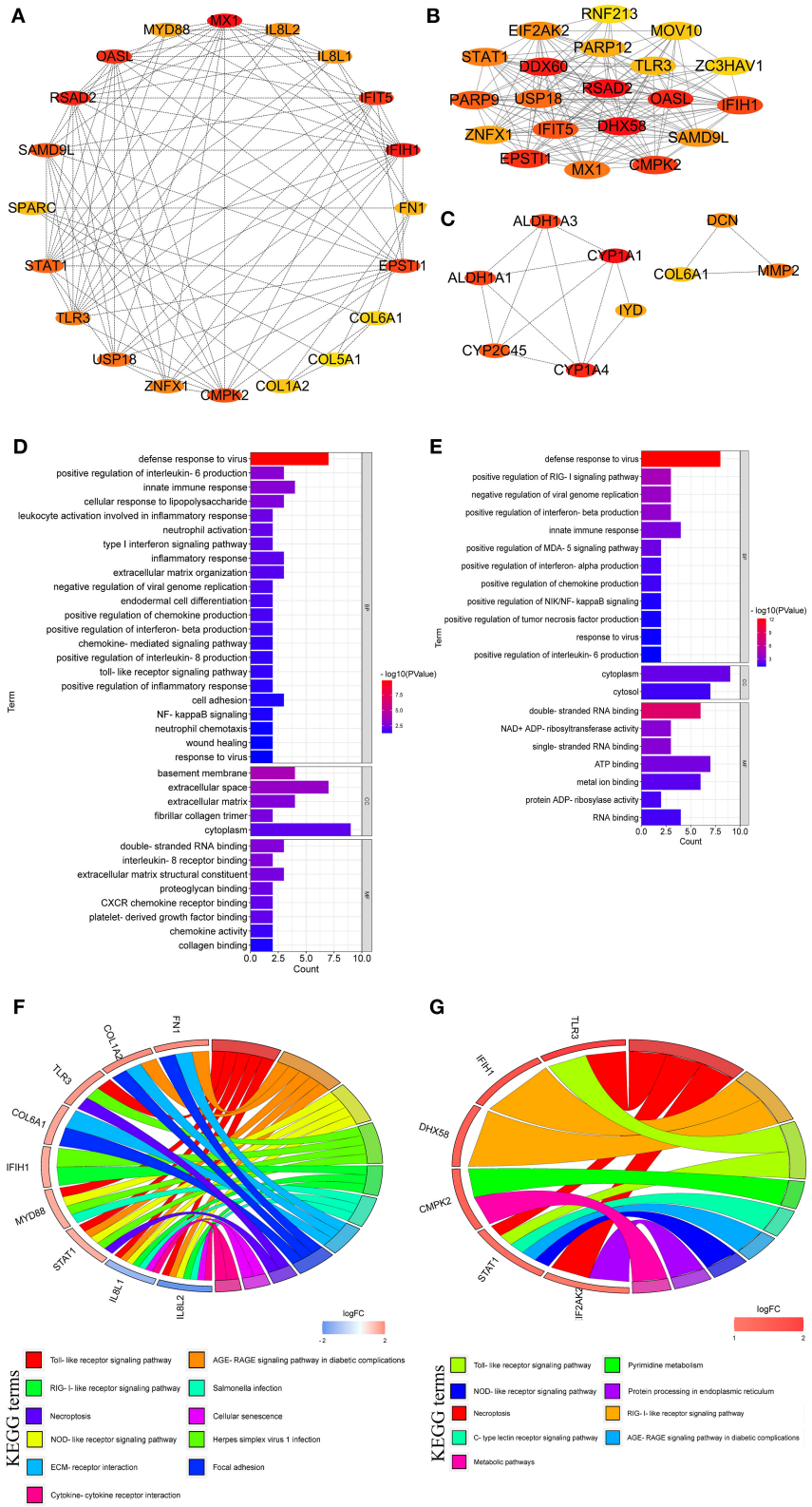

FIGURE 7 | Hub genes linked to the regulation of LPC on the jejuna morphology in broiler. **(A–C)** Hub genes identified by three comparisons (LPC1000 and LPC500 vs. control, and LPC1000 vs. LPC500, respectively); **(D,E)** GO enrichment for hub genes identified by two comparisons (LPC1000 and LPC500 vs. control, respectively); and **(F,G)** KEGG enrichment for hub genes identified by two comparisons (LPC1000 and LPC500 vs. control, respectively).

for the resistance to various infections (18). Additionally, *EPSTI1* was of great importance for antiviral activity mediated by IL-28A. *EPSTI1* actually restrained HCV replication without interferon, and *EPSTI1* knockdown resulted in viral enhancement. EPSTI1 could activate the *PKR* gene promoter and induce multiple *PKR*-dependent genes (such as *OAS1*, *IFIT1*, and *IFNβ*) responsible for the antiviral activity of *EPSTI1* (14).

CMPK2, known as cytidine/uridine monophosphate kinase 2, was a vital factor for innate immunity and infection. *CMPK2* played a critical role in dengue virus (DENV)-induced mitochondrial oxidative stress, cytokine release, and mitochondrial DNA (mtDNA) release to the cytosol. CMPK2 depletion could suppress the DENV-induced cell migration, TLR9 activation, and inflammasome pathway, and the increasing viral production (19). CMPK2 mediated the antiviral activity (19). In the present study, *CMPK2* was obviously up-regulated in chicken jejuna from LPC-treated groups which agree with the report that *CMPK2* was involved in mtDNA synthesis and antiviral immunity in animals (20). Multiple tissues exist for *CMPK2* expression, and bacterial infection could upregulate *CMPK2* expression in a time-dependent manner. *CMPK2* induced NLRP3 activation and mtDNA synthesis. *CMPK2* overexpression protected the intestinal barrier and hindered bacterial colonization (20).

IFIH1 (Interferon induced with helicase C domain 1) is an important virus cytosolic sensor encoding MDA5 protein. *IFIH1* variants were significantly enriched in children with Very Early Onset Inflammatory Bowel Disease (VEOIBD) compared to the control (21). Partial or complete MDA5 protein deficiency was linked to VEOIBD with variable expressivity and penetrance, implying a vital role for impaired intestinal viral sensing in the pathogenesis of Inflammatory Bowel Disease (IBD) (21). *IFIH1* also induced an antiviral Type I interferon (IFN) state. *IFIH1* overexpression increased Type I IFN activity. *FIH1* mutation might be related to the inflammatory cell infiltration into the intestinal epithelium and the thickened states and edema of the small intestine and colon (22). *IFIH1* was also the regulatory factor for facilitating M1 macrophage polarization by activating the*IRF3* gene. Viral RNAs stimulated the activation of IFIH1-IRF3. IFIH1-IRF3 activation induced by LPS was in a MyD88-dependent manner (22).

IFITs played a vital role in maintaining homeostasis and regulating immune responses. In HEK293T cells, *IFIT5* functioned as the negative regulatory factor in the IFN pathway. *IFIT5* might inhibit *IFNβ* promoter activities by targeting *IRF3*. IFIT5 reduced the IRF3 protein phosphorylation and restrained the *IRF3* nuclear translocation (23).

USP18, a mitochondria-localized deubiquitinase, specifically interacted with mitochondrial antiviral signaling protein (MAVS) and promoted K63-linked polyubiquitination and subsequent MAVS aggregation. *USP18* upregulated the IFN production and expression following virus infection (24). Mice with *USP18* deficiency were more susceptible to RNA virus infection. USP18, which served as a scaffold protein, could enhance the TRIM31 re-localization and facilitate the interactions between MAVS and TRIM31 in mitochondria (25). *USP18* was the negative regulatory factor for IFN signaling, and DENV infection significantly increased *USP18* expression (26).

STAT1, aka signal transducer and activator of transcription 1, might play a central role in the intestinal epithelium heath in a caspase-8-dependentmanner because it was located at the crossroad of multiple cell death-associated signaling pathways. In *CASP8*-deficient mice, *STAT1* activation in epithelium aggravated the sensibility toward bacterial-induced enteritis, intestinal inflammation, and lethality. *STAT1* depletion abrogated the intestinal barrier breakdown, epithelial cell loss, cell death, and systemic infection (27).

As discussed above, the hub genes, including *RSAD2*, *OASL*, *EPSTI1*, *CMPK2*, *IFIH1*, *IFIT5*, *USP18*, *MX1*, and *STAT1*, might be closely related to intestinal inflammation and infection, which affected the intestinal morphology characterized by the obvious changes in villus height and width, and crypt depth (28–31).

Signaling Pathways by Which LPC Increased the Jejuna Morphology in Broilers

In this research, we found that LPC regulated jejuna morphology via multiple signaling pathways, such as toll-like receptor (TLR), nod-like receptor (NLR), and necroptosis pathways. Previous studies indicated that TLR, NLR, and RILR were a variety of pathogen pattern recognition receptors involved in maintaining intestinal morphology, homeostasis and health (32–41). *TLR* mediated the inflammatory responses of intestinal mucosa macrophages to resist the pathogen invasion (32). *TLR* stimulation was enough to increase the MTDH expression, but MTDH depletion was also enough to restrain macrophages from producing inflammatory cytokines induced by TLR. Moreover, TLR could induce NFκB and MAPK signaling which were closely linked to the alleviated inflammatory reaction and the improved jejunal morphology after LPS stimulation (32, 33).

TLR2 might regulate several essential enteric physiological functions and pathological processes, including innate immune, peristaltic reflexes, intestinal serotonergic response, enteropathogenic infections, and gastrointestinal fluid absorption or secretion (34). *TLR2* activation might involve in the inflammatory response of the neuroendocrine cells. *TLR2* inhibition alleviates the oxidative stress and tissue damage of the intestinal tract, characterized by reduced pro-inflammatory cytokines production and restored SERT activity (34). When exposed to commensal or pathogenic bacteria, the intestinal innate immune cells would release the TLRs ligands (35). TLR signaling pathway mediated the intestinal microbiota disorder and contributed to the intestinal Graft-vs.-host disease development (35).

NLRs, a kind of cytosolic pattern-recognition receptors, played an important role in mucosal immune defense, intestinal infections, maintaining gut homeostasis and morphology, and shaping the microbiota. The current study showed that DEGs in chicken jejuna between LPC-treated groups and the control enriched in NLR signaling pathway which agree with the research that NLRs might function as the intestinal barrier guardian, given their association with NOD2 and inflammatory

bowel disease (36). NLRs mediated inflammatory cell pyroptosis, caspase-1 activation. NLRs might be involved in the common gastrointestinal bacterial pathogen infection in the small intestine (37). NLRs also regulate intestinal microbiota. NLR proteins, kind of cytoplasmic microbial sensors, were involved in various intestinal disorders, such as inflammatory bowel diseases (38). NLRs mediated gut protection by regulating the intricate interaction among immune, stromal, and epithelial cells. NLRs-mediated protection often needed the assistance of STAT3, MAPK, and NFκB pathways (39). Inflammation-related signaling pathways, including RILR, TLR, NLRs, NFκB, Jak-STAT, and TNF, mediated the intestinal epithelial cell (IEC) infection and severe inflammation induced by transmissible infection gastroenteritis virus (40).

Necroptosis, a kind of programmed cell death, was recently discovered. It combined the characteristics of necrosis and apoptosis, which was important in the intestinal injury and morphology. In our study, DEGs in chicken jejuna between LPC-treated groups and the control enriched in necroptosis signaling pathway which was consistent with the research that LPS resulted in typical cell necrosis characterized by enhanced expression of necroptosis protein (including RIP1, RIP3, MLKL, PGAM5, DRP1, and HMGB1) and the impairment of jejunal morphology in pig (41). Also, necroptosis dysregulation prevented the resolution of intestinal inflammation. E-type prostanoid receptor 4 suppressed the necroptosis by converging on receptor-interacting protein kinase 1 to reduce TNF-induced activation and the necroptosis effector membrane translocation in human and mouse IECs (42).

CONCLUSIONS

In conclusion, this study provides a valuable resource for identifying genes and signaling pathways associated with the regulation of LPC on the jejuna morphology in broiler.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94622.

ETHICS STATEMENT

The animal study was reviewed and approved by Anhui Science and Technology University (Bengbu, China) Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BY analyzed the results and prepared the tables and figures. XL wrote the manuscript. A-MA-M and NM revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Talent Introduction Program of Anhui Science and Technology University (No. DKYJ202003).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.911496/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 19 July 2022
doi: 10.3389/fvets.2022.946148

Mechanism of Lysoforte in Improving Jejuna Morphology and Health in Broiler Chickens

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Lysoforte (LFT) plays a vital role in maintaining broilers' health and intestinal morphology. However, the mechanism behind the effects of LFT improving intestinal morphology and health is still unclear. Therefore, this study was implemented to explore the central genes linked to the regulatory effect of LFT. Seventy-five newly hatched Cobb 500 male broilers were randomly divided into three groups: control, LFT500, and LFT1000 groups, with 25 chicks per group. The control chicks were provided with the basal diet, and the birds in LFT500 and LFT1000 groups were offered the same basal diet with 500 g/ton and 1,000 g/ton LFT, respectively. GSE94622 dataset consisted of the control and two LFT-treated groups (LFT500 and LFT1000). Jejuna samples were obtained from Gene Expression Omnibus (GEO). Totally 106–344 DEGs were obtained by comparing LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500. Gene ontology (GO) enrichment suggested that the DEGs are mainly related to the phosphatidylethanolamine biosynthetic process and neuron projection extension. KEGG analysis suggested the DEGs were enriched in AGE-RAGE, fatty acid elongation, ECM-receptor interaction (ECMRI), glycerophospholipid metabolism, focal adhesion, unsaturated fatty acids biosynthesis, and ABC transporters. Moreover, 29 genes, such as *REG4*, *GJB1*, *KAT2A*, *APOA5*, *SERPINE2*, *ELOVL1*, *ABCC2*, *ANKRD9*, *CYP4V2*, and *PISD*, might be closely related to promoting jejuna morphology in broilers. Taken together, our observation enhances the understanding of LFT in maintaining intestinal architecture and the general health of broiler chickens.

Keywords: gene, signaling pathway, Lysoforte, jejuna, broiler

OPEN ACCESS

Edited by:
Vinod Kumar Paswan, Banaras Hindu University, India

Reviewed by:
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Mahmoud M. Alagawany, Zagazig University, Egypt
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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: *17 May 2022*
Accepted: *21 June 2022*
Published: *19 July 2022*

Citation:
Li X, Shi X, Mesalam NM, Liu L, Chen Z and Yang B (2022) Mechanism of Lysoforte in Improving Jejuna Morphology and Health in Broiler Chickens. Front. Vet. Sci. 9:946148. doi: 10.3389/fvets.2022.946148

INTRODUCTION

Fats and oils, which are the most important dietary sources of energy, are excellent ways to accumulate the energy requirements for the optimized weight gain of broiler chickens (1, 2). Exogenous emulsifiers played an effective role in improving lipids utilization in broiler chickens, as the latter fail to gain lipids due to their poor emulsification in the gut (3). Lysoforte (LFT), a lysolecithin produced from lecithin that acts as an efficient emulsifier, could improve poultry growth, reproduction, and carcass quality by enhancing nutrient digestion and absorption, as well as reducing their mortality. In broiler chickens, LFT supplementation elevated average daily gain (ADG), final body weight, relative growth rate, dressing percent, carcass quality, net profit, total

return, economic efficiency, and reduced energy matrix value, FCR, and mortality rate (4, 5). Previous research has shown that LFT supplementation increased saturated fatty acids absorption. In addition, a synergistic effect has existed between LFT and enzymes in broilers (6). Papadopoulos et al. (7) reported that fat digestibility, digesta viscosity, and apparent metabolizable energy in chicken were improved by LFT supplementation.

LFT may also have a vital role in maintaining intestinal morphology and health in broilers. LFT supplementation decreased the mucosal thickness at 28 day and induced alterations in the duodenum morphology (7). LFT addition significantly increased the average villus length and width (8). LFT addition had the potential to improve the chicken jejunal morphology and health due to the changes inducted by LFT in the intestinal epithelium (8). However, the mechanism by which LFT improves intestinal morphology and health in broilers is unclear. Therefore, we obtained the microarray data of broiler jejuna treated with or without LFT from the GEO dataset (https://www.ncbi.nlm.nih.gov/geo/) and identified differentially expressed genes (DEGs) in birds' jejuna, aiming to explore the mechanism behind the regulation of LFT on the jejuna morphology and health in broilers.

MATERIALS AND METHODS

Ethics Statement

The present study was approved by the protocol from Anhui Science and Technology University (Bengbu, China) Institutional Animal Care and Use Committee (ECASTU-2015-P08).

Animals, Feed, and Tissue Collection

Seventy-five newly hatched Cobb 500 male broilers were randomly divided into three groups: control, LFT500, and LFT1000 groups, with 25 chicks per group. The control chicks were provided with the basal diet, and the birds in LFT500 and LFT1000 groups were offered the same basal diet with 500 and 1,000 g/ton LFT, respectively (8). The study lasted for 4 weeks. Ingredients and nutrient contents of the basal diet are presented in **Supplementary Table 1**. All birds were placed in the room with adjoining floor pens and weighed individually per week (8). On test day 10, five chicks per group were randomly chosen and killed *via* cervical dislocation. Pieces of ∼10 cm in length were collected from the middle of the jejuna (8).

RNA Extraction and Microarray Analysis

Given the LFT effects on chicken jejunal morphology (**Supplementary Table 2**), ∼50 mg of jejuna mucosa was homogenized with Tri Reagent (8). Total RNA was extracted using Directzol RNA columns, and the quality, purity, and integrity of RNA were assessed (8). Microarray analysis was performed with the chicken genome 1.0 array (8). The jejuna gene expression data were deposited in GEO (accession number: GSE94622) (8).

Microarray Data

GSE94622 consisted of the control ($n = 5$; GSM2479490, GSM2479491, GSM2479506, GSM2479507, and GSM2479523), LFT500-treated ($n = 5$; GSM2479533, GSM2479517, GSM2479516, GSM2479501, and GSM2479500), and LFT1000-treated ($n = 5$; GSM2479503, GSM2479520, GSM2479521, GSM2479537, and GSM2479536) broiler jejuna samples obtained at the 10th day of the experiment.

Data Processing

To obtain the DEGs between the jejuna samples treated with and without LFT, GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) was used to analyze the data from GSE94622. DEGs were identified as the genes with |log2-fold change (FC)| >1 and $P < 0.05$.

Analysis of KEGG and Genetic Ontology for DEGs

KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/kobas3/genelist/) was used to analyze the signaling pathways for DEGs. Regarding the genetic ontology (GO) analysis, DEGs were analyzed with DAVID (https://david.ncifcrf.gov/).

Protein Classification and Reactome Analysis for DEGs

Protein classification and Reactome analysis for DEGs were performed with the PANTHER (http://pantherdb.org/) and KOBAS 3.0.

Protein–Protein Interaction

STRING (https://string-db.org/) was employed to form protein–protein interaction (PPI). Cytoscape (version 3.8.0, http://www.cytoscape.org/) was used for further visualization.

Hub Genes and Their Functions

CytoHubba (http://apps.cytoscape.org/apps/cytohubba) was used to reveal hub genes from the PPI network, then the hub gene functions were summarized using GeneCards (https://www.genecards.org/), previous reports, and NCBI (https://www.ncbi.nlm.nih.gov/).

RESULTS

The Outline of Transcripts and Genes in Broilers Jejuna

A total of 38,535 transcripts and 14,086 genes were observed in the chicken jejuna. Transcripts expression density and UMAP are indicated in **Figures 1A,B**. **Figures 1C–E** represents the volcano plots for DEGs in the three comparisons of LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500, respectively. The jejuna diagram for DEGs in the three comparisons mentioned above is shown in **Figure 1F**. As shown in **Supplementary Table 3**, a total of 174–547 differentially expressed transcripts (DETs) and 106–344 DEGs were identified by the three comparisons. Compared with the control jejuna, 311 transcripts and 224 genes were upregulated, while 236

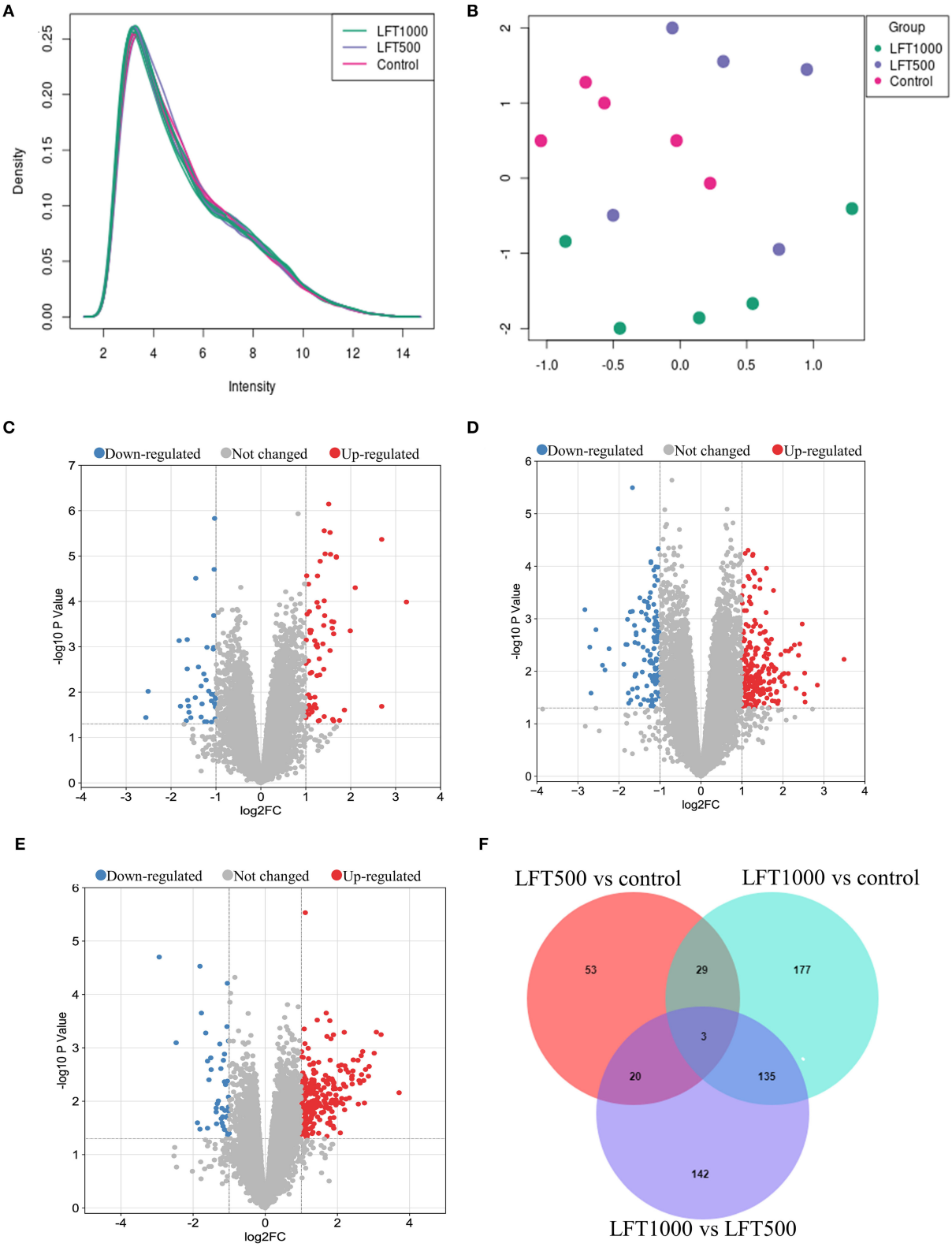

FIGURE 1 | Transcript and Gene Profiles in Broilers Jejuna Treated with and without LFT. **(A)** The density of transcripts expression; **(B)** UMAP; **(C–E)** Volcano plot of DEGs was identified by three comparisons (LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500, respectively). The red, gray, and blue spots represent the upregulated, unchanged, and downregulated genes. **(F)** Venn diagrams for the DEGs are identified in the three ways of comparisons mentioned above.

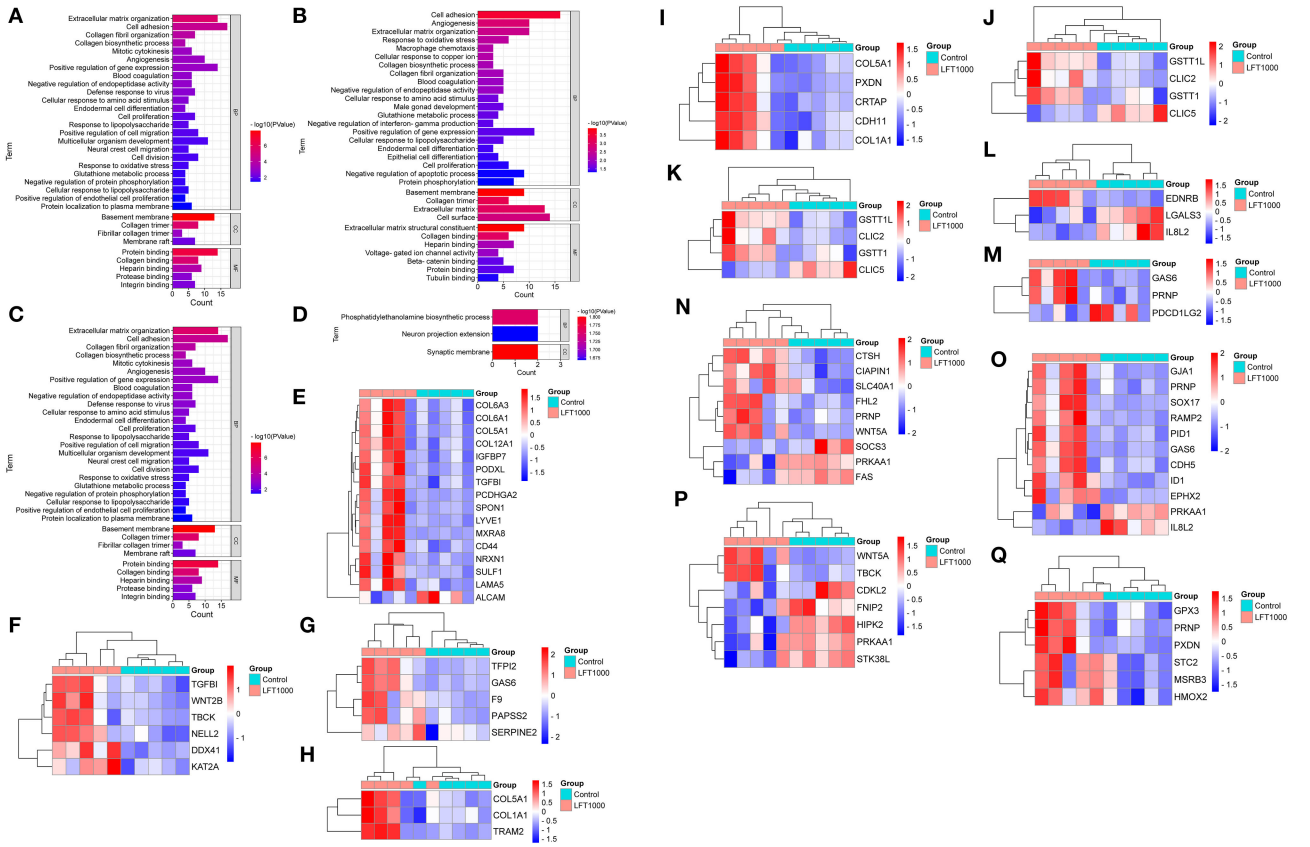

FIGURE 2 | GO Enrichment for DEGs in Broilers Jejuna Treated with and without LFT. **(A–C)** GO enrichment for DEGs identified in the three comparisons (LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500, respectively). **(D)** GO enrichment for the common DEGs in the two comparisons (LFT500 and LFT1000 vs. control). **(E–Q)** The heatmaps for DEGs in cell adhesion, cell proliferation, blood coagulation, collagen biosynthetic process, collagen fibril organization, glutathione metabolic process, macrophage chemotaxis, negative regulation of interferon-gamma production, positive regulation of gene expression, protein phosphorylation, and the response to oxidative stress.

transcripts and 120 genes were downregulated in LFT1000-treated jejuna (**Additional Files 1**, **2**); 98 transcripts and 68 genes were upregulated, while 76 transcripts and 38 genes were downregulated in LFT500-treated jejuna (**Additional Files 3**, **4**). The top 20 genes up- and downregulated in the three comparisons (LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500) were revealed in **Supplementary Tables 4–9**.

Twenty-nine common DEGs from the two comparisons (LFT1000 and LFT500 vs. control) are shown in **Supplementary Table 10**. These genes (including *REG4*, *GJB1*, *KAT2A*, *APOA5*, *SERPINE2*, *ELOVL1*, *ABCC2*, *ANKRD9*, *CYP4V2*, *PISD*, *PTGR1*, and *AKAP9*) might be closely related to promoting the jejuna morphology and health in broilers.

GO Analysis for DEGs

To reveal the biological processes associated with LFT regulation on broiler jejuna, GO analysis of DEGs in the three comparisons, including LFT1000 and LFT500 vs. control, and LFT1000 vs. LFT500 was illustrated in **Figures 2A–C** and **Additional Files 5–7**, respectively. DEGs obtained by comparing LFT1000 vs. control may participate in multiple biological processes, such as angiogenesis, blood coagulation, macrophage chemotaxis, glutathione metabolic process, collagen biosynthetic process, extracellular matrix (ECM) organization, the response to oxidative stress, and cell adhesion, proliferation, and differentiation.

DEGs between LFT1000 and the control may have a vital role in protein phosphorylation; leukocyte activation; adaptive immune response; antibacterial humoral response; inflammatory response; innate immune response; osteoclast differentiation; the negative regulation of viral genome replication and apoptotic process; and the positive regulation of production of chemokine, interferon-beta, interleukin-6, tumor necrosis factor; and the positive regulation of NIK/NF-kB pathway.

Twenty-nine common DEGs of two comparisons (LFT1000 and LFT500 vs. control) in the chicken jejuna were closely associated with phosphatidylethanolamine biosynthetic process and neuron projection extension (**Figure 2D**). In addition, **Figures 2E–Q** represents the heatmaps for DEGs in cell adhesion, cell proliferation, blood coagulation, collagen biosynthetic process, collagen fibril organization, glutathione metabolic process, macrophage chemotaxis, the negative regulation of

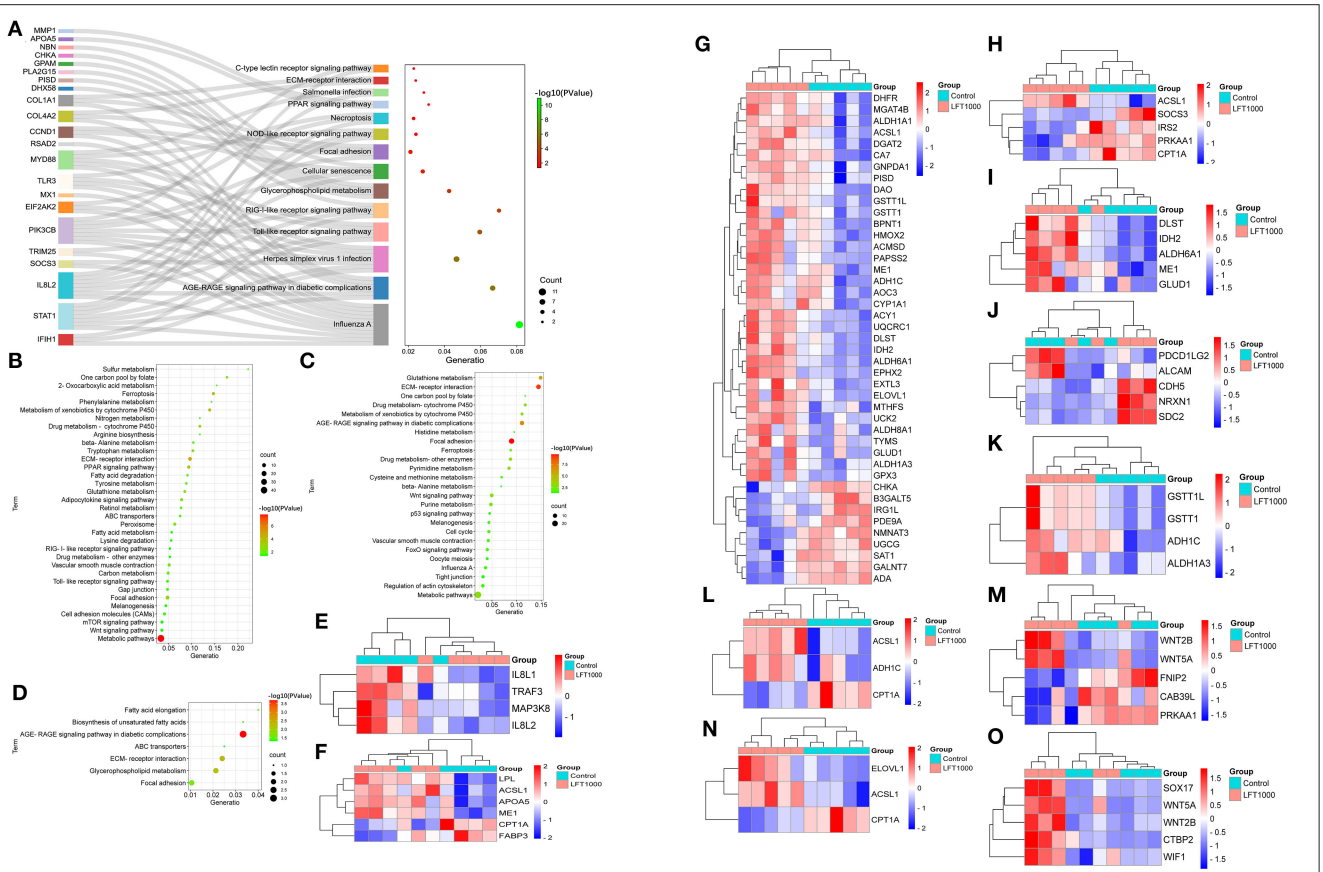

FIGURE 3 | KEGG Enrichment for DEGs in Broilers Jejuna Treated with and without LFT. **(A–C)** KEGG enrichment for DEGs was identified by three comparisons (LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500, respectively). **(D)** KEGG enrichment for the common DEGs was identified by two comparisons (LFT500 and LFT1000 vs. control). **(E–O)** Heatmaps for DEGs in TLR, PPAR, metabolism, adipocytokine, carbon metabolism, CAMs, drug metabolism-cytochrome P450, fatty acid degradation, mTOR, fatty acid metabolism, and Wnt signaling pathway.

interferon-gamma production, and the positive regulation of gene expression, protein phosphorylation, and the response to oxidative stress, respectively.

KEGG Enrichment for DEGs

To discover the pathways related to the regulation of LFT on broiler intestinal morphology and health, DEGs in the three comparisons, including LFT500 vs. control, LFT1000 vs. control, and LFT1000 vs. LFT500, were implemented in KEGG analysis, and the results were shown in **Figures 3A–C**, respectively (**Additional Files 8–10**). As illustrated in **Figure 3D**, the common DEGs of two comparisons (LFT500 and LFT1000 vs. control) are mainly linked to ABC transporters, AGE-RAGE, fatty acid elongation, focal adhesion, glycerophospholipid metabolism, ECMRI, and unsaturated fatty acids biosynthesis pathways. In addition, **Figures 3E–O** reveals toll-like receptor (TLR), PPAR, metabolism, adipocytokine, carbon metabolism, cell adhesion molecules, drug metabolism-cytochrome P450, fatty acid degradation, mTOR, fatty acid metabolism, and Wnt signaling pathway, respectively.

Reactome Enrichment and Protein Classification for DEGs

To further reveal the pathways related to LFT regulation on broiler jejuna heath, Reactome enrichment for DEGs in the three comparisons was performed. DEGs identified by comparing LFT1000 vs. the control may link to the metabolism, hemostasis, signal transduction, small molecules transport, carbohydrates metabolism, platelet activation, glycosaminoglycan metabolism, ECM organization, and neuronal system (**Figure 4A**). DEGs between LFT500 and the control related to hemostasis, signal transduction, cytokine signaling, neutrophil degranulation, TLR cascades, interleukin-2 family signaling, and platelet activation, signaling, and aggregation (**Figure 4B**).

Protein classification for DEGs in the three comparisons was performed. DEGs between LFT500 and the control groups might play an important role in acyltransferase, RNA helicase, kinase modulator, protease inhibitor, kinase activator, damaged DNA-binding protein, protein-binding activity modulator, ECM structural protein, and defense/immunity protein kinase (**Figure 4C**). DEGs between LFT1000 and the control

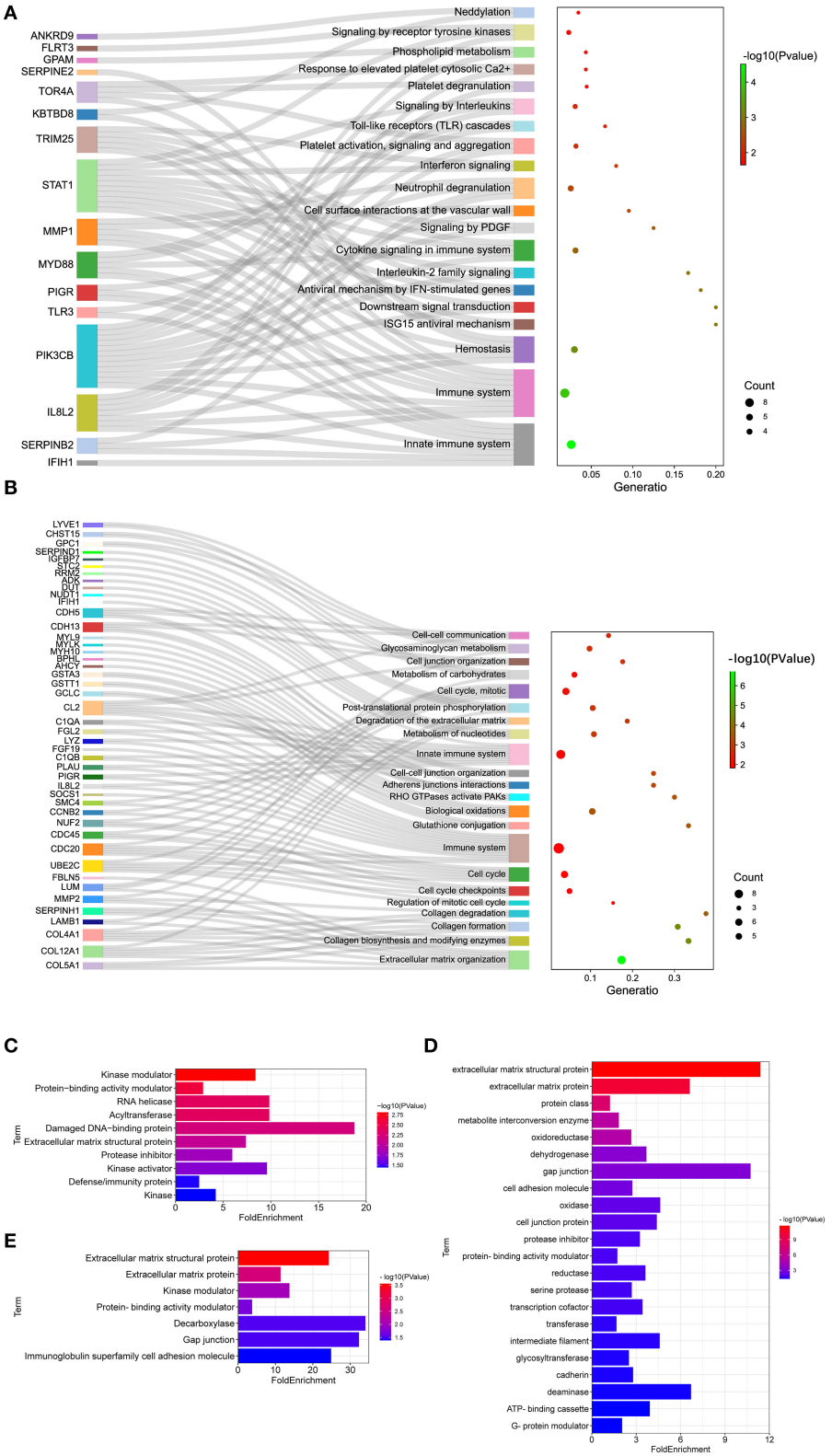

FIGURE 4 | Reactome Analysis and Protein Classification for DEGs. **(A,B)** Reactome analysis for DEGs was identified in two comparisons (LFT1000 and LFT500 vs. control). **(C–E)** Protein classification for DEGs was identified in two comparisons (LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500, respectively).

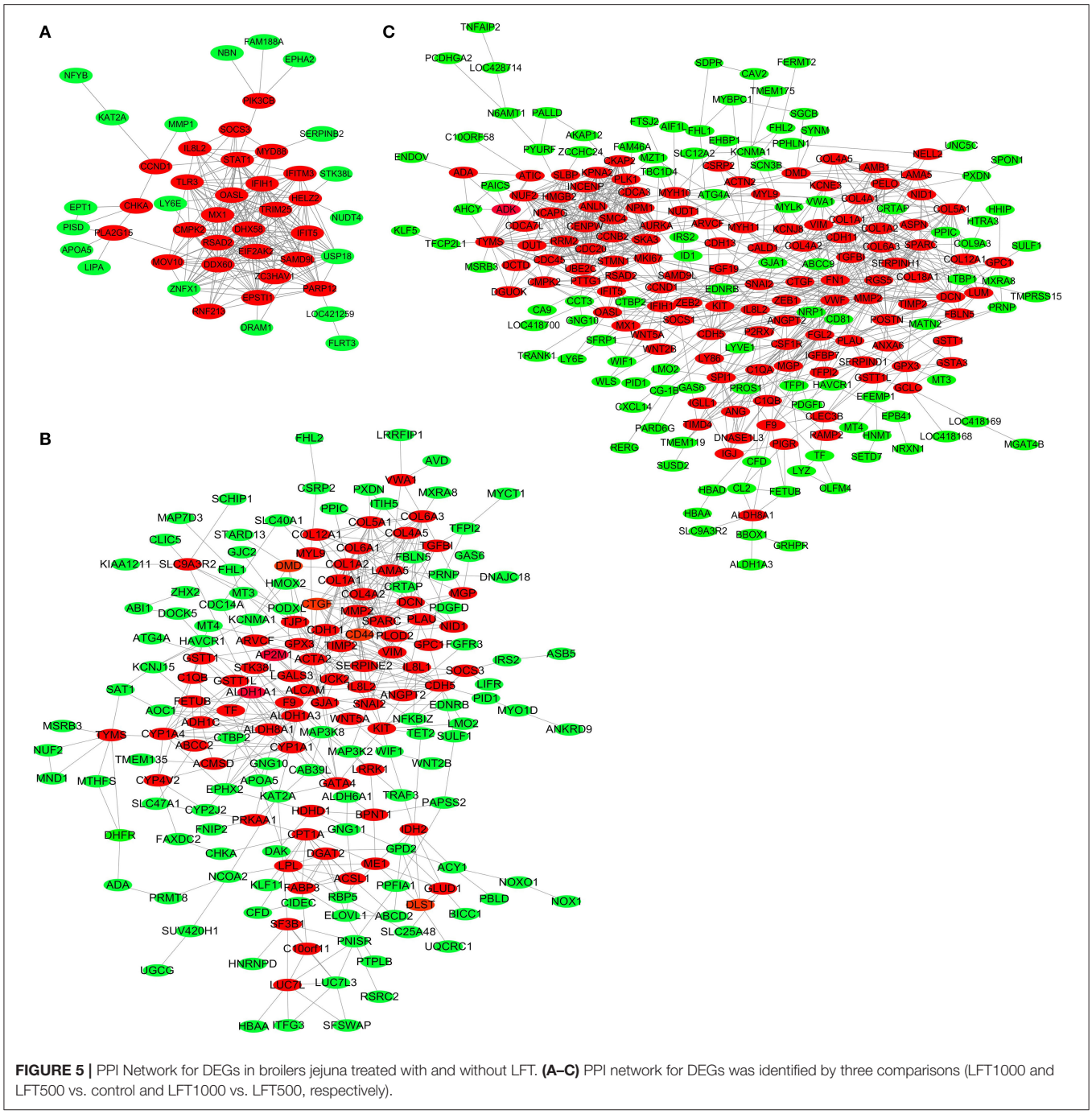

FIGURE 5 | PPI Network for DEGs in broilers jejuna treated with and without LFT. **(A–C)** PPI network for DEGs was identified by three comparisons (LFT1000 and LFT500 vs. control and LFT1000 vs. LFT500, respectively).

groups might contribute to reductase, oxidase, cadherin, deaminase, transferase, dehydrogenase, oxidoreductase, glycosyltransferase, gap junction, transcription cofactor, serine protease, intermediate filament, protease inhibitor, cell adhesion molecule, ECM structural protein, ECM protein, metabolite interconversion enzyme, cell junction protein, protein-binding activity modulator, and ATP-binding cassette G-protein modulator (**Figure 4D**). The common DEGs of two comparisons (LFT500 and LFT1000 vs. control) might link to decarboxylase, kinase modulator, gap junction, ECM protein, ECM structural protein, and protein-binding activity modulator, and immunoglobulin superfamily cell adhesion molecule (**Figure 4E**).

PPI Network

To further explore key genes, DEGs in the three comparisons, including LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500, were implemented in PPI networks analysis, and the results are shown in **Figures 5A–C**, respectively.

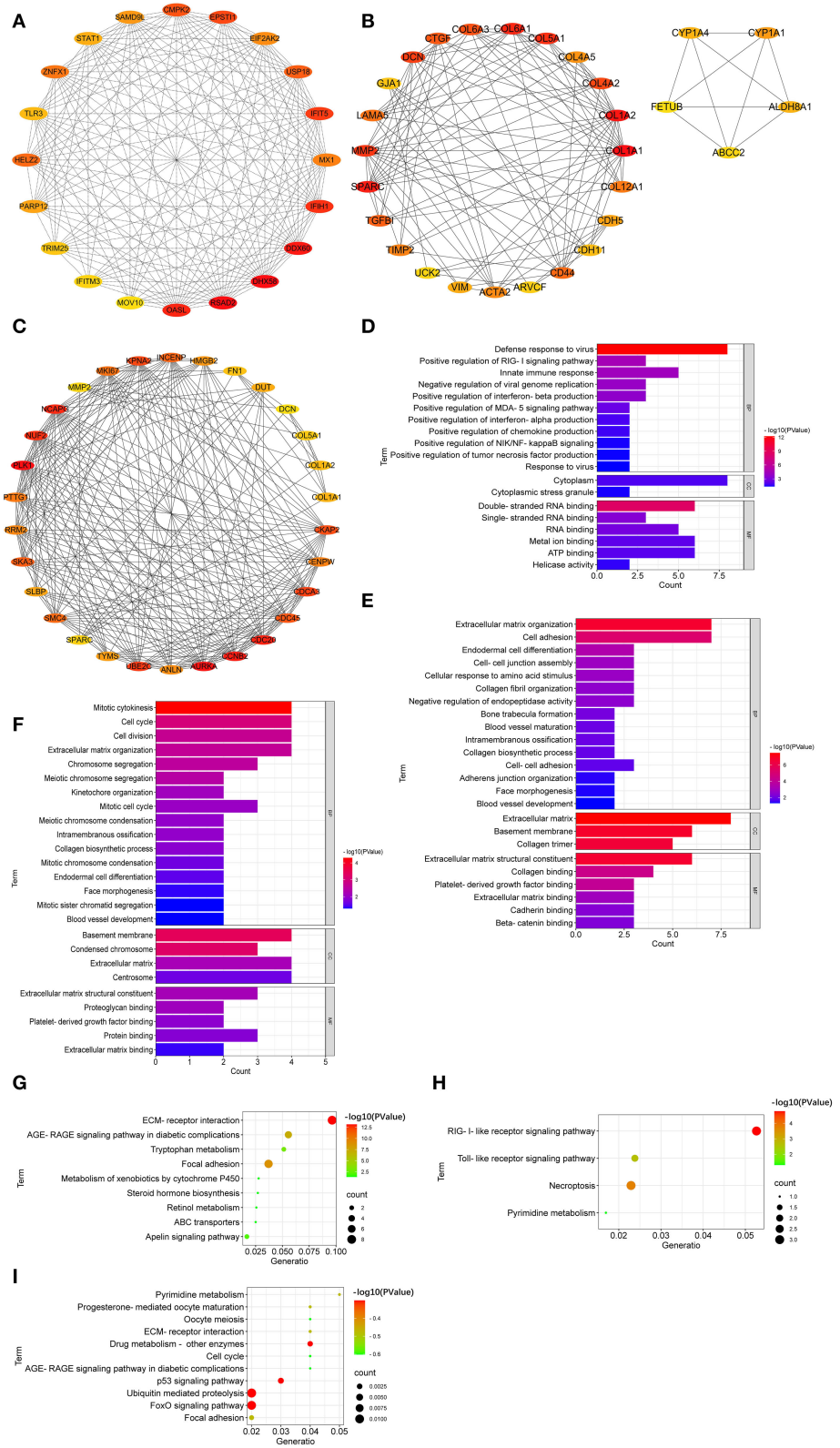

FIGURE 6 | Hub Genes Linked to the Regulation of LFT on the Morphology and Health in Broiler Jejuna. **(A–C)** Hub genes from DEGs were identified by three comparisons (LFT500 and LFT1000 vs. control and LFT1000 vs. LFT500, respectively). **(D–F)** GO and **(G–I)** KEGG enrichment for hub genes mentioned above.

Hub Genes and Their Function

The top 20 hub genes from DEGs between LFT500 and control groups included *RSAD2*, *DHX58*, *DDX60*, *OASL*, *IFIH1*, *IFIT5*, *EPSTI1*, *CMPK2*, *USP18*, and *HELZ2* (**Figure 6A**). The top 28 hub genes from DEGs between LFT1000 and control groups included *COL1A1*, *COL1A2*, *SPARC*, *COL6A1*, *COL5A1*, *MMP2*, *DCN*, *COL4A2*, *COL6A3*, and *CTGF* (**Figure 6B**). The top 30 hub genes from DEGs between LFT1000 and LFT500 groups included *PLK1*, *AURKA*, *CCNB2*, *CDC20*, *NCAPG*, *NUF2*, *UBE2C*, *CDCA3*, *KPNA2*, and *CKAP2* (**Figure 6C**).

GO enrichment suggested that the top 20 hub genes in the comparison of LFT500 vs. control related to innate immune response; the positive regulation of the production of chemokine, interferon-alpha, interferon-beta, and tumor necrosis factor; and the positive regulation of MDA-5, NIK/NF-kB, and RIG-I signaling pathway (**Figure 6D**). The top 28 hub genes from DEGs between LFT1000 and control groups participated in cell adhesion, endodermal cell differentiation, cell–cell junction assembly, intramembranous ossification, ECM organization, collagen fibril organization, blood vessel maturation, collagen biosynthetic process, blood vessel development, and the negative regulation of endopeptidase activity (**Figure 6E**). The top 30 hub genes from DEGs between LFT500 and LFT1000 groups are linked to cell division, cell cycle, mitotic cytokinesis, intramembranous ossification, kinetochore organization, chromosome segregation, blood vessel development, mitotic cell cycle, endodermal cell differentiation, mitotic chromosome condensation, collagen biosynthetic process, meiotic chromosome condensation, ECM, mitotic sister chromatid segregation, etc (**Figure 6F**).

KEGG enrichment suggested that the top 20 hub genes from DEGs between LFT500 and control involved in RIG-I-like receptor, pyrimidine metabolism, necroptosis, and TLR pathways (**Figure 6G**). The top 28 hub genes compared to LFT1000 vs. control linked to tryptophan metabolism, focal adhesion, retinol metabolism, ABC transporters, AGE-RAGE, apelin, and xenobiotics metabolism by cytochrome P450 signaling pathways (**Figure 6H**). The top 30 hub genes from DEGs between LFT1000 and LFT500 groups participated in p53, Fox O, AGE-RAGE, cell cycle, focal adhesion, drug metabolism, pyrimidine metabolism, and ECMRI signaling pathways (**Figure 6I**).

DISCUSSION

Hub Genes by Which LFT Maintains the Intestinal Morphology and Health in Broilers

In this study, multiple hub genes, such as *REG4*, *KAT2A*, *APOA5*, *SERPINE2*, *ELOVL1*, *ABCC2*, *ANKRD9*, *CYP4V2*, and *PISD1*, might participate in the regulation of LFT on intestinal morphology and health in broilers. For instance, *REG4*, a member of the small secretory protein family, was reported to participate in inflammatory bowel diseases and intestinal cancers (9–12).

In our study, 500 and 1,000 g/ton LFT treatment increased the *REG4* expression in the chicken jejuna (2.36- and 2.60-fold), which is consistent with Qi et al. who demonstrated that *REG4* was involved in membrane attack complexes killed inflammatory *Escherichia coli* (*E. coli*) to maintain gut health, and *REG4* gene knockdown increased the content of *E. coli* in the intestinal tract (9). *REG4* was obviously upregulated in colorectal cancer (CRC) tissue compared to the normal tissue. *REG4* expression in CRC tissue was linked to distant and lymph-node metastasis and histologic grade. *REG4* expression in CRC patients showed a worse prognosis (10). REG4 was the biomarker to predict concurrent chemoradiotherapy resistance in patients with rectal cancer. Previous research showed that the significant upregulation of the *REG4* gene was closely related to the undesirable outcome and the aggressive phenotype in rectal cancer patients (11). In our study, *REG4* played an important role in cell regeneration and proliferation. *REG4* expression was linked to higher overall survival and favorable clinicopathological parameters in CRC patients (12).

KAT2A, named lysine acetyltransferase 2A, inhibited the proliferation and growth of the intestinal cell, especially in CRC cells. *KAT2A*, succinylate, and succinyltransferase could decrease the α-KGDH complex entered the nucleus, reduce the gene expression, and inhibit the cell proliferation and growth in intestinal cancers (13). Histone acetyltransferase *KAT2A* could interact with long noncoding RNA LBX2-AS1 and RNA-binding protein PTBP1 and regulate the cell proliferation and invasion in CRC (14).

In humans and animals, *APOA5* was a vital gene for intestine chylomicron production and lipids metabolism. *APOA5* decreased the serum triglyceride (TG) by restraining ANGPTL3/8-mediated lipoprotein lipase inhibition (15). Variants in the *APOA5* gene affected TG concentrations and the entire lipoprotein subclass distribution and caused hypertriglyceridemia (16). Hypermethylation in exon 3 of the *APOA5* gene had a positive correlation with the lipoprotein profile and TG concentration linked to atherogenic dyslipidemia. The highest TG concentrations were observed in carriers with a high methylation percentage in the exon 3 of the *APOA5* gene (17). In our study, *APOA5* expression was obviously improved 1.65- and 2.05-fold in chicken jejuna by 500 and 1,000 g/ton LFT treatment, which was consistent with the previous finding that *APOA5* might control TG synthesis and secretion in the intestine. In the TC-7 cell line, saturated fatty acids stimulation obviously increased the *APOA5* gene expression; Similarly, fatty acid butyrate administration improved *APOA5* expression by ∼4 times; PPARα agonist treatment also enhanced the *APOA5* expression by 60% (18). In addition, PPARα has a vital role in lipid metabolism and improves ketogenesis and oxidation of fatty acid. PPARα activation reduced food intake and improved insulin sensitivity. Wy-14643 administration significantly increased the expression of *HMG-CoAS2* and *CPT1A* genes in the jejunum. The induction of *HMG-CoAS2* and *CPT1A* expression in the jejunum was linked to the decreased content of lipid droplet. *HMG-CoAS2* and *CPT1A* were two important enzymes for ketogenesis (19).

SERPINE2 might be a vital gene for intestinal health and disease, such as colorectal cancer. Intestinal epithelial cells (IECs), activation of oncogenic extracellular signal-related kinase (ERK), Ras, or BRAF strongly upregulated the SERPINE2 protein expression and secretion (20). *SERPINE2* gene expression was also dramatically increased in CRC cells compared with normal IECs; In the HCT116 cell, *SERPINE2* knockdown distinctly decreased the anchorage-independent growth, tumor formation, and cell migration in nude mice; *SERPINE2* mRNA level in CRC cell lines was markedly decreased by U0126 (a highly specific MEK1/2inhibitor) administration (21).

ELOVL1, a widely expressed gene in tissues from the ELOVL family, encoded the fatty acid elongase to produce C20–C28 fatty acids. In this study, *ELOVL1* was observably upregulated in chicken jejuna from LPC-treated groups, consistent with the report that *ELOVL1* regulated the very-long-chain fatty acid and sphingolipids synthesis, and was closely associated with the intestinal barrier function (22, 23). *ELOVL1* expression induced by inhibiting mTOR1 decreased fatty acids synthesis (22, 23). A previous study in mice indicated that *ELOVL1* knockout induced the defects in the epidermal barrier and the death after birth. In the epidermis of *ELOVL1* knockout mice, the content of C24 sphingomyelin was reduced, but the C20 sphingomyelin level was increased (23).

ABCC2, the gene encoding multidrug resistance protein 2 (MRP2), was located on the small intestinal epithelial brush border membrane. *ABCC2* had a vital role in regulating the absorption of nutrients and toxins (24–26). In this study, *ABCC2* expression was dramatically improved in chicken jejuna from LPC-treated groups. This result agreed with the report that *ABCC2* could limit the absorption of toxins, improve the endogenous xenobiotics and substances efflux, and mediate the beneficial effect of *Lactobacillus plantarum* on poultry intestines (24). The expression pattern of MRP2/ABCC2 in the small intestinal tract was tightly regulated. MRP2/ABCC2 expression in the small intestine was closely associated with ezrin phosphorylation status (25). *ABCC2*, *ABCC3*, and *ABCG2* were expressed in the intestine and could transport the glucuronidated compounds. *ABCC2* knockout significantly decreased the biliary excretion in mice (26). The exposure to thymeleatoxin reduced the amount of ABCC2 protein and the active ezrin. Moreover, cPKC activation weakened the interaction between ABCC2 and ezrin proteins (27).

Signaling Pathways by Which LFT Maintains the Intestinal Morphology and Health in Broilers

Our study found that LFT regulated the intestinal morphology and health in broilers *via* multifarious signaling pathways, including AGE-RAGE, ECMRI, focal adhesion, and ABC transporters. For example, *AGE* expression in the jejunal villi crypt as well as *RAGE* expression in the villi significantly enhanced the jejunal layer thickness, weight per length, and wall area (28). RAGE signaling was closely linked to intestinal permeability and inflammation. AGE-RAGE signaling and RAGE activation in the intestinal epithelium contributed to intestinal permeability and pathogenesis (29).

ECM, a vital component of the intestine, provided the structural framework and conveyed tissue-specific signals to the adjacent enterocytes. Porcine epidemic diarrhea virus (PEDV) infection resulted in extensive ECM remodeling in IECs. *SERPINE1* and *CD44*, two ECM-regulated genes, could enhance or inhibit the PEDV infection (30).

A previous research found that various signaling pathways were involved in intestinal schistosomiasis and trinitro-benzene-sulfonic acid-induced ileitis, such as ABC transporters, cell adhesion, ECMRI, antigen processing and presentation, TLR, and the response to chemical stimulus categories (31). The ABC-transporter mediated the cellular uptake, absorptive permeability, and intestinal absorption. For instance, the ABC-transporter-mediated efflux and the poor permeability were the major reasons for Rh2 poor absorption (32).

Fatty acid metabolism, such as fatty acid biosynthesis and elongation, might play a vital role in intestinal absorption and health. In our study, DEGs in chicken jejuna between LFT-treated groups and the control also enriched in fatty acid elongation and unsaturated fatty acids biosynthesis signaling pathways that were consistent with the report that polyunsaturated fatty acids, including oleic acid, linolenic acid, and conjugated linoleic acid (CLA), had a protective effect in the intestine morphology and health (33). In IECs, long-chain saturated fatty acids stimulated TG synthesis, and stearic acids and palmitic also stimulated phospholipid synthesis (34).

CLA potentially modulates gut microbiota and intestinal permeability. CLA increased intestinal permeability in the normal mice and obviously improved the tight junction proteins in the intestine of leptin-deficient mice (35). CLA increased the abundance of beneficial bacteria (such as *Roseburia*, *Dubosiella*, and *Anaerostipes*) and increased the abundance of pro-inflammatory bacteria (such as *Alistipes* and *Tyzzerella*) in eptin-deficient mice. In addition, gut microbiota was associated with intestinal permeability [39]. CLA increased the *SIgA* mRNA and SIgA protein content in the jejunal mucosa. CLA treatment significantly increased *PPARγ* expression in jejunum as well as lymphocyte proliferation, and the percent of T lymphocytes ($CD8^+$) in Peyer's node of broilers (36).

CLA addition could obviously enhance the immunity and antioxidant capacity of the intestinal mucosa in broilers. CLA supplementation at the level of 1.50% notably improved the $CD8^+$ T lymphocytes percentage in the duodenal epithelium, reducing the concentration of malondialdehyde and glutathione in the duodenal mucosa of the birds infected by *Eimeria acervuline* but had no effects on the activities of catalase and superoxide dismutase (37).

CONCLUSION

Taken together, signaling pathways (such as AGE-RAGE, fatty acid elongation, ECMRI, glycerophospholipid metabolism, focal adhesion, unsaturated fatty acids biosynthesis, and ABC transporters) and 29 genes (including *REG4*, *GJB1*, *KAT2A*,

APOA5, *SERPINE2*, *ELOVL1*, *ABCC2*, *ANKRD9*, *CYP4V2*, and *PISD*) might be closely related to promoting jejuna morphology in broilers. Our observation enhances the understanding of LFT in maintaining intestinal architecture and the general health of broiler chickens.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Protocol from Anhui Science and Technology University (Bengbu, China) Institutional Animal Care and Use Committee (ECASTU-2015-P08).

AUTHOR CONTRIBUTIONS

BY conceived the study. XL wrote the manuscript and prepared the figures. XS, NM, LL, and ZC prepared the tables and analyzed the results. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Talent Introduction Program of Anhui Science and Technology University (No. DKYJ202003) and the National Natural Science Foundation of China (No. 31560642).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.946148/full#supplementary-material

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

frontiers | Frontiers in Veterinary Science

TYPE Brief Research Report
PUBLISHED 05 August 2022
DOI 10.3389/fvets.2022.942159

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 12 May 2022
ACCEPTED 12 July 2022
PUBLISHED 05 August 2022

CITATION
Li X, Abdel-Moneim A-ME, Hu Z, Mesalam NM and Yang B (2022) Effects of chronic hypoxia on the gene expression profile in the embryonic heart in three Chinese indigenous chicken breeds (*Gallus gallus*). *Front. Vet. Sci.* 9:942159. doi: 10.3389/fvets.2022.942159

Effects of chronic hypoxia on the gene expression profile in the embryonic heart in three Chinese indigenous chicken breeds (*Gallus gallus*)

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Hypoxia exposure (HE) has adverse impacts on the embryonic development of chicken, whereas the mechanism underlying the response of the heart to HE during embryo development in birds is still unclear. Therefore, our study was designed to reveal the hub genes and the signaling pathways linked to chronic hypoxia stress. Thus, the gene expression microarray GSE12675, downloaded from the GEO database, included 12 embryonic heart samples in hypoxia and normoxia of three Chinese indigenous chicken breeds [Shouguang (SG), Tibetan (TB), and Dwarf Recessive White (DRW) chickens]. A total of 653 to 714 breed-specific differentially expressed genes (DEGs) were detected in each pairwise comparison. Gene ontology (GO) showed that the DEGs were mainly involved in biological processes, including vasoconstriction, cell differentiation, and the positive regulation of vasoconstriction. KEGG enrichment revealed that the DEGs were mainly enriched in MAPK, PPAR, insulin, adrenergic signaling in cardiomyocytes, etc. Moreover, 48 genes (e.g., *SGCD*, *DHRS9*, *HELQ*, *MCMDC2*, and *ESCO2*) might contribute to the response of the heart to HE. Taken together, the current study provides important clues for understanding the molecular mechanism of the heart's response to HE during the embryonic period of chicken.

KEYWORDS

signaling pathway, gene, chronic hypoxia, embryonic heart, chicken

Introduction

Hypoxia exposure (HE), a frequent natural event during the embryonic stage, has adverse impacts on the development and growth of chicken. HE could cause right ventricular hypertrophy, reduce the body weight, and increase the mortality of chicken embryo, and might be linked to cardiac defect and sudden death syndrome in chicken (1–4). HE altered the developmental trajectories and modified the phenotypes of the developing embryos (2). Also, HE improved the contents of lactic acid, tri-iodothyronine, corticosteroid, and thyroxine

in the plasma of chicken during the embryonic stage (3). Villamor et al. reported that HE (15% O_2) decreased the tolerance of the pulmonary artery to thromboxane A mimetic U-46619, norepinephrine, potassium chloride, endothelin-1, and electrical-field stimulation at embryonic day 19 (4). Inversely, hypoxic hearts showed an increase in left and right ventricular thickness and wall area in chicken embryo (4). In White Leghorn chicken, HE reduced the femoral flow but increased femoral artery resistance by regulating the nitric oxide production at embryonic day 19 (5).

Crossley et al. found that exposure to the three levels of hypoxia (15, 10, and 5% O_2) depressed the heart rate (HR) and blood pressure (BP) in chicken embryos (6). HE might induce the release of adrenaline and muscarine from autonomic nerve terminals and chromaffin tissue. In embryonic chicken, the tolerance of cardiovascular to hypoxia was involved in the central nervous system and the cholinergic regulation of arterial pressure (6). Severe hypoxia (5% O_2) resulted in significant HR changes. *In ovo*, the tolerance of HR to severe hypoxia consisted of an initial reduction in HR and the following partial HR recovery. Some factors of the egg, such as catecholamines, might be vital for the survival and response of the chicken embryo to hypoxia (7). HE also reduced the number of cardiomyocytes, restricted growth and heart development, and showed signs of cardiac insufficiency in the chick embryo (8). Hypoxic incubation increased the ratio of the ventricle to body mass on hatching days 11, 15, 17, and 19 and reduced absolute heart mass on hatching day 19. HE affected the proliferation rate and absolute mass of heart at hatching day 15 (8).

Previous studies have shown that a set of genes were involved in the tolerance and adaptation of chicken heart to hypoxia. To enhance the chicken cardiomyocytes in adapting to the hypoxia, the growth factors, stress proteins, and enzymes that were associated with anaerobic glycolysis were observably upregulated. Then, antioxidants and stress proteins were increased in the cardiomyocyte which was reoxygenated after hypoxia (9). HE enhanced the expression of multiple genes including cardiac troponin T, heme oxygenase, and hypoxia upregulated protein 1 in the chicken embryonic heart. Cardiac troponin T was associated with binding tropomyosin to regulate calcium binding and contractility of the heart muscle (10).

It was well-known that Shouguang (SG), Tibetan (TB), and Dwarf Recessive White (DRW) chickens were three Chinese indigenous chicken breeds with many excellent traits, such as outstanding meat quality and excellent environmental adaptability. For example, TB chickens, which inhabited the high-altitude Qinghai-Tibet Plateau for more than 1,000 years, had an adaptive ability to hypoxia (11). In this regard, it is of great significance to reveal potential candidate genes and signaling pathways for those traits. However, the mechanisms of the response to hypoxia in Chinese indigenous chicken breeds during the embryonic period are limited. In the previous study from Li and Zhao (11), the breed-specific differentially expressed gene (DEGs) in the hearts of SG and DRW chickens, and the common DEGs in the heart among the three breeds (SG, TB and DRW chickens) between hypoxia and normoxia have not been analyzed in depth. Therefore, based on the aforementioned research, this study was conducted to clarify the genes and signaling pathways linked to the response of the heart to chronic hypoxia in the three abovementioned chicken breeds.

Materials and methods

Sample collection, RNA extraction, and probe hybridization

The current research was performed based on the experiment that was completed by Li and Zhao from China Agricultural University (11). In total, 680 fertilized eggs were obtained from the China Agricultural University's Experimental Chicken Farm. Since the first day of the incubation, 120 SG, 120 DRW, and 100 TB chicken fertilized eggs were kept in the incubator with hypoxia (13% O_2) or normoxia (21% O_2) at a temperature of 37.8°C with 60% humidity. At embryonic day 17, the heart tissues from 5 chickens per group were mixed for further pool genome array analysis. Each pool was replicated two times. Subsequently, the total RNA of pool samples was extracted using TRIzol reagent. The hybridized probe array was stained with streptavidin-phycoerythrin in the GeneChip® Fluidics Station 450 (11).

Differential expression analysis

To explore the DEGs associated with the heart's response in chickens exposed to hypoxia, the data from GSE12675 were analyzed using the GEO2R software (http://www.ncbi.nlm.nih.gov/geo/geo2r). GSE12675, collected from the abovementioned study, included 12 embryonic heart samples in hypoxia and normoxia of three Chinese indigenous chicken breeds (SG, TB, and DRW chickens) with four per breed (11). A p-value of < 0.05 with $|\log_2 \text{Fold Change (FC)}| > 1$ was identified as the standard for the DEGs.

Gene ontology and KEGG analysis of the DEGs

To clarify the biological processes closely associated with the DEGs, gene ontology (GO) analysis was conducted using the DAVID database (https://david.ncifcrf.gov/summary.jsp). To annotate the signaling pathways for the DEGs, KOBAS (http://kobas.cbi.pku.edu.cn/kobas3/genelist/) was used for the KEGG analysis of DEGs.

Reactome analysis and protein classification

Reactome analysis for the DEGs was also implemented with the KOBAS 3.0 software. Moreover, protein classification analysis for the DEGs was tested using the PANTHER classification system (http://pantherdb.org/).

Protein-protein interaction network

The protein-protein interaction (PPI) network and its further visualization for the DEGs between the two groups were conducted using the STRING database (https://string-db.org/) and the Cytoscape 3.8.0 software (http://www.cytoscape.org/), respectively.

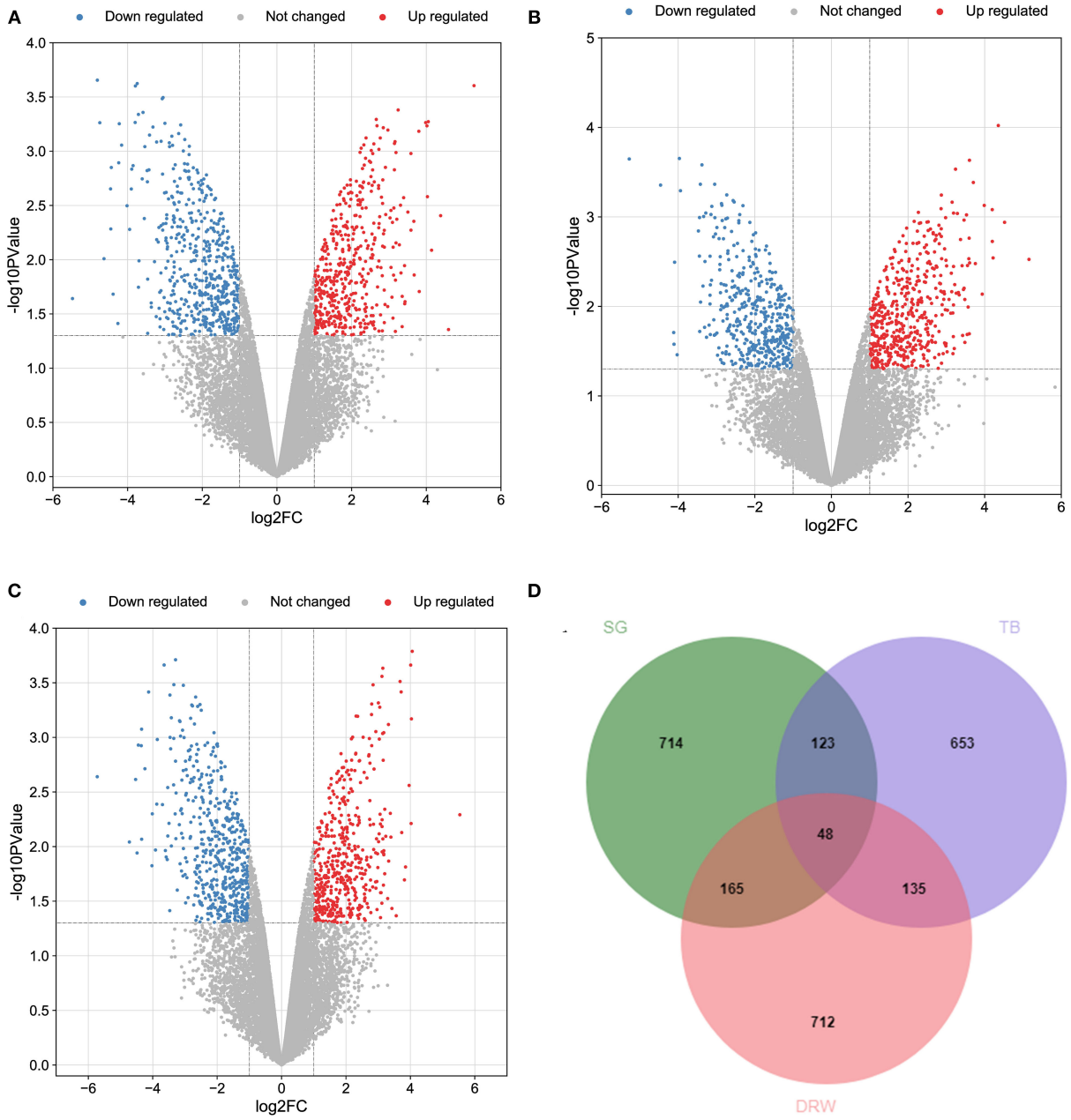

FIGURE 1
The profile of transcripts and genes in the hearts of chickens treated with hypoxia and normoxia. **(A–C)** revealed the volcano plot of the DEGs in the hearts of SG, TB, and DRW chickens treated with hypoxia and normoxia. The red and blue points represent the upregulated and downregulated genes. The gray spots mean unchanged genes. **(D)** indicated the Venn diagram for the DEGs mentioned above.

Hub genes and their functions

The CytoHubba plugin (http://apps.cytoscape.org/apps/cytohubba) was used to obtain the Hub genes. To evaluate the hubs of each gene, the Matthews correlation coefficient (MCC) algorithm was used to calculate the gene connectivity in the PPI network. Then, a new network was created. In the network, the hub nodes were colored according to their importance: from red (the most important) to yellow (the least important) (12). Hub gene functions were summarized with the help of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.-gov/), GeneCards (https://www.genecards.org/), STRING, and previous literature.

Results

Overview of the genes in chicken embryonic heart

Totally 14,086 genes were obtained from the heart samples of the three Chinese indigenous chicken breeds (SG, TB, and DRW chickens). Figures 1A–C represent the volcano plots for the DEGs in SG, TB, and DRW for the comparison of hypoxia vs. normoxia. The Venn diagram for the DEGs identified from the hearts of the three breeds is shown in Figure 1D. The DEGs (Supplementary Files 1–3) of the three breeds' hearts under hypoxia are shown in Supplementary Table 1. A total of 959 to 1,060 DEGs were identified in the three chicken breeds. Compared with the hearts in normoxia, 487, 508, and 491 genes were upregulated, and 463, 451, and 569 genes were downregulated in the SG, TB, and DRW chickens exposed to hypoxia, respectively (Supplementary Table 1).

The breed-specific DEGs in three chicken breeds' hearts under hypoxia are indicated in Supplementary Table 2. Totally 653 to 714 breed-specific DEGs (Supplementary Files 4–6) were identified in the three chicken breeds. Compared with the hearts in the normoxia groups, 330, 347, and 720 genes were upregulated, whereas 384, 306, and 392 genes were downregulated in the hypoxia groups of SG, TB, and DRW chickens, respectively (Supplementary Table 2).

In total, 48 common DEGs in three chicken breeds' hearts between hypoxia and normoxia are displayed in Supplementary Table 3. The common genes included *SGCD*, *BIRC7*, *HAVCR1*, *DHRS9*, *HELQ*, *DMRT1*, *SVOPL*, *DZANK1*, *LUZP2*, *MCMDC2*, *ESCO2*, and *CSF2*. Most of these genes may be associated with the response of the heart to HE. For example, the protein encoded by *SGCD* is a subcomplex of the dystrophin-glycoprotein complex (DGC). DGC forms a link between the F-actin cytoskeleton and the extracellular matrix. This protein is abundantly expressed in the skeletal and cardiac muscles. *SGCD* mutations are associated with autosomal recessive limb-girdle muscular dystrophy and dilated cardiomyopathy.

Moreover, the top 20 upregulated and downregulated breed-specific DEGs in SG, TB, and DRW chicken hearts under hypoxia are reported in Supplementary Tables 4–9 .

GO enrichment for the DEGs

As shown in Figure 2A, the special DEGs in SG chicken heart were involved in many biological processes, including cell differentiation, lipid biosynthetic process, TOR signaling, cell-matrix adhesion, platelet aggregation, protein polymerization, plasminogen activation, actin cytoskeleton organization, the positive regulation of cell division, synaptic transmission, glutamatergic, and the defense response to Gram-positive bacterium.

Also, as indicated in Figure 2B, the special DEGs in TB chicken heart were involved in various biological processes, such as the positive regulation of vasoconstriction, cell-cell signaling, modulation of synaptic transmission, nerve growth factor signaling pathway, regulation of neuron differentiation, cell proliferation in the forebrain, positive regulation of cytokinesis, anterior/posterior pattern specification, and lipid phosphorylation.

In addition, as revealed in Figure 2C, the special DEGs in DRW chicken heart may play a key role in various biological processes, such as vasoconstriction, animal organ morphogenesis, the response to interleukin-18, macrophage activation, immune response, cellular response to calcium ion, the regulation of angiogenesis, cellular response to tumor necrosis factor, and extracellular matrix organization.

Furthermore, the breed-special DEGs in the regulation of transcription from RNA polymerase II promoter and cell differentiation biological processes in SG chicken are indicated in Figures 2D,E, respectively. The breed-special DEGs in cell-cell signaling, modulation of synaptic transmission, positive regulation of vasoconstriction, and regulation of systemic arterial BP by vasopressin biological processes in TB chicken are shown in Figures 2F–I, respectively; The breed-special DEGs in animal organ morphogenesis, cellular response to tumor necrosis factor, cellular response to calcium ion, extracellular matrix organization, immune response, vasoconstriction, and the regulation of angiogenesis biological processes in DRW chicken are revealed in Figures 2J–P, respectively.

KEGG enrichment for the DEGs

As shown in Figure 3A, the breed-special DEGs in SG chicken heart were mainly involved in adrenergic signaling in cardiomyocytes, PPAR, MAPK, FoxO, calcium, apelin, toll-like receptors, neuroactive ligand-receptor interaction (NLRI), ECM-receptor interaction (ERI), RIG-I-like receptor, AGE-RAGE, fatty acid biosynthesis,

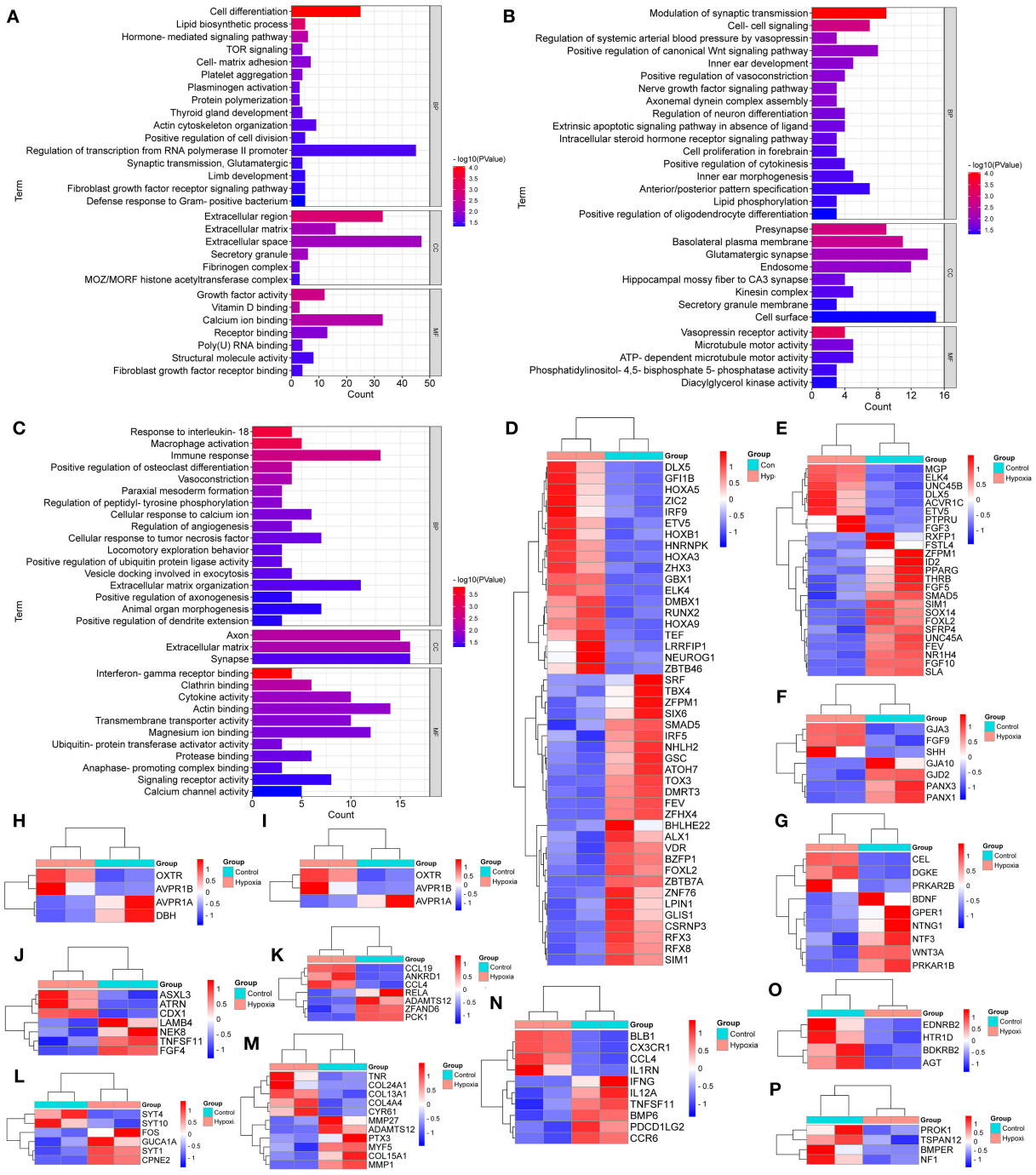

FIGURE 2
GO Enrichment for the breed-special degs in the hearts of chickens treated with hypoxia and normoxia. **(A–C)** indicated GO enrichment for the breed-special DEGs in the hearts of SG, TB, and DRW chickens treated with hypoxia and normoxia. **(D,E)** indicated the heatmaps of the breed-special DEGs in cell differentiation and the regulation of transcription from RNA polymerase II promoter in SG chicken, respectively. **(F–I)** revealed the heatmaps of the breed-special DEGs in cell-cell signaling; the modulation of synaptic transmission; the positive regulation of vasoconstriction; and the regulation of systemic arterial BP by vasopressin biological processes in TB chicken. **(J–P)** showed the heatmaps of the breed-special DEGs in animal organ morphogenesis, cellular response to tumor necrosis factor, cellular response to calcium ion, extracellular matrix organization, immune response, vasoconstriction, and the regulation of angiogenesis biological processes in DRW chicken.

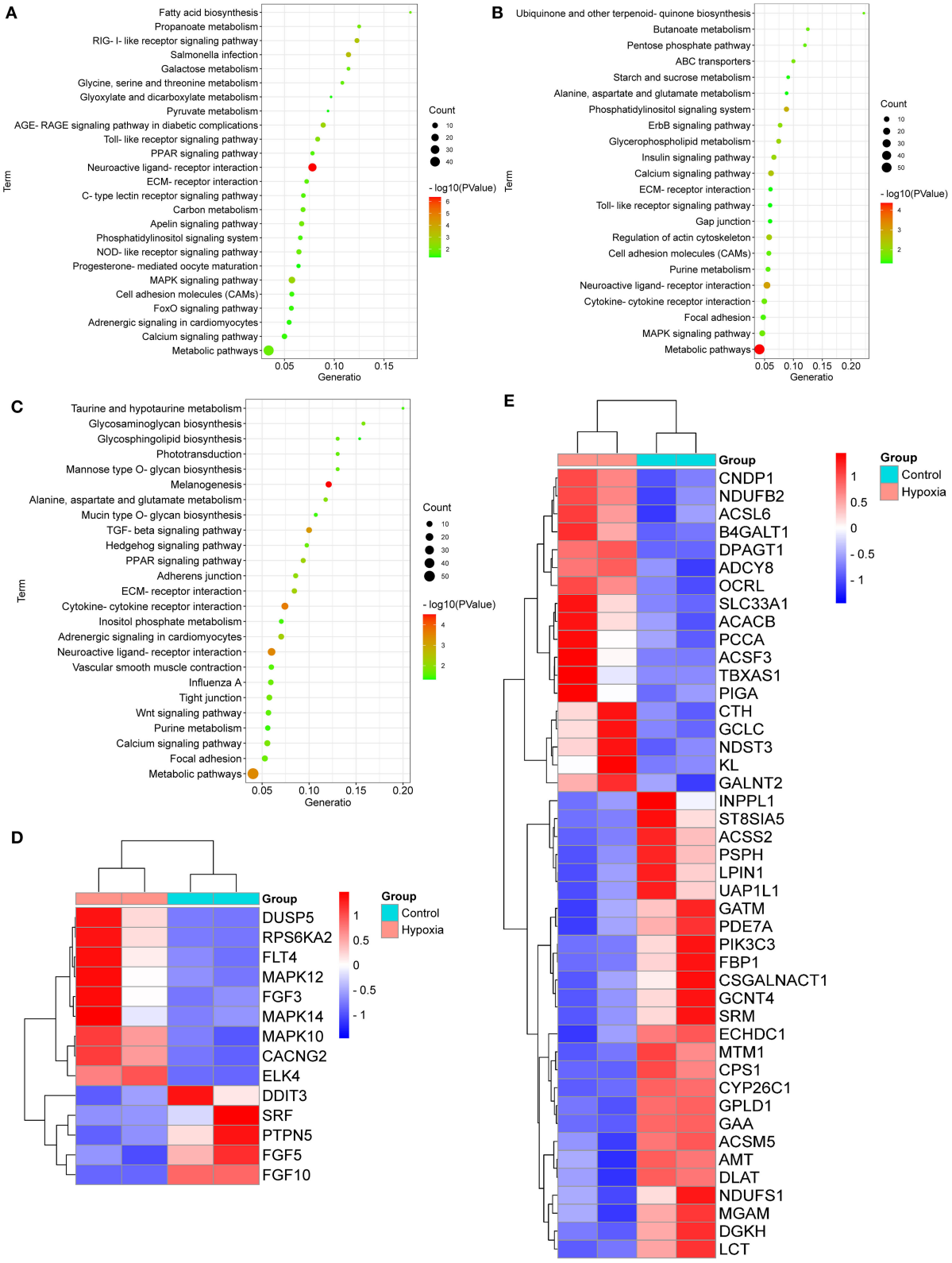

FIGURE 3
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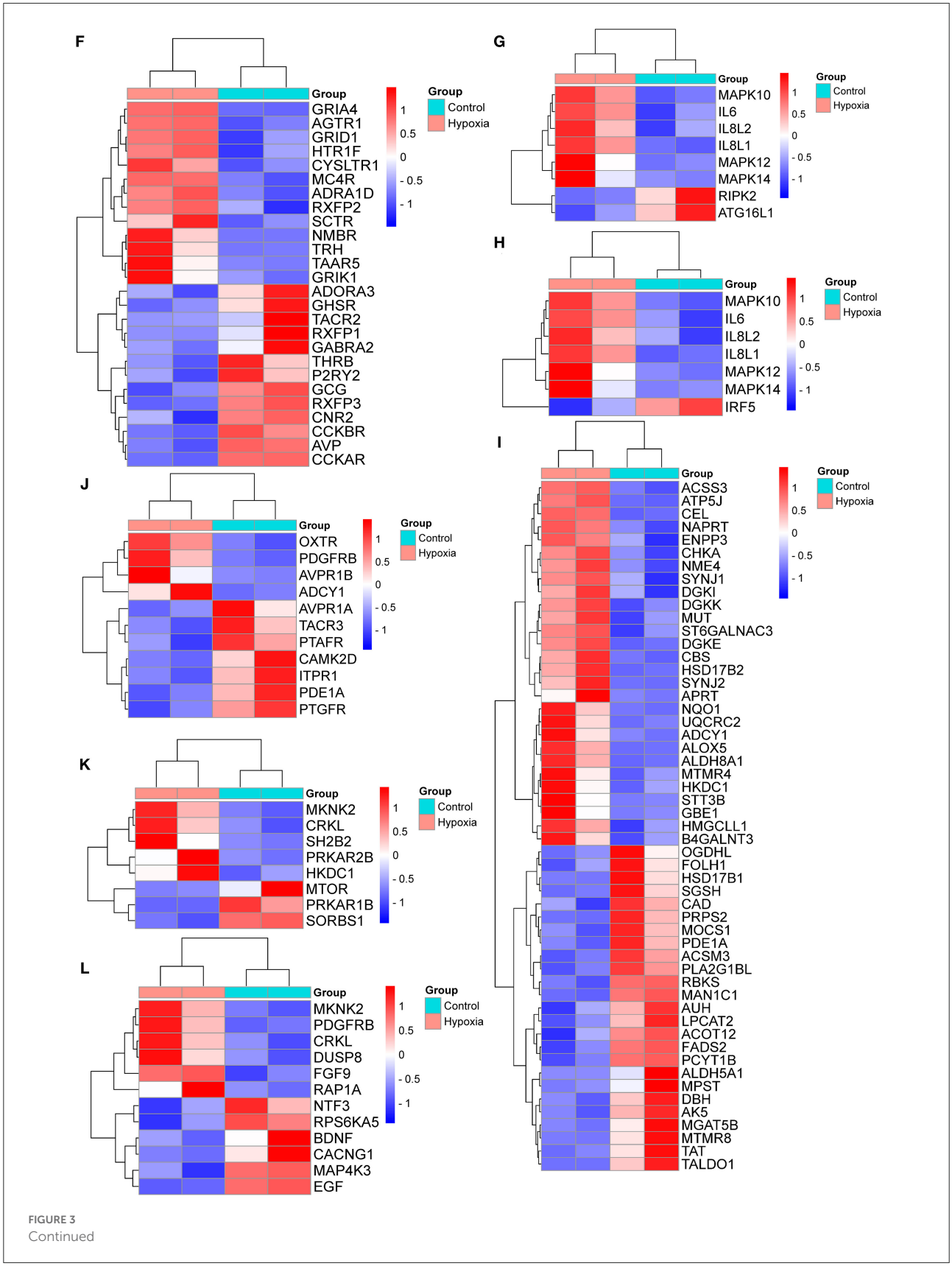

FIGURE 3
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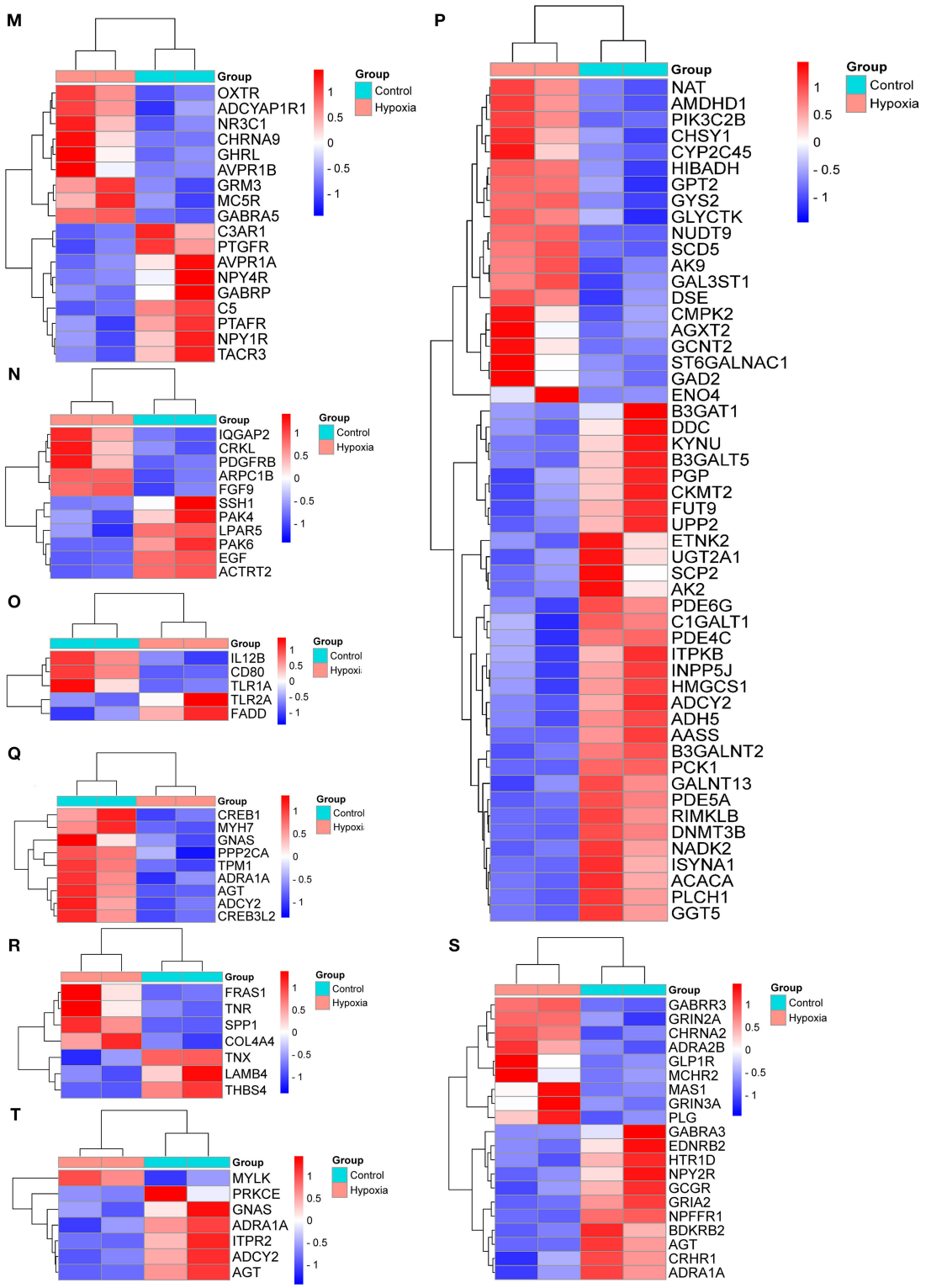

FIGURE 3
KEGG analysis for the breed-special DEGs. **(A–C)** indicated the KEGG analysis for the breed-special DEGs in the hearts of SG, TB, and DRW chickens treated with hypoxia and normoxia. **(D–H)** showed the heatmaps of the breed-special DEGs in metabolic pathways, MAPK, NLRI, NOD-like receptor, and toll-like receptor signaling pathways in SG chickens. **(I–O)** revealed the heatmaps of the breed-special DEGs in metabolic pathways; calcium, insulin, MAPK; NLRI; regulation of actin cytoskeleton; and toll-like receptor signaling pathways in TB chicken. **(P–T)** showed the heatmaps of the breed-special DEGs in metabolic pathways; adrenergic signaling in cardiomyocytes, ERI, NLRI, and vascular smooth muscle contraction signaling pathways in DRW chicken.

metabolic pathways, carbon metabolism, glyoxylate and dicarboxylate metabolism, and progesterone-mediated oocyte maturation.

Also, the breed-special DEGs in TB chicken heart were mainly involved in calcium, ErbB, MAPK, insulin, metabolic pathways, ABC transporters, Gap junction, cell adhesion molecules, glycerophospholipid metabolism, cytokine-cytokine receptor interaction, alanine, aspartate and glutamate metabolism, and toll-like receptor signaling pathways (Figure 3B).

Moreover, the breed-special DEGs in DRW chicken heart were primarily enriched in adrenergic signaling in cardiomyocytes, vascular smooth muscle contraction, ERI, calcium, hedgehog, PPAR, melanogenesis, metabolic pathways, TGF-beta, tight junction, adherens junction, glycosphingolipid biosynthesis, glycosphingolipid biosynthesis, purine metabolism, and Wnt signaling pathways (Figure 3C).

Furthermore, the heatmaps for the breed-special DEGs in metabolic pathways, MAPK, NLRI, NOD-like receptor, and toll-like receptor signaling pathways in SG chicken are shown in Figures 3D–H, respectively; The heatmaps for the breed-special DEGs in metabolic pathways, calcium, insulin, MAPK, NLRI, regulation of actin cytoskeleton, and toll-like receptor signaling pathways in TB chicken are shown in Figures 3I–O, respectively; The heatmaps for the breed-special DEGs in metabolic pathways, adrenergic signaling in cardiomyocytes, ERI, NLRI, and vascular smooth muscle contraction signaling pathways in DRW chicken are revealed in Figures 3P–T, respectively.

Reactome analysis and protein classification for the DEGs

As shown in Figure 4A, the breed-special DEGs in SG chicken heart were mainly enriched in myogenesis, striated muscle contraction; muscle contraction, signal transduction, small molecules transport, signaling by receptor tyrosine kinases, biological oxidations, PI3K/AKT signaling, RAF/MAP kinase cascade, MAPK1/MAPK3 signaling, MAPK family signaling cascades, and PIP3 activates AKT signaling.

In addition, the breed-special DEGs in TB chicken hearts might play an important role in signal transduction; metabolism; SLC-mediated transmembrane transport; neuronal system; GPCR ligand binding; neutrophil degranulation; nucleotides metabolism; transmission across chemical synapses; and Gap junction assembly (Figure 4B).

Moreover, the breed-special DEGs in DRW chicken hearts were mainly involved in striated muscle contraction; signal transduction; muscle contraction; GPCR downstream signaling; G alpha signaling events; adaptive immune system; neuronal system; extracellular matrix organization; Rho GTPase cycle; collagen degradation; activation of matrix metalloproteinases; and platelet homeostasis (Figure 4C).

The DEGs performed protein classification using PANTHER (Figures 4D–G). As shown in Figure 4D, the breed-special DEGs in SG chicken hearts were mainly involved in hydratase, ligase, cysteine protease, extracellular matrix glycoprotein, calmodulin-related protein, helix-turn-helix transcription factor, and membrane trafficking regulatory protein.

The breed-special DEGs in TB chicken might have vital roles in the transporter, kinase, neurotrophic factor, gap junction, microtubule binding motor protein, non-motor microtubule-binding protein; growth factor, zinc finger transcription factor, microtubule or microtubule-binding cytoskeletal protein, and transmembrane signal receptor (Figure 4E).

The breed-special DEGs in DRW chicken might be involved in the transporter, kinase, DNA photolyase, growth factor, DNA methyltransferase, intermediate filament, cytokine, transfer/carrier protein, nucleotide kinase, amino acid kinase, extracellular matrix protein, intercellular signal molecule, and secondary carrier transporter (Figure 4F). Of particular note was that the common DEGs among three chicken breeds might play a vital role in acetyltransferase, protease inhibitor, and protein phosphatase (Figure 4G).

PPI network and hub genes and their functions

To further reveal the core genes, the DEGs in the three chicken breeds, namely SG, TB, and DRW, were implemented in the analysis of the PPI networks, and the results are shown in Figures 5A–C, respectively. As shown in Figure 5A, many genes such as *FETUB*, *HGFAC*, *GAL8*, *FGG*, *GC*, and *DDX49* might be related to the response of SG chicken heart to chronic hypoxia. Similarly, *ALDH8A1*, *C3AR1*, *ACSM3*, *EXOC3L4*, *TAT*, *RAP1A*, *APOB*, and *GAL13* might be closely associated with the response of TB chicken heart to chronic hypoxia (Figure 5B). Many genes, including *LBFABP*, *STXBP1*, *SLC6A12*, *NPR1*, *CDON*, *GYS2*, *PLG*, *C6*, *SERPINA5*, *KYNU*, and *PDE5A* might be closely linked to the response of DRW chicken heart to chronic hypoxia (Figure 5C).

As shown in Figure 5D, the top 25 hub genes from the DEGs in SG chicken included *GC*, *F13B*, *FGA*, *TM4SF4*, *FETUB*, *HGFAC*, *GAL8*, *FGG*, and *ANGPTL3*. The top 20 hub genes from the DEGs in TB chicken included *ACSM3*, *EXOC3L4*, *APOB*, *ALDH8A1*, *C3AR1*, *TAT*, *RAP1A*, *GAL13*, and *MOSPD2*

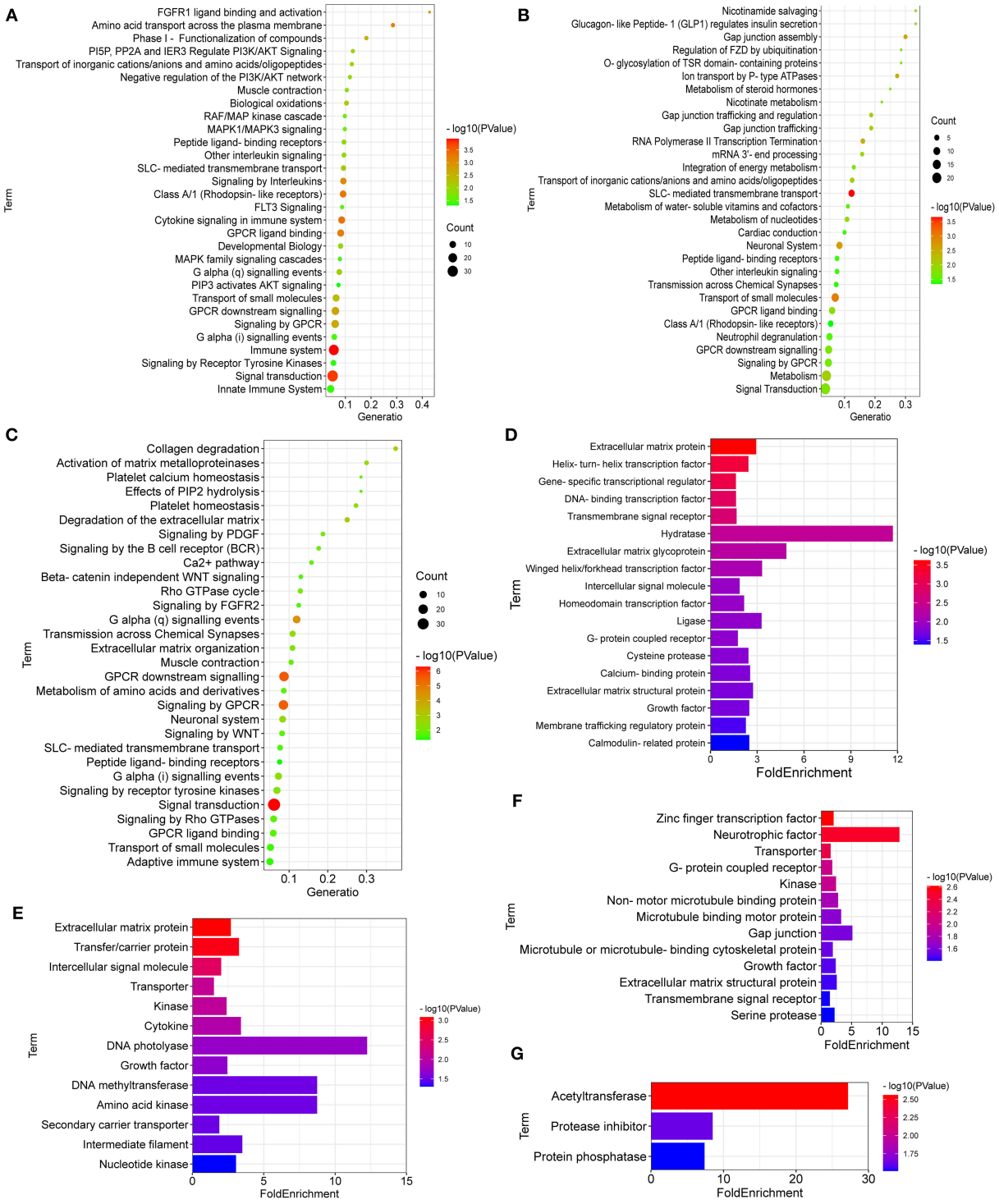

FIGURE 4
Reactome enrichment and protein class for the breed-special DEGs. **(A–F)** showed Reactome enrichment and protein class for the breed-special DEGs in the hearts of SG, TB, and DRW chickens. **(G)** showed protein class of the common DEGs among the three chicken breeds.

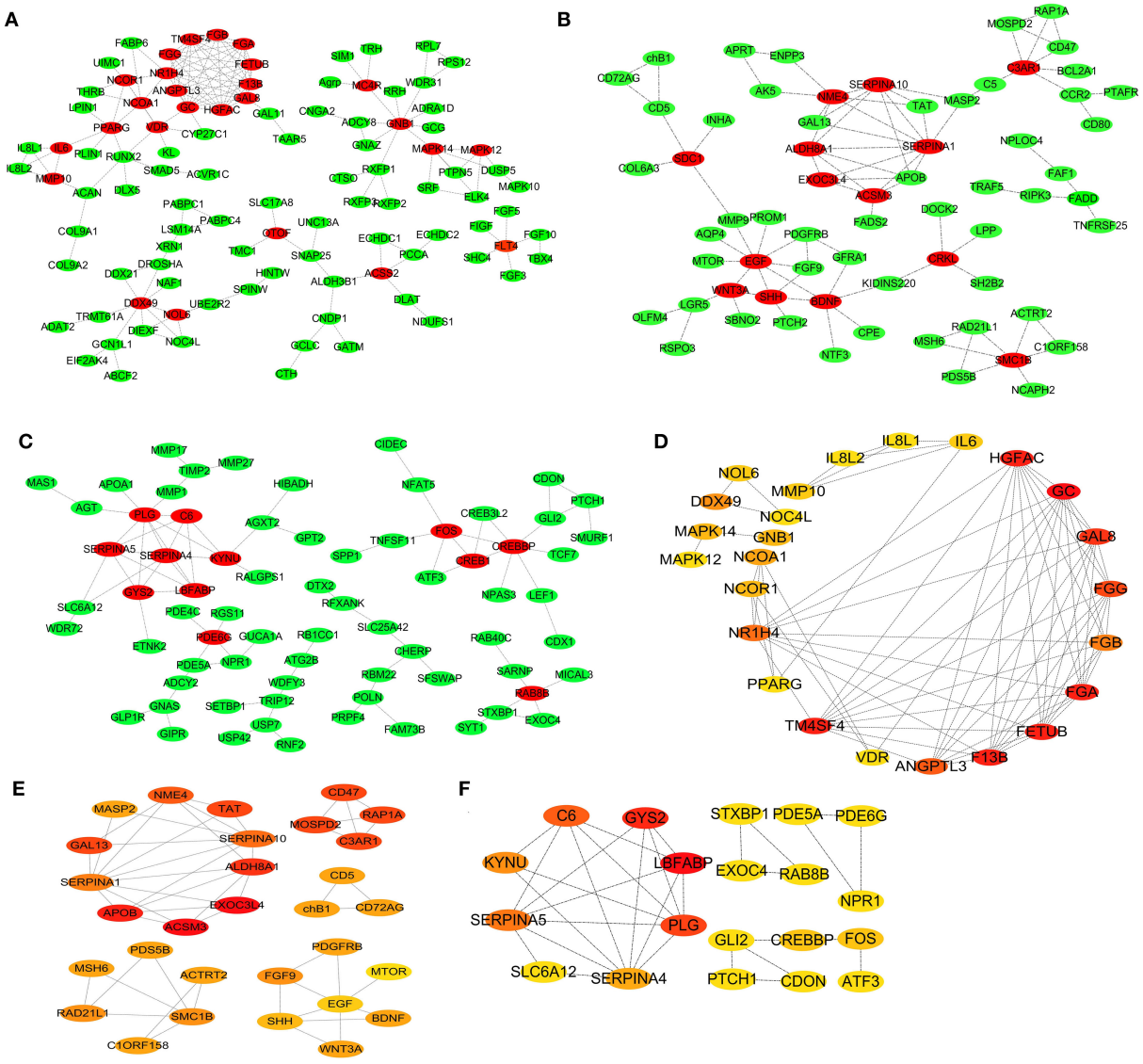

FIGURE 5
PPI Network and hub genes for the breed-special DEGs. **(A–F)** represented the PPI network and the Hub genes analysis for the breed-special DEGs in the hearts of SG, TB, and DRW chickens. The significance of genes highlighted in red, orange, and yellow was gradually decreased in the response of the hearts exposed to hypoxia.

(Figure 5E). The top 20 hub genes from DEGs in DRW chicken included *LBFABP*, *GYS2*, *PLG*, *C6*, *SERPINA5*, *KYNU*, *SERPINA4*, *FOS*, and *CREBBP* (Figure 5F).

Discussion

Genes related to the response of chicken heart to chronic hypoxia

In this study, we found 48 common DEGs in the heart of three chicken breeds between hypoxia and normoxia, including *SGCD*, *DHRS9*, *HELQ*, *MCMDC2*, and *ESCO2*. That is to say, those genes are likely to be closely associated with the response of the chicken heart to chronic hypoxia. For example, our data showed that the expression of *SGCD* was significantly downregulated in the hearts of SG (decreased by 89%) and DRW (decreased by 66%) chickens, but upregulated in TB (increased by 230%) chickens under hypoxia, which was consistent with the report that *SGCD* might be involved in chicken cardiomyopathy and muscular dystrophy. *SGCD* gene knockout could result in progressive heart muscle dysfunction in drosophila (13). Besides, TGF beta activation and SMAD signaling played a

vital role in cardiac muscle function and injury in *SGCD* null flies (13).

DHRS9, named dehydrogenase/reductase 9, is a powerful biomarker for human regulatory macrophages. *DHRS9* expression distinguished Mregs from a set of antigen-presenting cells, such as DC-10 and PGE2-induced myeloid-derived suppressor cells (14). In our study, the expression of *DHRS9* in the hearts of SG, TB, and DRW chickens in hypoxia was significantly higher than that in normoxia, which agrees with the report that *DHRS9* was obviously upregulated in patients with the cardiorenal syndrome, and it might serve as a potential biomarker for predicting the cardiorenal syndrome (15). Deniz et al. analyzed the gene expression profiles of patients with degenerative mitral regurgitation (DMR) in sinus rhythm and atrial fibrillation and found that *DHRS9* might have a structural remodeling role in the extracellular matrix and cellular stress response (16).

HELQ, a DNA helicase, played an important role in DNA lesions repair. The antitumor activities of *HELQ* might be associated with upregulated expression of the DNA damage-related proteins CHK1 and RAD51 (17). During the homologous recombination of cells, *HELQ* deficiency compromised the end-joining and single-strand annealing pathways and resulted in the bias toward the long-tract gene conversion tracts (18).

MCMDC2, named minichromosome maintenance domain containing 2, might play an important role in the formation and stabilization of DNA strands. During meiotic recombination, MCMDC2 promoted homolog alignment and provided the basis for inter-homolog crossover formation (18). MCMDC2 played a crucial function in meiotic recombination (19). MCMDC2 was vital for homologous sequences invasion by stabilization of recombination intermediates following strand invasion, both of which were needed to drive stable DSB repair *via* recombination (20). In this study, the expression of *MCMDC2* gene in the hearts of SG, TB, and DRW chickens under hypoxia was obviously higher than those under normoxia, hinting that *MCMDC2* might enhance homolog alignment and DNA strand stabilization in chicken heart development under hypoxia during the embryonic stage.

ESCO2, an acetyltransferase, is required for neuronal differentiation and sister chromatid cohesion. *ESCO2* overexpression enhanced the differentiation of neural progenitor cells and P19 embryonic carcinoma cells. On the contrary, the *ESCO2* knockdown blocked the differentiation of the above-mentioned cells. Importantly, Notch protein mediated the *ESCO2* effects (21). The *ESCO1*-dependent modification of SMC3 regulated the cohesin activities, such as transcriptional control, DNA repair, chromosome loop stabilization, and formation (21). *ESCO2* was upregulated during fin regeneration and specifically within the blastema. *ESCO2* knockdown significantly reduced the *CX43*/*GJA1* expression, which was required for cell-cell communication (22).

Signaling pathways linked to the response of chicken heart to chronic hypoxia

The response of the chicken heart to chronic hypoxia was mainly associated with multiple signaling pathways, including MAPK, PPAR, insulin, metabolic pathways, ERI, adrenergic signaling in cardiomyocytes, and vascular smooth muscle contraction. The MAPK signaling pathway was likely to participate in cardiac function regulation under hypoxia. Research showed that the MAPK signaling pathway mediated the hypoxia/reoxygenation (H/R)-induced injury in cardiomyocytes (23). The key proteins in MAPK/JNK signaling pathway, which were inhibited by the miR-155 inhibitor, were significantly upregulated in the H/R cardiomyocytes, and BAG5 overexpression enhanced the protective effect of those proteins on the cell injury induced by H/R. Moreover, the *HIF1a* expression patterns were altered following different treatments (24). The MAPK signaling pathway also had a vital role in regulating DNMT1/HMGB1-mediated cardiac progenitor cell apoptosis during the hypoxia process (25).

PPAR, a vital regulator for lipid metabolism, was linked to maintaining the homeostasis of myocardial energy metabolism, cardiac function, and tissue structure (26–30). Hypoxia increased myocardial lipid accumulation and mitochondrial dysfunction. Hypoxia downregulated the myocardial lipid metabolism-related genes, including *PPARα* (26). WY14643, a kind of *PPARα* activator, reduced the lipid accumulation in the myocardium induced by hypoxia, enhanced the left ventricular systolic and mitochondrial functions, and upregulated the *PPARα*, *PPARGC1A*, and *CPT1A* genes, and downregulated *ACC2* (26). In our study, the PPAR signaling pathway might mediate chicken cardiac response to hypoxia, which was consistent with the report that *PPARα* also played a vital role in regulating cardiac metabolic remodeling in response to both hypoxia and supplementation of nitrate in diet (27). Metabolism intervention might offer new approaches to the treatment of heart failure (29). *PPARγ* also powerfully modulated the signaling disorders in the heart and pulmonary vascular wall (30). Chronic hypoxia could result in cardiomyocyte hypertrophy, right ventricle hypertrophy (RVH), and right ventricle systolic pressure. Pioglitazone, a kind of *PPARγ* agonist, relieved the RVH, pulmonary hypertension, and cardiomyocyte hypertrophy induced by chronic hypoxia (30).

Insulin can eliminate the adverse effects of hypoxia and improve cardiomyocyte viability. Hypoxia reduced the cardiomyocyte viability, increased the autophagy and apoptosis and endoplasmic reticular (ER) stress pathway-associated apoptotic responses accompanied by an increase of pro-apoptotic transcriptional factor, and apoptosis in myocardial cells. In this study, the insulin signaling pathway might have a vital role in chicken cardiac response to hypoxia, which was similar to the report by Liu et al. who found that insulin could effectively relieve autophagy and ER stress and prevent hypoxia-induced cellular apoptosis *via* PI3K/Akt signaling pathway (31). An unusual HR, blood glucose, and mean arterial BP induced by hypoxia could be restored to the normoxia state by an acute insulin supplement (32).

ECM-receptor interaction (ERI) may be related to the response of the chicken heart to hypoxia. The ERI pathway regulated cell proliferation, apoptosis, growth, and differentiation. The dysregulation of the pathway was responsible for cell death (33). Qi et al. reported that yak was well-adapted to the hypoxia and high altitude. To clarify the underlying mechanism behind the adaptation, they conducted a transcriptomic experiment for yaks and found that the heart was the key organ showing adaptive transcriptional changes. Multiple cell proliferation and survival-associated signaling pathways, including ERI, PI3K-Akt, HIF-1, and focal adhesion, might be involved in the adaptation and response of the heart to hypoxia (34). The study from San et al. identified the DEGs in crureus and pectorales between the Arbor Acres and the Zhuanghe dagu chickens and indicated that the ERI pathway was co-enriched in both the tissues. In addition, ERI could regulate the metabolism of intramuscular adipocytes (35).

Adrenergic signaling in cardiomyocytes is very important for maintaining cardiac physiological function and preventing cardiac diseases. Beta-adrenergic stimulation highly restricted the cAMP distribution and promoted the phosphorylation of proteins for the contractile responses of cardiomyocytes. Moreover, the interaction between beta-adrenergic signaling and other receptor-stimulated signaling cascades changed the beta-adrenergic signaling for proper contractility in the myocardium (36). Beta-adrenergic stimulation rapidly increased G alpha palmitoylation in cardiomyocytes. This palmitoylation kinetics was temporally consistent with the downstream production of cAMP and contractile responses. The plasma membrane-localized palmitoyl acyltransferase DHHC5 is an important mediator of the stimulus-dependent palmitoylation in cardiomyocytes (37).

However, there are two deficiencies in the present study: (1) the lack of verification for the transcriptome sequencing results, and (2) the lack of further studies on the mechanisms of the important genes and signaling pathways potentially relevant to the chronic hypoxia-induced chicken embryonic heart.

Conclusion

In the study, we found diverse signaling pathways (including MAPK, PPAR, insulin, ERI, and adrenergic signaling pathways) and many genes, such as *SGCD*, *DHRS9*, *HELQ*, *MCMDC2*, and *ESCO2* might contribute to the response of the chicken heart to HE. This study provided a valuable clue for the in-depth understanding of the molecular mechanism of the adaptability of the chicken heart to chronic hypoxia.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (ECASTU-2015-P08) of Anhui Science and Technology University, China.

Author contributions

XL analyzed and visualized the results and wrote the manuscript. BY and ZH conceived the study. NM and A-MA-M revised the scientific English. All authors reviewed the manuscript.

Funding

This study was supported by the Natural Science Foundation of Anhui Provincial Education Department (No. KJ2020A0083) and the Talent Introduction Program of Anhui Science and Technology University (No. DKYJ202003).

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.2022.942159/full#supplementary-material

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TYPE Original Research
PUBLISHED 15 August 2022
DOI 10.3389/fvets.2022.948502

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 19 May 2022
ACCEPTED 21 July 2022
PUBLISHED 15 August 2022

CITATION
Salgado Pardo JI, Navas González FJ, González Ariza A, Arando Arbulu A, León Jurado JM, Delgado Bermejo JV and Camacho Vallejo ME (2022) Traditional sexing methods and external egg characteristics combination allow highly accurate early sex determination in an endangered native turkey breed. *Front. Vet. Sci.* 9:948502. doi: 10.3389/fvets.2022.948502

Traditional sexing methods and external egg characteristics combination allow highly accurate early sex determination in an endangered native turkey breed

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Early sex determination methods are not only crucial in the worldwide massive poultry industry, but also for small-holder producers. The profitability of sexing techniques must be accounted for when aiming to boost management, nutrition, and conservation practices in endangered poultry breeds. This becomes pivotal when the local breed dealt with belongs to an understudied species, such as the turkey. So, the main objective of this study is to identify which method combination may report a higher likelihood of successful sex determination in poults across the three-pattern varieties of the Andalusian turkey breed. A total of 84 one to two days old Andalusian turkey poults (42 black, 28 black-roan, and 14 bronze-roan) were evaluated in this study. Sex determination was performed using 15 methods, which included testing external egg metrics and eggshell color, poult morphological appraisal and phaneroptics, and behavioral traits. Possible differences across plumage varieties and the interaction between sex and plumage were observed when external egg quality was measured. Sex determination through behavioral methods in black base feathered (black and black-roan) male sex individuals showed seven times higher sensitivity when compared to the rest of the studied individuals ($\chi^2 = 7.14$, df = 1, $P < 0.01$). In contrast, for the black-roan plumage females, the method based on the color of down feathers was approximately four times more sensitive ($\chi^2 = 3.95$, df = 1, $P \leq 0.05$). For the bronze-roan pattern, none of the sexing techniques was reported to efficiently predict sex itself. However, the most proper method combination to determine sex, independent of plumage color, was physical external egg characteristics, the color of down feathers, and behavioral approaches ("English method" and "slap technique"). The specificity values were found to be 49.12, 93.33, and 100%,

while the sensitivity values were observed to be 74.64, 91.03, and 100%, which translated into accuracy of 63.10, 92.26, and 100% in black, black-roan, and bronze-roan poults, respectively. Our results suggest that the method combination tested in this study could be considered a highly accurate, simple, and affordable alternative for sex determination in turkeys. This could mean a pivotal advance for small producers of turkeys, as early sex detection can help to plan timely conservational management strategies, which is of prominent importance in the context of endangered poultry breeds.

KEYWORDS

external egg quality, poult morphological, sex determination, preincubation, post-hatching, native breed, behavior

Introduction

Early sex determination plays a pivotal role in the turkey farming specialization, since two different lines are commonly used: a heavy line, which comprises males, and a laying line, which sources dams (1). The difference in body weights between these two strains is the basis for the differentiation of farms to ensure basic animal management and nutrition (2). Thus, hatching poults need to be separated by sexes to be raised independently, depending on the commercial strategy chosen by breeders (3). Apart from its critical economic impact, the possibility of sex detection before hatching is also interesting in terms of both animal welfare and ethical issues by the early separation of the different sexes (4).

Sexing chicks during the first day of life could be a critical step not only in the commercial poultry industry but also in the design of conservational and breeding programs for endangered native breeds, as described by (5). The use of reliable sexing techniques in endangered avian breeds is of special importance in breeding programs, since it could avoid lowing hatching rate problems or copulation problems due to side effects derived from high inbreeding in such minority populations (6). In these terms, native poultry breeds, such as the Andalusian turkey, could benefit from the early sex determination of poults. The Andalusian turkey breed is a Spanish endangered autochthonous population distributed around the Southeast Iberian Peninsula and might be the direct descendants of the first turkeys imported from Mexico to arrive in Seville's port during the early 16th century (7).

Andalusian turkey is raised in semi-grazing conditions by backyard producers in the Guadalquivir Basin and is characterized by great adaptability to the environment. However, during the 20th century, the number of individuals belonging to this native breed drastically decreased as a consequence of the introduction of commercial hybrid strains in Spain (8). This situation promoted local genotype displacement and hybridization, which suggested the need for urgent conservation measures to be taken.

The implementation of a standardized accurate method for the sex determination of 1-day-old poults could mean a crucial improvement for breeders, making it possible to take proper management decisions at hatching instead of waiting for 4–5 months, when animals start to display sexual dimorphism characters (9). As a consequence, Andalusian turkey males may be aimed toward the maximization of their meat production while letting hen for the laying aptitude (10). These sexing methods can also be a beneficial tool when management strategies to preserve genetic diversity are designed, since sex distribution across the population is possibly biased (5).

Sexual dimorphism is defined as the differences in external appearance, among other traits, between the two genders of one species and is influenced by both genetic and environmental factors (11). Generally, males and females differ in size, color, shape, and appendage development (such as feathers, wattle or appendage, caruncles, beard, and spurs). On the other hand, sexual dimorphism can also be manifested by scent or courtship vocalizations, behavior, and cognition (12). Recent advances in poultry genetics have made it possible to obtain, based on the crosses of given parental strains, offspring showing specific phenotypic traits that make both sexes distinguishable in the early stages of life (13). Genes that modify feather growth have also been described and reported to permit early sex determination (14). However, its implementation in breeding programs was discarded due to a negative impact on the production traits (15). More recent technologies have developed new tools, in which algorithm wing edge detection is used. For this, computational imaging of external wing feathers growth is employed (16). Again, these methods may be difficult to implement in local poultry populations, as morphological and phaneroptic traits may broadly vary across different breeds and varieties.

In avian species, sexual dimorphism is caused by several secondary phenotypical traits that can be recognizable even in

the laid egg until the 1-day-old poult (5). Several sex-influenced phenotypical traits in these early stages have been reported, including egg size (17, 18), the opacity of the eggshell (19), feather color, morphology and distribution (20), appendicular skeleton dimensions, focusing on tarsus-metatarsus length (21), head length and size (22, 23), tail inclination (24), and the behavioral performance of the individuals (22, 25).

Considering the aforementioned premises, this study aims to establish which method combination may offer the most efficient and accurate method to determine sex at the early stages of life across the three plumage varieties of the Andalusian turkey breed. This information will be processed to tailor specific non-invasive sexing methods for poult from local turkey populations. The identification of the proportions of individuals belonging to each sex when working with endangered populations can contribute to the improvement and progress of the genetic management tasks carried out in these genotypes. Thus, the tool developed in this study can be a complement to the more commonly used techniques, which have been widely tested on a commercial scale but are sometimes inefficient due to the implicit diversity found in local populations.

Materials and methods

Animals and sample size

The present research was conducted in a public hatchery located at the Agropecuary Provincial Center of Diputación of Córdoba (Andalusian, Spain). A total of 18 turkey hens and 3 toms, aged between 12 and 16 months, coming from the base population of the Andalusian turkey breed were reared in three different groups according to plumage color (black: 1M/6F; roan-black: 1M/6F; and bronze-roan: 1M/6F), and were involved in the egg production.

Taking advance of the breeding season (from February to April 2019), eggs were collected daily and stored at 17–18 °C and 70–75 % humidity in incubating platters until their incubation. All eggs were individually numbered, and external egg metrics and eggshell color were determined before incubation.

Eggs intended for incubation were kept for a maximum of 7 days since oviposition. A total of 311 eggs were incubated and divided into seven different incubation periods to ensure sufficient birds are included in the study. An incubator with automatic egg turning (Masalles, M240-I, Barcelona, Spain) was used for 26 days at 37.2 °C and 55 % RH. On the 26th day of incubation, eggs were transferred to a hatchery cabinet (Masalles, 25-N HLC, Barcelona, Spain) maintained at 36.7 °C and 60 % RH until hatching (2 more days). A total of 162 poults hatched, and then were wing-banded and placed in a room with an electric stove to help them regulate their body temperature until performing the sex determination tests.

A random sample of 162 turkey poults (76 black, 58 black-roan, and 28 bronze-roan) was used for sexing. Finally, of the total animals subjected to the sexing tests, we were able to determine the sex of 84 individuals (42 black, 28 black-roan, and 14 bronze-roan). This was due to the fact that some animals died, and others were donated to local farms (as part of an Andalusian turkey breed recovery program) before the sexual dimorphism of the individuals became evident. In the literature, it has been reported that samples of around 100 or even fewer individuals report 95% sexing accuracy in other local poultry species (5, 9, 26). Therefore, of the total of 972 observations that were obtained, only 506 observations were used in the analyzed database, of which the individual sex was confirmed and a complete sexing determination procedure of three appraisers was collected.

Bird management was directed under the European Union Direction regulations (2010/63/EU) as transposed to Spanish Royal Decree-Law 53/2013. This study did not need to be subjected to evaluation by the Ethics Committee of Animal of the University of Córdoba, since it is not part of the legislation for the protection of animals used for scientific purposes.

External egg metrics and eggshell color

Before incubation, external egg quality was determined in each egg:

- M1 and M2 (major and minor diameters). These measurements were determined using a digital caliper (precision, ±0.01 mm; Electro DH M 60.205, Barcelona, Spain).
- M3 [shape index (SI)]. This index was computed using the following formula (27):

$$SI = \left(\frac{ØM}{Øm} \right) * 100$$

where ØM is the major diameter and Øm is the minor diameter.

If the egg is long and pointed, the individual will be taken as a female, while wide and flat eggs are assigned to males (28). To establish the limits to consider an egg long or flat and wide or broad, the shape index and the median of the diameters were calculated (non-normal distribution, $p > 0.05$), to set over and below the median categories.

- M4 (egg weight). Eggs were weighed individually using an electronic scale (precision ±0.01 g; Cobos, CSB-600C, Barcelona, Spain).
- M5, M6, and M7 (eggshell L*, eggshell a*, and eggshell b*). Eggshell color was assessed using a portable spectrophotometer (CM 700d, Konica Minolta Holdings Inc., Tokyo, Japan), and the results of eggshell color were

expressed according to the International Commission on Illumination (CIE) L*a*b* system color profile.

Poult morphological appraisal and phaneroptics, behavioral traits, and handling for sexing assignation methods

To carry out the sex determination procedure, each turkey poult was held by the neck during the examination, with the index and middle fingers of the sexer, keeping the poult's head down. Defecation of the animal was caused by applying pressure on the abdominal cavity. Finally, the cloaca was cleaned with a piece of tissue paper (5).

Various sexing methods based on the poult morphological appraisal and phaneroptics and behavioral traits were performed by three sexers through eight methods, and a dichotomous scale (male or female) was used to classify the animals (5, 29) (Figure 1). The examination tests were performed after hatching, considering it as days 1 and 2 since hatching, and were carried out by three different non-trained evaluators. The different methodologies employed are described as follows:

- M8 (English method). The bird is suspended for 5 s by holding the beak with two fingers, thus analyzing the acquired behavior. If the bird stands still, it is considered male, and if the bird kicks, it will be considered female.
- M9 (Tail inclination method). The turkey poults will be taken as a female when the direction of the tail feathers is toward the ground. However, it will be considered as male if the tail is straight.
- M10 (Japanese method or cloaca examination). As described in the "Introduction" section, vent sexing starts from the basis of the appreciation of morphological visual distinction of genital anatomical structures between sexes in hatched poults by trained experts. Cloaca needs to be externalized by carefully applying pressure with the fingers, and then focusing on the central and ventral parts of it. An individual can be considered a male if it shows a unique outline in the cloaca, or a female if two little bulges are observed.
- M11 (General coloring of down feathers method). This method involves observation of the color pattern displayed by the down feathers on both sides. Individuals displaying a uniform coloration will be considered females, while the poults that exhibit heterogeneous coloring of down feathers will be considered males.
- M12 (Fan-shaped wings and general wing metrics determination). It is based on the growth of primary and secondary reminge feathers of the wings. In this regard, a female is identified when all primary and secondary reminge feathers of the wing show a parallel growth and describe a uniform fan edge. In contrast, a wing that exhibits feathers at a different growth stage, describing an irregular contour, will be considered a characteristic of males.
- M13 (Body size and head morphology method). Males have been described to present proportionally smaller and more rounded heads compared to females, showing a bigger and more angular head shape. To state the limits to consider whether the head of a poult is big or small, the median sizes were computed (the sample was not normally distributed, $p > 0.05$), to set over and below the median categories.
- M14 [Leg length method (from femorotibial joint to the end of the medial phalange)]. Male poults are considered to have long legs when compared to female ones. To state the limits to consider whether the legs are long or short, the average measure of the sizes was computed to set over and below the median categories, since the sample was not normally distributed ($p > 0.05$). The complete leg was considered, and not only the shanks.
- M15 (Behavior/coping styles or slap technique method). Hands are clapped at a prudent distance of 20 cm from the animal. This technique is applied individually for each poult in an isolated place, distant from the rest of the poults. Two different reactions can be observed: freezing (male) and fleeing or attempting to escape (female).

All the methods used in Sections External egg metrics and eggshell color and Poult morphological appraisal and phaneroptics, behavioral traits, and handling for sexing assignation methods of the present work are depicted in Figure 1.

Sex confirmation

After achieving 25 weeks of age, Andalusian turkey breed females and males exteriorized the secondary sexual characters that enabled the confirmation of the real sex of the individuals.

Statistical analysis

Binary logistic regression

Binary logistic regression was used to fit the statistical model described below. This model represents how the chance of an animal belonging to one of the two possible categories (sexes) may depend on the results for covariates or predictors (sexing methods). In this context, Y was defined as a binary outcome with two categories (sex).

Ordinary least squares (OLS) on a dichotomous dependent variable and binary logistic regression are the two alternatives that can be considered in the case of regressing binary outcomes.

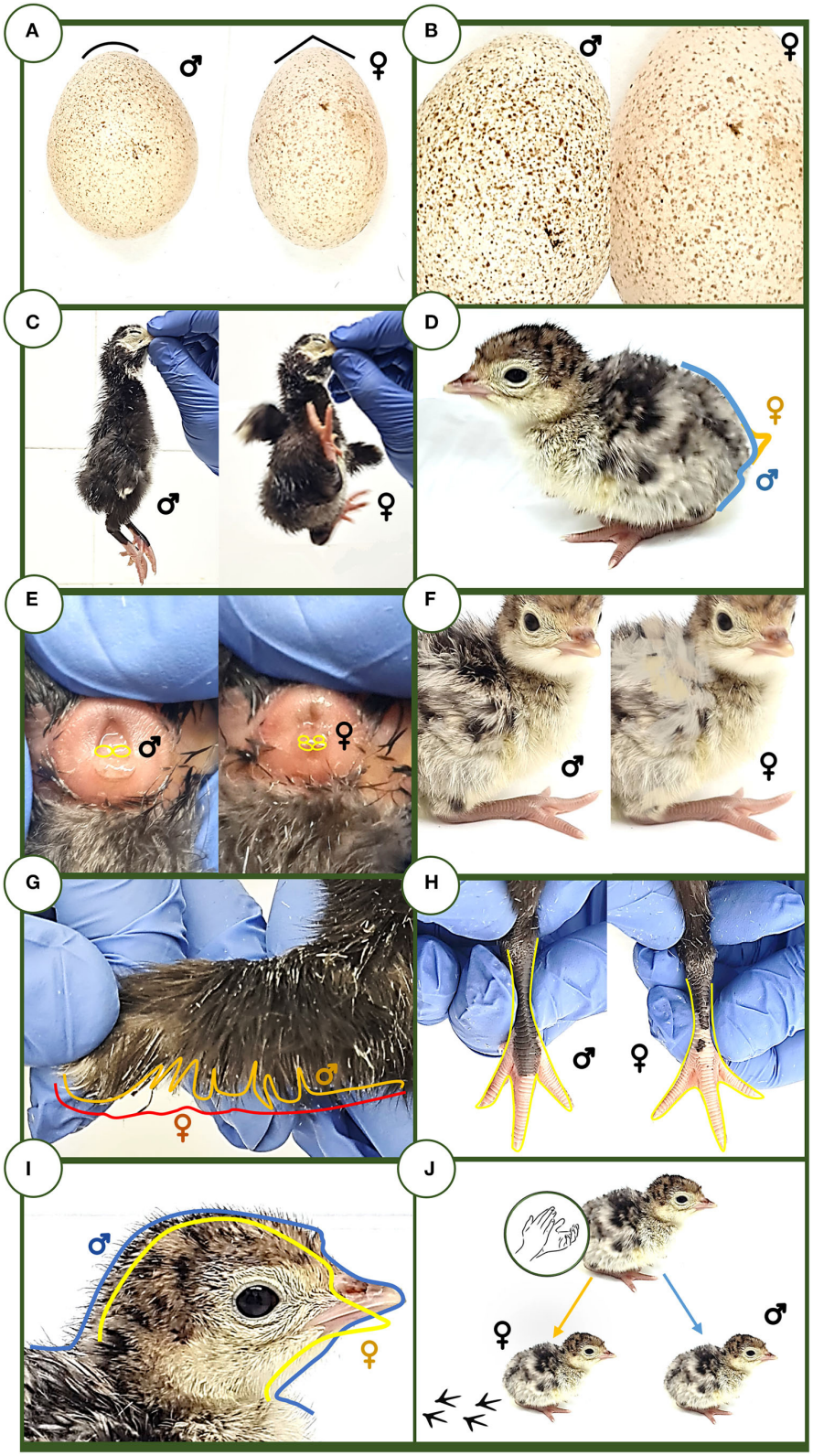

FIGURE 1
Sex assignment methods. **(A)** Egg length and width test. **(B)** Eggshell color. **(C)** English test. **(D)** Tail inclination. **(E)** Cloaca. **(F)** Side feathers. **(G)** Wing fan. **(H)** Legs. **(I)** Head size and morphology. **(J)** Behavior/coping style.

OLS is a type of linear least squares method that is used to estimate the unknown parameters in a linear regression model.

Particularly, OLS selects the parameters of a linear function of a set of explanatory variables (sex determination methods) by the principle of least squares, minimizing the sum of the squares of the differences between the observed dependent variable (values of the variable being observed) in the given dataset and those predicted by the linear function of the independent variable.

However, there are three assumptions that must be met prior to running the analyses. First, the error terms need to be heteroskedastic. Thus, the variance of the dependent variable and independent variables must be different, the error terms must not distribute normally, and the predicted probabilities can be > 1 or < 0, which can be a problem for subsequent analysis. The "logit" model solves these problems:

$$\ln[p/(1-p)] = a + BX$$

where p is the probability that Y for cases equals 1, p (Y=1), 1-p is the probability that Y for cases equals 0, 1–p(Y=1), p/(1-p)" is the odds, and ln[p/1-p] is the log odds, or "logit."

In logistic regression, we predict Z, not p, because of Z's convenient mathematical properties. Z is a linear function of the predictors, and we can translate that prediction into a probability. The natural log of the odds is called the "logit" = "Z." Z can be described as follows;

$$Z = \log(p/1-p) = B_0 + B_1 \cdot X_1 + B_2 \cdot X_2 + B_3 \cdot X_3 \ \ldots$$

B's in logistic regression are analogous to b's in OLS, B1 is the average change in Z per one unit increase in X1, controlling for the other predictors, and so on.

The set of independent covariates and categorical predictors (B) consisted of the external egg metrics and eggshell color and sexing methods outcomes using the logistic regression procedure of the Modeling Data Package in XLSTAT Version 2014.5.03 (30). A single model was performed for each of the varieties (black, black-roan, and bronze-roan). The Hosmer–Lemeshow test was used to determine the goodness of fit of the logistic regression model. Essentially, it is chi-square goodness of fit test. When $P > 0.05$ (assuming $\alpha = 0.05$), we conclude that the logistic regression model is a good fit.

Interpreting logistic coefficients

Once significant covariates and predictors have been identified, the sign of Bs will determine the changes in the log odds of the dependent variable, but not changes in the dependent variable (as in OLS). If B for a specific predictor is positive, a unit change in its related x will raise the odds of the event happening, after controlling for the other predictors, while if B is negative, the odds of the event decrease with a unit increase in x.

Exp(B) means "e to the power B" or e^B. It is called the "odds ratio" (Gr. symbol: Ψ), e is a, mathematical constant used as the "base" for natural logarithms. In logistic regression, eB is the factor by which the odds change when X increases by one unit.

$$\text{New odds/Old odds} = eB = \text{odds ratio}$$

Odds ratios > 1 indicate a positive relationship between IV and DV (event likely to occur)

Odds ratios < 1 indicate a negative relationship between IV and DV (event less likely to occur)

The significance of logistic coefficients is determined by a Wald test. Wald is χ^2 with 1 df and equals a two-tailed t^2 with a *p*-value exactly the same.

The knowledge of the distribution of sex yielded the likelihood of the sample. To estimate the B parameters of the model (the coefficients of the linear function), the likelihood function was maximized. As opposed to linear regression, an exact analytical solution does not exist; hence, an iterative algorithm had to be applied.

Maximization of the likelihood function was performed using the Newton–Raphson algorithm with 100 iterations and a convergence level of 0.000001, which are given as default by XLSTAT Version 2014.5.03 (30).

Specificity and sensitivity

Sensitivity, true positive rate, or the recall measured the proportion of individuals correctly attributed to sex, and specificity (also called the true negative rate) measured the proportion of individuals incorrectly attributed to sex. These two parameters were computed using the logistic regression procedure of the Modeling Data Package in XLSTAT Version 2014.5.03 (30).

Results

Table 1 displays the existing correlations across external egg traits. In this case, as the probability of these variables modeling for real sex determination was lower than 0.001 (Tables 1, 2), the variables chosen were concluded to statistically significantly condition and model for real sex determination. Table 3 determined whether the set of variables evaluated in this study may have significantly conditioned (i.e., have been responsible for) real sex determination by comparing the model as it was defined with a simpler model with only one intercept.

Table 2 provides several indicators of the quality of the model (or goodness of fit). These results were equivalent to R^2 and the analysis of the variance table in linear regression and ANOVA. The most important value was the probability of the chi-square test on the log ratio. This is equivalent to Fisher's F test, and it is used to evaluate whether the variables bring significant information by comparing the model when it

TABLE 1 Correlation matrix for external egg characteristics across Andalusian turkey variety pairs.

	Variable	Egg weight	Major diameter	Minor diameter	Shape index	Eggshell L*	Eggshell a*	Eggshell b*
Black	Egg weight	1.0000	0.8810	0.6971	−0.1932	0.0665	−0.0815	−0.1485
	Major diameter		1.0000	0.5155	−0.4339	0.1677	0.0017	−0.1494
	Minor diameter			1.0000	0.3759	0.0023	−0.1902	−0.2408
	Shape index				1.0000	−0.2572	−0.0034	0.0767
	Eggshell L*					1.0000	−0.4750	−0.6504
	Eggshell a*						1.0000	0.5871
	Eggshell b*							1.0000
Black–roan	Egg weight	1.0000	0.8042	0.9735	0.0650	−0.1428	−0.1892	0.0767
	Major diameter		1.0000	0.6712	−0.4920	−0.1941	−0.0002	0.2944
	Minor diameter			1.0000	0.2549	−0.0417	−0.2482	−0.0425
	Shape index				1.0000	0.1398	−0.3061	−0.3788
	Eggshell L*					1.0000	0.2621	−0.5066
	Eggshell a*						1.0000	0.3564
	Eggshell b*							1.0000
Bronze–roan	Egg weight	1.0000	0.8677	0.5874	−0.5105	0.2303	0.4855	−0.0066
	Major diameter		1.0000	0.6595	−0.6107	0.1625	0.4832	0.2456
	Minor diameter			1.0000	0.1919	0.0869	0.5166	0.3301
	Shape index				1.0000	−0.1142	−0.0915	0.0380
	Eggshell L*					1.0000	0.3305	−0.3803
	Eggshell a*						1.0000	0.0317
	Eggshell b*							1.0000

TABLE 2 Goodness of fit statistics for each Andalusian turkey variety.

	Black		Black–roan		Bronze–roan	
Statistic	**Independent**	**Full**	**Independent**	**Full**	**Independent**	**Full**
Observations	252	252	168	168	84	84
Sum of weights	252.0000	252.0000	168.0000	168.0000	84.0000	84.0000
df	251	241	167	157	83	74
−2 Log (Likelihood)	347.0570	323.2748	232.0396	118.2362	114.7286	0.0000
R^2 (McFadden)	0.0000	0.0685	0.0000	0.4904	0.0000	1.0000
R^2 (Cox and Snell)	0.0000	0.0901	0.0000	0.4921	0.0000	0.7448
R^2 (Nagelkerke)	0.0000	0.1204	0.0000	0.6572	0.0000	1.0000
AIC	349.0570	345.2748	234.0396	140.2362	116.7286	20.0000
SBC	352.5864	384.0985	237.1635	174.5998	119.1594	44.3082
Iterations	0	6	0	8	0	24

Df, degrees of freedom; AIC, Akaike's Information Criterion; SBC/BIC, Schwarz's Bayesian Criterion/Bayesian Information Criterion.

is defined with a simpler model with only one constant. In this case, as the probability was lower than 0.0001 (Table 1), we could conclude that data could be significantly modeled by the set of variables chosen.

Parameter analysis

Table 4 provides details on the model and presents a measure of the effect of the variables considered on the categories of the response variable. There is one intercept for each category of the response variable and one set of coefficients, since the parallel curves hypothesis is supposed to be met.

When the regression coefficient for a specific category within a variable was equal to 0.000, this indicated that the said category was taken as the reference to measure the higher or lower repercussions of the subsequent categories in the same variable. The standardized regression coefficient measured the number of times that a certain level or category had a higher (positive

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TABLE 3 Test of the null hypothesis (Black: Y = 0.5476, Roan–black: Y = 0.4643, and Roan–bronze: Y = 0.5714).

	Black			Black–roan			Bronze–roan		
Statistic	**df**	**Chi–square**	**Pr > Chi²**	**df**	**Chi–square**	**Pr > Chi²**	**df**	**Chi–square**	**Pr > Chi²**
−2 Log (Likelihood)	10	23.7822	0.0082	10	113.8034	< 0.0001	9	114.7286	< 0.0001
Score	10	22.9502	0.0109	10	88.5760	< 0.0001	9	52.2329	< 0.0001
Wald	10	21.2797	0.0192	10	43.1063	< 0.0001	9	8.9880 E^{-5}	1.0000

standardized coefficient) or lower (negative standardized coefficient) repercussion.

The interpretation of parameters was not immediate. Based on the results in Table 3, it was concluded that the model equation for each variety was as follows:

Black variety

Pred(REAL SEX) = 1/[1 + exp(-(-9.01007+0.00635*Egg Weight+0.09983*Egg Length+0.01051*Egg Width+0.03087*Shape index-0.00983*L*-0.02763*a*-0.04581*b*-0.19432*English method-1+0.65821*General coloring of down feathers method-2+0.72086* Behavior/coping styles or slap technique method))]

Black-roan variety

Pred(REAL SEX) = 1/[1 + exp(-(-166.45357+0.54722*Egg Weight+1.49114*Egg length-3.68572*Egg Width+2.43531*Shape index+0.22455*L*+0.04886*a*+0.36373*b*-0.15040*English method-1-1.09496*General coloring of down feathers method-1-0.19976* Behavior/coping styles or slap technique method))]

Bronze-roan variety

Pred(REAL SEX) = 1 / [1 + exp(-(-42494-233.76746*Egg Weight+337.60584*Egg length o+822.15956*Egg Width+6.06954*L*-20.84484*a*-13.93728*b*+0.21860*English method-1-0.85095*General coloring of down feathers method-1+0.68119* Behavior/coping styles or slap technique method))]

Specificity and sensitivity

Specificity values were 49.12, 93.33, and 100%, while sensitivity values were 74.64, 91.03, and 100%, which translated into the accuracy of 63.10, 92.26, and 100% in black, black–roan, and bronze-roan poults, respectively. A detailed report of the classification table for the estimation sample used to compute the aforementioned parameters is presented in Table 5.

Discussion

High variability in the ability of the different methods used to predict sex across the different plumage varieties was found. However, the combination of external characteristics of egg, the coloring of down feathers, and behavioral techniques ("English method" and slap technique) reported the best sexing performance with 63.10, 92.26, and 100 % of individuals being correctly classified as black, black-roan, and bronze-roan varieties, respectively (Table 5).

Our results suggest that larger turkey eggs, and hence heavier turkey eggs, are more likely to develop into black and bronze-roan female poults. Literature references have reported a significant relationship between egg metrics and poult sex determination in hens (5, 31). In line with these results, (32) suggested that male turkey poults display higher weights at hatching, as a result of the smaller difference existing between male poult weight and egg preincubation weight than in females. This finding has also been reported for the eggs of other species, such as those of the white-crowned sparrow. In this particular case, the male-containing eggs were larger than the eggs containing females (17) would ascribe this early live sexual dimorphism finding to an adaptive mechanism background in the species (17). This would also be supported by the findings in our study that although male-containing eggs were slightly lighter than the ones containing females in the previously named plumage varieties, no influence of egg size on the adult weight of bird has been reported (33).

Although black Andalusian eggs were larger than those laid by the rest of the plumage varieties, greater difficulties were encountered during the sexing of black Andalusian turkey poults based on the external characteristics of egg. This translates into a disadvantage at the time of sexing, considering the external appearance. Such difficulties may rely on the lower existing variability across eggs of this variety. Indeed, a low genetic variability was reported by (34) in the black plumage variety of the Spanish turkey population. These authors described low values for the number of nucleotides and haplotypes estimated in this population, which is indicative of populations originating from a small number of founders (35). In contrast, high variability in the products of roan varieties may evidence potential traces of hybridization with other nearby Spanish

TABLE 4 Summary of the results for strength of association between the plumage varieties of the Andalusian turkey breed and the ability to succeed or fail when assigning sex for the different methods using the chi–square independence test.

	Source	df	Chi–square (Wald)	Pr > Wald	Chi–square (LR)	Pr > LR
Black	Egg weight	1	0.0114	0.9149	0.0114	0.9149
	Major diameter	1	0.4698	0.4931	0.4708	0.4926
	Minor diameter	1	0.0037	0.9514	0.0037	0.9514
	Shape index	1	0.1405	0.7078	0.1408	0.7075
	Eggshell L*	1	0.0907	0.7632	0.0908	0.7632
	Eggshell a*	1	0.0189	0.8906	0.0189	0.8906
	Eggshell b*	1	1.0740	0.3000	1.0720	0.3005
	English test	1	0.4082	0.5229	0.4078	0.5231
	Down feathers	1	3.8028	0.0512	3.9067	0.0481
	Coping styles	1	7.1448	0.0075	7.2311	0.0072
Black–roan	Egg weight	1	1.5166	0.2181	1.6092	0.2046
	Major diameter	1	0.8368	0.3603	3.8218	0.0506
	Minor diameter	1	1.8873	0.1695	3.3790	0.0660
	Shape index	1	2.7296	0.0985	35.1506	< 0.0001
	Eggshell L*	1	7.6134	0.0058	14.3921	0.0001
	Eggshell a*	1	0.0706	0.7905	0.0706	0.7904
	Eggshell b*	1	11.1091	0.0009	15.7778	< 0.0001
	English test	1	0.0754	0.7837	0.0753	0.7837
	Down feathers	1	3.9462	0.0470	4.2696	0.0388
	Coping style	1	0.1649	0.6847	0.1653	0.6843
Bronze–roan	Egg weight	1	$7.18741E^{-5}$	0.9932	2811.4050	< 0.0001
	Major diameter	1	$7.27825E^{-5}$	0.9932	3460.1907	< 0.0001
	Minor diameter	1	$7.09201E^{-5}$	0.9933	2378.8811	< 0.0001
	Shape index	1	$7.77541E^{-6}$	0.9978	2234.7065	< 0.0001
	Eggshell L*	1	$2.66893E^{-5}$	0.9959	2595.1430	< 0.0001
	Eggshell a*	1	$5.57253E^{-5}$	0.9940	2595.1430	< 0.0001
	Eggshell b*	1	$2.24125E^{-9}$	1.0000	2595.1430	< 0.0001
	English test	1	$1.0971E^{-8}$	0.9999	2595.1430	< 0.0001
	Down feathers	1	$2.05692E^{-8}$	0.9999	2595.1430	< 0.0001
	Coping style	1	$7.18741E^{-5}$	0.9932	2811.4050	< 0.0001

breeds, such as the Oscense and the Minorcan Gall D'Indis turkey breeds, with which Andalusian roans share a similar plumage pattern (7, 36).

Regarding eggshell color, being the descendants of reptiles, ancestral birds were thought to have laid white eggs at first (37). Eggshell pigmentation may have appeared as a mechanism to hide the nest from antipredators, prevent parasitic infestations, or protect the embryo from light-filtering harmful irradiations (38). Furthermore, some species have developed different eggshell pigmentations as a response to other features, such as reinforcement mechanisms for weak shell structure (39), as cooling mechanisms, due to protoporphyrin's ability to reflect infrared light (40), or by the hen to act as a sexual decoy for mating (41).

While eggs containing male poults displayed significantly intenser pigmented eggshells, the color of female-containing eggs was less intense and brighter in the Andalusian turkey breed due to lower pigment depositions. These results are supported by (42), who reported a strong significant association between shell pigmentation intensity and increased male hatching numbers in the barn swallow species. These authors suggested that visual cues about brood sex ratio before egg hatching may let parents prepare for provisioning a highly energy-demanding male-biased brood. Indeed, (43) suggested that turkey hens could adjust the water vapor conductance of eggshells by manipulating carcass porosity, and proposed that secretor cells could adjust the carcass pore number to match embryo metabolism. This maternal ability to influence the egg functional characteristics of turkey, probably mediated by thyroid or iodine metabolism, could affect shell pigment deposition as well, as a response to early sex dimorphism properties in the egg.

10.3389/fvets.2022.948502

TABLE 5 Classification table for the estimated sexes according to different plumage varieties.

	From\To	Male	Female	Total	% correct
Black	Male	56	58	114	49.12%
	Female	35	103	138	74.64%
	Total	91	161	252	63.10%
Black–roan	Male	84	6	90	93.33%
	Female	7	71	78	91.03%
	Total	91	77	168	92.26%
Bronze–roan	Male	36	0	36	100.00%
	Female	0	48	48	100.00%
	Total	36	48	84	100.00%

The possibility of a hormonal basis in the correlation between sex ratio and egg color could be presumed. However, the mechanism of eggshell color deposition mediated by eggshell glands remains unclear. Contextually, although no influence of blood estrogen or ovulation mechanisms has been determined (44), higher progesterone blood levels prior to ovulation have been proved to influence the accumulation of colored substances in the shell gland, thus in the shell that will eventually be deposited (45), *via* their implication in the activation of the δ-aminolevulinic acid synthetase (46). However, references that either contrast (47) or support our results can be found in the literature, which may provide pieces of evidence of a multifactorial nature for the aforementioned correlation.

According to the Trivers–Willard hypothesis, hens in good metabolic conditions could bias the sex ratio of their progeny toward males, while hens exhibiting poor conditions tend to bias toward females (48–50). In line with this finding, females in good body condition maintain eggshell color to limit visible changes and conceal their eggs in anti-predator behavior. Nevertheless, food-restricted females in lower body conditions modify biliverdin and protoporphyrin concentrations in the eggshell (51). This reinforces the results obtained in the present study, since the ratio of different sexes correlates with the shell color. After the turkey poults are hatched, the present research not only reports acceptable results for the down feather's color method, especially for the black and black-roan patterns, but also allows for a rather efficient early identification of females (better fit). Although this method had been successfully used before for sex determination in hybrid and local fowl strains (13, 52, 53), our study constitutes the first to report its application in the early sex determination of turkeys.

Feather color is strongly influenced by the endocrine system, with thyroid hormone activity being considered one of the most highly conditioning elements of the system (54). Parallelly, pituitary hormones like the α-melanocyte-stimulating hormone (a-MSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are also involved in the plumage coloring process (55), but are thought to be less related to sexual dimorphism and chick feather pigmentation. Sex hormones also influence plumage pigmentation, particularly in terms of sexual dimorphic color pattern, probably acting at the level of melanoblast differentiation (56). Contextually, (54) described that although the expression of feather color is mostly influenced by genetics, estrogen or testosterone levels produce alterations in the plumage pattern of the embryo. In this regard, estrogen has been described to have a high impact on feather color in Brown Leghorn's birds (5). This finding was supported by (52) who was able to differentiate male and castrated female chicks from the New Hampshire x Light Sussex cross with a high success rate, suggesting that early endocrine sexual dimorphism may determine down feather color differences across sexes.

The bronze-roan variety did not report satisfactory results when the down feather's color technique was used. In this way, differences in sexual dimorphism patterns across different genotypes of a single breed population were suggested (5). The most extended feather varieties of Andalusian turkey are black and black-roan, which are originally presented in the ancestral domesticated turkey in Mexico (57). The presence of bronze-roan plumage in the Andalusian breed population may derive from the hypothetical hybridization of individuals belonging to this breed with other similar breeds, which may have translated into the interferences impeding the efficiency of the method.

Although less frequently approached, behavior-based sexing methods, which have barely been included in scientific reports, have reported scarce but interesting results. This gap of knowledge is even larger in turkeys, a species for which worldwide animal production integration is relatively recent. The behavior of this species is comparable to that of other birds that had been domesticated earlier in history (58). The first reference to the scientific application of the "English method" (or "inversion test") dates back to the past century in Argentina and reported nearly 70% accuracy in the sex determination of hen chicks (29, 59). These results and those in the present article are in line with those reported by (5), who confirmed the significant accuracy of the method for chick sexing in the Utrerana hen breed, a local breed from Spain.

Despite the fact that differences in the reaction to acoustic stimuli between the sexes have been thoroughly studied in chicks (60–67), the behavior/coping style or "slap" technique has scarcely been reported as a sex discriminant technique. It was only (5) who evaluated its applicability in a chick sex-determining study. In line with these results, the "slap technique" reports significant results supporting its feasible applicability for sex determination in domesticated *Meleagris gallopavo* which had never been described in turkey species prior to this study.

The influence of sex on chick behavior has been a widely studied topic during the second half of the 20th century (62, 63, 67, 68). Chick fear was known to inhibit general activity, and scared animals were described to perform both low activity and peeps (62). When 7-day-old chicks were submitted to new stimuli in an 'open field' test, males were less active and displayed rather fearful responses, displaying freezing, sitting, lying, and eye-enclosure patterns more frequently than females (68).

Chick behavior studies have also focused on isolated animals. In this case, chicks react to loud noises, such as the ring of a bell, and females were much more reactive than males, displaying higher walking and peeping activities and lower freezing, sitting, and eye-enclosure responses (64). Thus, the "English method" can be analogous to this open-field reaction test research made on chicks. The results of the present study showed a lack of activity in males and a higher response when exposed to a new environment, like the hand of the observer, in females. In the "slap technique," similar results were obtained. Males showed a significant decrease in walking and peeping activity, and thus a significantly decreased reactivity when compared to females. This finding is in contrast to the outcomes of previous research, as males tended to experience increased physiological fear responses, which were reduced after medical tranquilization to the same fear levels displayed by females (64).

Alternative theories propose that higher rates of activity in females (ambulation and distress calls), when compared to males, might not be ascribed to lower fear reactions but to a stronger need to reinstate social contact with conspecifics (69). In addition, male nestlings have shown higher exploration of unfamiliar objects than females when it is required to separate them from their partners, reinforcing less social attachment behavior among males (70). These different responses across chick sexes reflect adult sexual behavior and social organization (71). This way, newly hatched Japanese quail females displayed fear reactions less frequently when a male chick was present (72). This behavior relates to the adult social organization of a certain avian species, where a single male guards a small female group. In this regard, Andalusian turkey females show high social attachment due to their flock idiosyncracies, while males may display lower social needs owing to their solitary nature (73).

The aforementioned sex-related chick responses might be the consequence of early endocrine modulation of post-hatching sexual dimorphism behavior. Contextually, the presence of first steroid hormones in the egg has a maternal origin and plays an important role in the offspring's sex establishment, since they influence post-hatched chick behavior (74). Although the endocrine system is not matured yet, the hormonal synthesis in embryos begins during the egg developmental stage (75, 76). Indeed, the establishment of the hypothalamic–hypophyseal–adrenal axis is known to take place during days 17 and 18 of early development in turkeys, and hormonal activity increases during hatching (77). Hence, differences in the embryonal hormone profiles between sexes could suggest that endocrine sexual dimorphism might influence gallinaceous chicks right from the egg stage.

The aforementioned fact particularly concerned sexual hormones. On the one hand, testosterone has been reported to be the first hormone that is present equally in the plasma of the embryos of both sexes until day 7 of incubation, although a significant increase in the testosterone levels in males is observed (78). On the other hand, (79) described that the embryo ovary produces higher levels of estrogen than the testes during egg development. This produces a higher estrogen/androgen ratio in females, which was suggested to determine the reproductive behavior of adult Japanese quails. Additionally, steroids have proved their influence on both hormone receptors' tissular density and hormone-secreting cell distribution during early development and have direct implications in showing distinct sensitivity to hormones in adulthood (80).

Apart from its influence on physiological fear responses, the influence of hormones on anatomical neural development explains a female's greater reactions and activity (5) explained that a chick's sexual dimorphism behavior may be a direct consequence of the impact of different steroids on the development of visual vias' lateralization. Neural and behavioral lateralization has been reported to play a fundamental role in brain organization, where androgens, especially testosterone, are involved (81). In this sense, several functions are lateralized in avian species (82), and two *vias* are described. First, the 'right-eye system' is specialized to see large distant objects, whereas the 'left-eye system' is skilled to analyze the changes in special relations and positions (70) suggested that the specialization of the left-eye system is lower in female chicks than in male ones. This particularly lower space sight could explain the female's particular closeness to the hen and its relatives, and therefore their higher partnership needs described above. When considering the early feeding rates of female chicks after hatching and their higher willingness to eat novel colored food (71), greater development in the right-eye system of female chicks is suggested (83). This phenomenon could support the major reactiveness of females to fear-generating stimuli, which can be attributed to the right-eye system's implication in fixing large and distant objects, similar to the human observer (70).

In the present research, the 'slap test' not only performed efficiently in black-feathered poults, but this method was also seven times more significant than the other techniques for this plumage. The effects of plumage color on behavior have been extensively studied with contradictory results. Indeed, while in hens, white-feathered individuals have been suggested to be more aggressive than black and gray ones (84, 85). In an indigenous turkey breed, more aggressive behavior has been reported in black- and lavender-feathered individuals when compared to white individuals (86). In line with these results, as aggression can frequently be initiated by a fear-producing stimulus, (87)

described white-feathered turkeys may be less fearful than bronze birds.

The basis for this observation may stem from the fact that a significant relationship has been reported between skin pigmentation and certain conduct patterns in many species, such as Norway rats, lions, and wild foxes (88–90). The link between different physiological mechanisms and dark feather patterns has been described to be the result of broad pleiotropic effects of the gene network encoding melanin synthesis or its transport and deposition (42, 91). Genes controlling the deposition of plumage pigments, such as the agouti signaling protein gene, melanocortin-1 gene, or the tyrosine gene, have been proposed to also affect the hormonal status and influence sexual behavior or aggressiveness (92). Thus, darker animals may tend to be more aggressive, possibly due to a higher release of self-stimulating pheromones and a greater secretion of exocrine glands that melanocortins promote (86).

Due to plumage color selection during domestication and its well-studied relationship with behavior (91), higher primitive behavioral gene preservation in black-feathered turkeys should be considered. At this concern, behavior has been closely related to animal domestication, being directly and indirectly modified by humans, since individuals that show better tolerance to human presence also showed the highest production (25, 93, 94). This can be attributed to a lower hypothalamic–hypophyseal–adrenal axis reactivity, a consequence of genetic modifications that selection for docility achieved during domestication (25). For instance, in an open-field test, black-feathered poults displayed greater reactiveness to fear (86). It has been reported that black plumage performed fear-avoidance behaviors (escaping, jumping, and flight) more frequently than lighter-colored individuals.

The effect of early selection practices along with the domestication process of turkeys may be more relevant in the Andalusian turkey breed. The Andalusian turkey breed is a very rudimentary population that has barely been submitted to selection or improvement. This population has often been described as a living representation of the first turkeys that arrived in Europe in the 16th century, and before that, these birds had only accomplished 1,300 years of domestication (10), while comparatively, the chicken species may have probably been domesticated for 4,700 years by that time (95, 96). Therefore, black-feathered Andalusian turkeys could present a greater degree of relatedness to wild ancestors than the individuals presenting one of the remaining plumage patterns.

Bertin and Richard-Yris (97) reported that despite thousands of years of domestication, the free-range-reared domestic animals showed behaviors that still closely resembled those of their wild ancestors. Contextually, the increased frequencies of sex-biased fear responses to strong human stimuli may be the reason why statistically significant reliability in the "slap test" was only reached in black-feathered poults. This hypothesis is supported by previous research in which a non-selected fowl genotype shared more behavioral patterns with a wild ancestor than a highly selected strain (98).

Conclusion

Conclusively, the combination of egg external characteristics, down feather coloring, and two behavioral techniques ("English method" and slap technique) allows effective sexing in newly hatched poults belonging to the Andalusian turkey breed, chiefly for the two roan varieties (black-roan and bronze-roan). Sexual dimorphism is not very evident in egg size, since egg dimensions do not influence adult weight in turkeys. Early sexual dimorphism is significant when eggshell color is considered, since female-containing eggs were less intensely colored and brighter due to a lower pigment deposition. Color differences of the bronze-roan variety with the predominant black-based colors render this method significantly invalid for sex determination in this plumage pattern. Behavioral techniques like the "English method" and the "slap test" presented high discriminatory power. In any case, the development of this battery of tests, their high predictive potential, and the ease of implementation in non-industrialized farms allows a reliable determination of sexual relationship in a population due to their low economic cost of implementation and the relative improvement in efficiency at data collection, which deems this tool a time and resource-economic alternative to other methods.

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the animal study because Bird management was directed under the European Union Direction regulations (2010/63/EU) as transposed to Spanish Royal Decree-Law 53/2013. This study did not need to be subjected to evaluation by the Ethics Committee of Animal of the University of Córdoba, since it is not part of the legislation for the protection of animals used for scientific purposes.

Author contributions

JS, FN, and AG: conceptualization and original draft preparation. FN, AG, and AA: methodology and data

curation. FN and AA: software and visualization. JL, JD, and MC: validation and formal analysis. JS, FN, AG, and AA: investigation. JD and MC: resources and funding acquisition. MC: project administration. FN and JL: reviewing and editing. FN: supervision. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by FEDER project P20_00893.

Acknowledgments

This work would not have been possible without the assistance of ACPA (Asociación de Criadores del Pavo Andaluz), IFAPA (Instituto de Investigación y Formación Agraria y Pesquera), Diputación de Córdoba, and PAIDI AGR 218 research group.

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.2022.948502/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 22 August 2022
DOI 10.3389/fvets.2022.944063

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 14 May 2022
ACCEPTED 25 July 2022
PUBLISHED 22 August 2022

CITATION
Dang DX, Zhou H, Lou Y and Li D (2022) Effects of *in ovo* feeding of methionine and/or disaccharide on post-hatching breast development, glycogen reserves, nutrients absorption parameters, and jejunum antioxidant indices in geese. *Front. Vet. Sci.* 9:944063. doi: 10.3389/fvets.2022.944063

Effects of *in ovo* feeding of methionine and/or disaccharide on post-hatching breast development, glycogen reserves, nutrients absorption parameters, and jejunum antioxidant indices in geese

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We investigated the effects of *in ovo* injection of methionine (Met) and/or disaccharide (DS) on breast muscle and small intestine development, and the aspect of the glycogen contents, digestive enzymes activities, and jejunal antioxidant parameters in geese after incubation. A total of 600 fertilized eggs were used in this study to be employed in a 2 × 2 factorial experiment. Eggs were randomly assigned to 4 groups, 6 replicates per group, and 25 eggs per replicate. Factors in four groups included non-injection, Met injection (5 g/L Met dissolved in 7.5 g/L NaCl), DS injection (25 g/L maltose and 25 g/L sucrose dissolved in 7.5 g/L NaCl), and DS plus Met injection (25 g/L maltose, 25 g/L sucrose, and 5 g/L Met dissolved in 7.5 g/L NaCl). As a result, birth weight, relative weight of breast muscle, diameter of myofiber, glycogen contents, jejunal villus and surface area, and jejunal digestive enzymes activities improved, while liver glucose-6-phosphatase activity decreased, by DS injection. Additionally, DS administration upregulated the expression of myogenic factor-5 (Myf-5) from breast muscle and sodium/glucose cotransporter protein-1 (SGLT-1) from jejunum. *In ovo* delivery of DS has long-term effects on the improvement of jejunal glucose transporter-2 (GLUT-2) and sucrase-isomaltase expression. *In ovo* feeding of Met improved the relative weight of breast muscle and small intestine, diameter of myofiber, length of small intestine, jejunal villus width, jejunal sucrase, Na^+/K^+ATPase and alkaline phosphatase activities, and jejunal glutathione (GSH) concentration, and decreased the jejunal glutathione disulfide (GSSH) and the ratio of GSSG to GSH, in early-life post-hatching. The breast muscle Myf-5 and myostatin expression, jejunal villus height and surface area, jejunal glutathione peroxidase concentration, and the expression of GLUT-2 in jejunum long-term improved by *in ovo* delivery of Met. Moreover, *in ovo* feeding of DS plus Met mixture synergistically improved the diameter of myofiber, jejunal villus height and width, jejunal sucrase, and alkaline phosphatase

activities in early-life post-hatching, but long-term upregulated the expression of jejunal GLUT-2. Therefore, we concluded that *in ovo* injection of Met plus DS is an effective way to improve the development of gosling during post-hatching stages.

KEYWORDS

goose, *in ovo* injection, intestinal health, post-hatching development, nutrient absorption

Introduction

Geese are seasonal egg producers, its eggs are mainly used for eating, reproduction, and research purposes. It becomes more important to improve gosling quality during post-hatching stages in commercial geese farming. In modern gosling hatcheries, birds are always taken out of the incubator at the same time, which means premature birds have to wait for other late-born birds to ensure maximum hatching over the same period. Additionally, commercially hatched gosling faces entirely different post-natal environments in comparison with geese in natural brooding; they could not have immediate access to feed and water. A series of operations such as packaging and shipping also inevitably delays the access to feed and water. Therefore, gosling faces a fasting challenge during its initial crucial period of life.

The vigorous growth and metabolism activities during the fasting periods lead to an insufficient supply of energy and protein and the risk of oxidative stress, resulting in retarded growth, limited gut development, and stunted breast muscle (1–4). The *in ovo* injection approach provides a method in overcoming these problems and filling the gap between the hatch and first feed access (5).

Amnion has been proven to be an effective site for implementing *in ovo* injection techniques (6). The embryo orally consumes the amniotic fluid before pipping the air cell under natural conditions, thus consuming the supplemented nutrients which are presented in the enteric tissues (7). This presents an opportunity to inject nutrients into the amnion to uplift the status of growth and development in the embryo (8).

Digestible disaccharides (DS) are possible candidates for exogenous energy provision, it plays an important glucose precursor for late-term bird embryo catabolism and could alleviate energy deficiency by stimulating glucose anabolism (9, 10). Methionine (Met) as the first limiting amino acid for birds has been reported to decrease oxidative stress and uplift the protein supply (11, 12). To our knowledge, studies on the effects of *in ovo* injection of DS and/or Met on the performance in geese were still limited. In our previous study, *in ovo* injection of DS and/or Met had positive effects on the development of the embryo of goslings (13). We hypothesized that the administration of DS and/or Met into the amnion of the late-term embryo may serve as a tool to provide energy for small intestine and embryo activity, in turn alleviating energy and protein lack, and improving breast muscle and intestine development in geese during post-hatching periods. To test this hypothesis, we conducted this study to investigate the effects of *in ovo* injection of DS and/or Met on the post-hatching breast muscle parameters, glycogen reserves, glucose-6-phosphatase (G-6-Pase) activity, myogenic factor-5 (Myf-5) and myostatin (MSTN) expression in breast muscle, small intestine parameters, jejunum morphology, jejunum digestive enzymes activities, jejunum antioxidant indices, jejunum sodium/glucose cotransporter protein-1 (SGLT-1), glucose transporter-2 (GLUT-2), and sucrase-isomaltase (SI) expression.

Materials and methods

Experimental design, animals, and housing

The fertilized eggs (in total 1,000) were purchased from the Dekun Poultry Food Co., Ltd (Meihekou, Jilin). The breed of the egg was Jilin White geese. All eggs were laid on the same day, represented in the same weight class. Fertilized geese eggs were incubated in the standard condition in an incubator (Keyu CFZ microcomputer automatic incubator, Dezhou, Shandong). Before transiting to the incubator, eggs were pre-heated to 30°C for 12 h, disinfected with 37% formaldehyde and potassium permanganate (2:1), and distributed into incubator tray levels. The incubation period included three phases (phase 1, days 1–14; phase 2, days 15–28, and phase 3, days 29–31). During phase 1, the temperature was 38°C and the humidity was 65%; during phase 2, the temperature was 37.5°C and the humidity was 55%; and during phase 3, the temperature was 37.2°C and the humidity was 70%. All eggs were turned once per 2 h for 180 s.

Eggs were candled checking for containing embryonated eggs on day 23 of incubation. A total of 600 fertilized eggs were selected from the embryonated eggs obtained above and randomly assigned into 4 groups with 6 replicates per group and 25 eggs per replicate. This experiment was a two-factor design, which included DS injection or Met injection. Eggs treatment

in four groups was divided into non-injection, Met injection (5 g/L Met dissolved in 7.5 g/L NaCl), DS injection (25 g/L maltose and 25 g/L sucrose dissolved in 7.5 g/L NaCl), and DS plus Met injection (25 g/L maltose, 25 g/L sucrose, and 5 g/L Met dissolved in 7.5 g/L NaCl). Injection solutions and paraffin were prepared on the day of injection (day 24 of incubation) and were treated at 121°C for 15 min. The injection was conducted when the solutions and paraffin are cooled down to room temperature.

A 70% of ethanol was used for egg surface sterilization. Cleaned eggs were put onto a holder with the large end on top. The position of amnion was identified by candling. The upper side of the eggs (the air space) was pierced by an egg-shell punch. Injections were performed with a disinfected injector. About 1.5 mL of each solution was injected into the amnion of each egg to a depth of 20 mm, without hurting the embryo. After each inoculation, the needle was routinely disinfected to minimize the risk of infection. All eggs were held outside the incubator for <5 min while injecting, including the non-injected control eggs. Immediately after the injection, the hole was sealed using paraffin, and the eggs were returned to the incubator and incubated in line with the routine procedure until hatched.

After hatching, birds were transported to a temperature-controlled room with continuous lighting and distributed into cages according to the replicates. Feeds were provided immediately after transportation. The raising period was until day 28 of age (Table 1). Uniform management was conducted during this period. The temperature of the room was maintained at 30°C during the first 3 days and then reduced by 2°C per week.

The Animal Care and Use Committee of Jilin Agricultural University (Changchun, China) supervised the procedure of this experiment.

TABLE 1 Composition and nutrient levels of the experimental basal diet (%, as-fed basis).

Ingredients, %	
Corn	60.00
Soybean meal	29.11
Wheat bran	6.00
Fish meal	2.00
Lysine-HCl	0.20
Methionine	0.23
Dicalcium phosphate	0.84
Limestone	0.82
Sodium chloride	0.30
Vitamin and trace mineral premix[a]	0.50
Total	100.00
Calculated value, %	
Metabolizable energy, MJ/kg	11.67
Available phosphorus	0.40
Analyzed composition, %	
Crude protein	19.78
Methionine	0.50
Total sulfate amino acid	0.77
Lysine	1.08
Calcium	0.78
Crude fiber	0.31
Neutral detergent fiber	1.09
Acid detergent fiber	0.35

[a]Provided per kg of complete diet: vitamin D_3, 200 IU; vitamin A (retinyl acetate), 1,500 mg; vitamin E (DL-α-tocopheryl acetate), 12.5 mg; vitamin K_3, 1.5 mg; thiamine, 2.2 mg; riboflavin, 5 mg; nicotinic acid, 65 mg; folic acid, 1 mg; pantothenic acid, 15 mg; pyridoxine, 2 mg; biotin, 0.2 mg; choline, 1,000 mg; Fe, 90 mg; Cu, 6 mg; Mn, 85 mg; Zn, 85 mg; I, 0.42 mg; Se, 0.3 mg; Co, 2.5 mg.

Feed analysis

After homogeneous mixing, feed samples were collected from each dietary group. All feed samples were dried in a 70°C constant temperature oven for 72 h. Subsequently, feed samples were ground and sieved with a 1-mm sieve. The collected feed powder is with a diameter of <1 mm for feed composition analysis. According to the procedure established by the AOAC (14), the dry matter (method 930.15), crude protein (nitrogen × 6.25; method 968.06), and crude fiber (method 991.43) composition in the diet were analyzed. Then, the representative feed samples in each group were hydrolyzed with 6 N HCl for 24 h at 110°C. An amino acid analyzer (2690 Alliance, Waters, Inc., Milford, MA) was used for determining amino acid contents in the diet. In addition, the contents of neutral detergent fiber and acid detergent fiber in the diet were measured according to the method provided by Mertens (15).

Sample collection

On hatching day, day 7 post-hatching, and day 28 post-hatching, three birds were randomly selected from each replicate group, weighed, and slaughtered by cervical dislocation for further analysis.

The liver was removed from the carcass and the adherent material of the liver was carefully removed under ice-cold saline. About 100 mg of liver samples were taken and homogenized in 0.25 mol l^{-1} sucrose solution for G-6-Pase activity analysis. The remaining sample of the liver was frozen as aliquots in liquid nitrogen, and stored at −80°C for measuring the liver glycogen contents.

Both sides of breast muscle were stripped after being slaughtered. One side of the breast muscle sample was weighed and fixed with 10% neutral-buffered formalin for analyzing myofiber traits. Another side of the breast muscle sample was frozen as aliquots in liquid nitrogen, and stored at −80°C for

measuring breast muscle glycogen contents and breast muscle development-related gene expression situation.

The whole small intestine was removed and the adherent material of the small intestine was carefully removed under ice-cold saline, weighed, and separated into the duodenum, jejunum, and ileum. The duodenum was defined as the portion of the small intestine composed of the duodenal loop; the jejunum was considered as the part between the duodenum and ileum; and the ileum as a distal segment before ileo-cecal junction equal to the length of the cecum. The jejunum sample was used for measuring antioxidant parameters first, and then about 1-cm long segment from the middle of the jejunum was taken in duplicate and placed in two separate tubes. One sample was fixed with 10% neutral-buffered formalin solution for histology and the other sample was frozen in liquid nitrogen, and then stored at −80°C for measuring digestive enzyme activities and nutrients transport gene expression.

Experimental parameters measurement

Growth performance parameters analysis

The body weight of goslings was checked after incubation to calculate the birth weight and correspondingly calculated the mortality rate.

Breast muscle and small intestine parameters analysis

The relative weight of breast muscle and small intestine were calculated using the following equation:

$$Organ\ index\ =\ \frac{Organ\ weight}{Live\ body\ weight}\ \times\ 100\ \%.$$

The length of the small intestine was measured by a dividing ruler.

Myofiber traits analysis

After slaughtering the geese, the breast muscle samples were cut into small pieces and fixed with 10% neutral buffered formalin for 12 h, followed by dehydration in increasing concentrations of alcohol (70, 80, 90, 95, and 100%) and xylene. Consequently, samples were embedded in paraffin and stored in an oven at 60°C. About 12 h later, samples were removed from the oven and histological cassettes. Fragments were placed in "paper boxes" and covered with paraffin. After the paraffin solidified into blocks, the "papers" were removed and the blocks were kept under refrigeration until the cuts were realized (16).

Serial tissue sections (3 μm thickness) were excised perpendicular to the direction of the myofibers using a cryostat. After sectioning, put the paraffin section ribbon on the coating slide glass. Dried slides were kept in an oven at 60°C for 2 h to eliminate any excess paraffin. The next step consisted of paraffin removal and slide hydration, using xylene, and different concentrations of ethanol. Samples were then stained following the hematoxylin and eosin staining protocol (16).

Samples were then dehydrated again and mounted. In each specimen, the diameters of muscle fibers were measured under a light microscope equipped with a ScopePhpto (LY-WN 300, Hangzhou Scopetek Opto-Eletric Co., Ltd.).

No less than 150 intact, well-oriented muscle fibers cross-sectional area of five fields of vision were measured under 40 times the objective lens. With muscle fibers assumed to be round, the muscle fiber cross-sectional area (A) was converted to diameter (D) by the formula, $D = 2\sqrt{A/\pi}$. The average value was calculated to represent the diameter of the muscle fibers (17).

Glycogen reserves analysis

About 0.1 grams sample of liver and breast muscle were stored at 1 ml of 8% HClO4, homogenized (in ice) for 45 s, and centrifuged at 7,700 rpm at 4°C for 16 min. A 10-μl aliquot of the supernatant was transferred to a clean polypropylene tube, along with 0.4 ml of 8% perchloric acid and 2.6 ml of iodine color reagent made of 1.3 ml of solution A (0.26 g iodine + 2.6 g potassium iodide dissolved in 10 ml of distilled water) in 100 ml of 67.8% saturated calcium chloride. All samples were read at a wavelength of 450 nm. The amount of glycogen present in the sample solution was determined by the preparation of a known glycogen standard curve.

G-6-Pase activity analysis

Livers were taken to a total weight of 100 mg and homogenized in 0.25 mol l^{-1} sucrose solution for analyzing G-6-Pase activity. G-6-Pase was assayed (in 20 mmol l^{-1} Tris–HCl, pH 7.3 for 10 min at 37°C) by complex formation of inorganic phosphate (Pi) liberated from G-6-Pase (0.1 mol l^{-1}) and subtracting the amount of Pi produced from para-nitrophenylphosphate (20 mmol l^{-1}) under the same conditions of the assay. The protein concentration in liver homogenization was assayed using the method described by Bradford (18). G-6-Pase activity was expressed in micromoles of released inorganic phosphorus per min per milligram protein.

Muscle growth-related gene expression analysis

Total RNA was isolated from muscle samples using RNAiso Reagent (TaKaRa, Dalian, Liaoning, China). The RNA integrity was assessed by electrophoresis on a 1% agarose gel containing formaldehyde. The RNA concentration was measured using a Beckman DU-640 spectrophotometer (Beckman). The sequences of primers for the genes tested were specifically designed according to the sequences located in

TABLE 2 Primers used for quantitative real-time PCR.

Gene	Accession[a]	Product (bp)[b]	Primer sequences
β-Actin	M26111	158	Forward GCCCAGCACGATGAAGAT
			Reverse ATTTACGGTGGACGATGGAC
MSTN	AY448009	133	Forward GTGGCTCTTGATGACGGTAGT
			Reverse GCAGTGTGCTGAGGATTTGA
Myf-5	KU744843	147	Forward GCGTTTGAGACCCTGAAGAG
			Reverse TCCCGGCAGGTGATAGTAGT
SGLT-1	KU744842	126	Forward GTAACATTGGCAGCGGACAT
			Reverse TGGGTACAAACAGCCATCCT
GLUT-2	KU744841	118	Forward CAGTTCTTCCTGCTCCTGCT
			Reverse TCATCGGGTCACAGTTTCCT
SI	KU744844	193	Forward CGTCACCTTCCCTCTTTGG
			Reverse GGATTATGCTTCACTTCCACTTTG

SGLT-1, sodium/glucose cotransporter protein-1; SI, sucrase-isomaltase; Myf-5, myogenic factor-5; GLUT2, glucose transporter-2; MSTN, myostatin.
[a]Accession number refer to Genbank (NCBI).
[b]PCR product size (base pairs).

GenBank (Table 2). The total RNA samples were purified and subjected to reverse transcription using the Takara PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) and processed for cDNA synthesis as per Takara PrimeScript RT instructions (19).

The relative expression levels of Myf-5 and MSTN genes in pectoral muscle were analyzed by RT-PCR, which was performed in a 10-μL reaction mix containing 1 μL 2 × SYBR Premix Ex Taq II (TakaRa, Dalian, China), 3 μL dH_2O, 0.5 μL of the upstream and downstream primers, and 1 μL cDNA using a Bio-Rad CFX-96 thermocycler (Bio-Rad, CA). The reaction conditions were as follows: initial denaturation at 95°C for 30 s and 44 cycles of amplification at 72°C for 30 s. The annealing was carried out for 40 s at temperatures specific to each target gene. At the end of the amplification, step-wise melting curves were performed to confirm the product specificity. The cytoskeletal protein, β-actin, was used as the internal reference (20). All the reactions were performed in triplicate. The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The relative level of each mRNA normalized to the ΔCt (β-actin) gene was calculated using the following equation:

$$Fold\ change = \frac{2^{Ct\ target\ gene\ (control)\ -Ct\ target\ gene\ (treatment)}}{2^{Ct\ housekeeping\ gene\ (control)-Ct\ housekeeping\ gene\ (treatment)}}$$

Jejunum morphology analysis

The jejunum samples were cut into small pieces and further treated by fix and dehydration according to the methods described above to prepare paraffin blocks.

A microtome was used to make five cuts that were 5 μm. The cuts were stained with hematoxylin-eosin. The values were measured using a light microscope. Measurements of villus height and width were determined at a magnification of 10X. A minimum of five measurements per slide were made for each parameter and averaged into one value (21). Villus surface area was calculated from the villus height (from the tip of the villi to the villus crypt junction) and width at half height (22). Values presented means from 10 adjacent villi and only vertically oriented villi were measured.

Digestive enzymes activities analysis

Enzyme activities were assayed in homogenized jejunum tissue. Samples were thawed at 4°C and homogenized in 10 times the volume of cold normal saline. The homogenates were then centrifuged at 20,000 ×g for 20 min at 4°C and the supernatant was collected for enzyme assays. Sucrase [Enzyme Commission (EC) 3.2.1.48] and maltase (EC 3.2.1.20) activity were assayed colorimetrically using sucrose and maltose as substrates, respectively. The activity was expressed as micromoles of glucose released per minute per gram of jejunum wet tissue. Alkaline phosphatase (EC 3.1.3.1) activity was determined by measuring the hydrolysis of p-nitrophenol at 37°C, and the unit of activity was expressed as per min per gram of jejunum wet tissue. Na^+/K^+ATPase (EC 3.6.1.3) activity was determined by measuring the liberation of phosphate from ATP-Na2 (No. A7699, Sigma-Aldrich) in two media: medium I (all ATPases system) and medium II (Na^+/K^+ATPase restrained system); and the activity of Na^+/K^+ATPase was calculated as the difference between phosphates liberated by each homogenate in the two media and was expressed as micromoles of phosphates per milligram homogenate protein or per milliliter of serum per h.

Jejunum antioxidant parameters analysis

The activities of glutathione peroxidase (GPX), glutathione (GSH), and glutathione disulfide (GSSG) in the mucose were determined using commercial kits (Nanjing JianCheng Bioengineering Institute, Nanjing, P. R. China) according to the instructions of the manufacturer. All results were normalized against total protein concentration in each sample for inter-sample comparison. The GSSG/GSH ratio was determined according to the data on GSSG and GSH activities in mucose.

Jejunum nutrients transport gene expression analysis

The abundance of SGLT-1, GLUT-2, and SI mRNA isolated from geese jejunum tissues (approximately 50 mg) was analyzed by the above step. The sequences of primers for the genes tested were specifically designed according to the sequences located in

GenBank (Table 2). The cytoskeletal protein, β-actin, was used as the internal reference (20).

Statistical analysis

The data were analyzed as a two-way ANOVA factorial arrangement of treatments using the GLM procedure in SPSS18.0 software, with treatment as the fixed effect and the replicate cage as the experimental unit. Factors involved were *in ovo* injection of DS and/or Met. The data are presented as the means ± standard deviation (SD). Results were considered significant at $P < 0.05$.

Results

Growth performance parameter

Birth weight was increased by feeding DS into the embryo ($P < 0.05$). The *in ovo* injection strategies had no significant effects on the mortality rate (Table 3).

Breast muscle and myofiber trait parameter

In ovo injection of DS increased the relative weight of breast muscle ($P < 0.01$) and the diameter of myofiber ($P < 0.01$) on the day of hatching. Met administration increased the relative weight of breast muscle on the day of hatching ($P < 0.01$) and day 7 post-hatching ($P < 0.05$) and increased the diameter of myofiber on day 7 post-hatching ($P < 0.01$). In addition, *in ovo* delivery of Met plus DS synergistically increased the diameter of myofiber on the day of hatching ($P < 0.01$) (Table 4).

Glycogen reserves

As shown in Table 5, *in ovo* injection of Met had no significant effects on the glycogen reserves. However, *in ovo* feeding of DS increased breast muscle glycogen content on the day of hatching ($P < 0.01$) and day 7 post-hatching ($P < 0.01$), liver glycogen content on the day of hatching ($P < 0.01$), and total glycogen content on the day of hatching ($P < 0.01$).

G-6-Pase activity

DS injection decreased the G-6-Pase activity on the day of hatching ($P < 0.01$). However, Met injection had no significant effects on the G-6-Pase activity (Table 6).

Muscle growth-related gene expression

The expression of Myf-5 from breast muscle was upregulated by DS injection on the day of hatching ($P < 0.05$). Moreover, Met injection upregulated the Myf-5 and MSTN expression on the day of hatching ($P < 0.01$), day 7 post-hatching ($P < 0.01$), and day 28 post-hatching ($P < 0.01$) (Table 7).

Small intestine parameter

Met injection increased the relative weight of the small intestine on the day of hatching ($P < 0.05$) and the length of the small intestine on day 7 post-hatching ($P < 0.05$). However, DS injection had no significant effects on the small intestine parameters (Table 8).

Jejunum morphology

The jejunal villus height ($P < 0.01$), villus width ($P < 0.05$), and villus surface area ($P < 0.01$) on day of hatching, villus height ($P < 0.01$) on day 7 post-hatching, and villus height ($P < 0.01$) and villus surface area ($P < 0.01$) on day 28 post-hatching were increased by *in ovo* injection of Met. DS injection increased jejunal villus height and villus surface area on the day of hatching ($P < 0.01$) and day 7 post-hatching ($P < 0.01$). Additionally, a synergistic effect of DS plus Met injection on the jejunal villus height ($P < 0.01$) and villus surface area ($P < 0.01$) was observed on the day of hatching (Table 9; Figures 1–3).

Digestive enzymes activities

The Na^+/K^+ATPase ($P < 0.05$) and alkaline phosphatase ($P < 0.01$) activities on the day of hatching and sucrase ($P < 0.01$) and alkaline phosphatase ($P < 0.01$) activity on day 7 post-hatching were increased by *in ovo* Met delivery. *In ovo* feeding of DS increased maltase ($P < 0.01$), sucrase ($P < 0.01$), Na^+/K^+ATPase ($P < 0.05$), and alkaline phosphatase ($P < 0.01$) activities on the day of hatching, also increased sucrase ($P < 0.01$) and alkaline phosphatase ($P < 0.01$) activities on day 7 post-hatching. Additionally, *in ovo* injection of Met plus DS synergistically increased the sucrase activity ($P < 0.01$) on day 7 post-hatching and the alkaline phosphatase activity ($P < 0.01$) on the day of hatching (Table 10).

Jejunum antioxidant parameters

The administration of Met increased GSH ($P < 0.01$) and GPX ($P < 0.01$) levels, whereas decreased GSSG ($P < 0.01$) levels

TABLE 3 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on growth performance parameters of geese during the early-life post-hatching[a,b].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
Birth weight, g	95.93 ± 5.14	101.64 ± 7.27	99.35 ± 3.28	105.68 ± 9.98	0.199	0.045	0.912
Morality rate, %	3.47 ± 1.44	2.68 ± 2.70	3.65 ± 1.65	2.66 ± 0.04	0.937	0.399	0.919

[a]The data are presented as the means ± standard deviation.
[b]The "−" means without nutrients injection, while "+" means with nutrients injection.

TABLE 4 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on breast muscle parameters of geese during the early-life post-hatching[1,2].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
Relative weight of breast muscle, %							
Day of hatching	0.67 ± 0.05	0.91 ± 0.13	0.92 ± 0.08	1.03 ± 0.06	<0.001	<0.001	0.059
Day 7 post-hatching	0.70 ± 0.02	0.71 ± 0.06	0.74 ± 0.09	0.79 ± 0.07	0.031	0.206	0.425
Day 28 post-hatching	1.31 ± 0.13	1.22 ± 0.16	1.34 ± 0.33	1.23 ± 0.22	0.830	0.280	0.945
Diameter of myofiber, μm							
Day of hatching	5.31 ± 0.09[c]	6.37 ± 0.36[a]	5.88 ± 0.36[b]	6.24 ± 0.26[a]	0.080	<0.001	0.007
Day 7 post-hatching	7.02 ± 0.32	7.02 ± 0.32	7.63 ± 0.84	7.96 ± 0.34	0.001	0.431	0.431
Day 28 post-hatching	12.55 ± 1.34	12.43 ± 0.49	12.93 ± 1.07	12.51 ± 1.04	0.586	0.524	0.720

[1]The data are presented as the means ± standard deviation.
[2]The "−" means without nutrients injection, while "+" means with nutrients injection.
[a,b,c]Different superscripts within a row indicate a significant difference ($p < 0.05$).

TABLE 5 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on glycogen reserves of geese during the early-life post-hatching[a,b].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
Breast muscle glycogen contents, mg/g							
Day of hatching	1.73 ± 0.17	1.95 ± 0.09	1.73 ± 0.24	2.12 ± 0.17	0.274	<0.001	0.240
Day 7 post-hatching	2.00 ± 0.10	2.40 ± 0.20	2.14 ± 0.29	2.54 ± 0.17	0.112	<0.001	0.991
Day 28 post-hatching	1.53 ± 0.14	1.53 ± 0.09	1.61 ± 0.16	1.55 ± 0.22	0.478	0.690	0.681
Liver glycogen contents, mg/g							
Day of hatching	3.31 ± 0.29	4.08 ± 0.17	3.48 ± 0.51	4.21 ± 0.53	0.374	<0.001	0.926
Day 7 post-hatching	64.58 ± 4.48	63.96 ± 2.00	66.20 ± 4.42	65.34 ± 2.03	0.300	0.607	0.933
Day 28 post-hatching	43.77 ± 4.10	45.68 ± 3.12	46.16 ± 4.00	42.15 ± 3.06	0.699	0.484	0.058
Total glycogen contents, mg/g							
Day of hatching	0.10 ± 0.01	0.14 ± 0.02	0.12 ± 0.01	0.15 ± 0.03	0.110	<0.001	0.665
Day 7 post-hatching	2.90 ± 0.47	2.81 ± 0.38	2.92 ± 0.15	2.88 ± 0.21	0.727	0.642	0.890
Day 28 post-hatching	1.34 ± 0.14	1.29 ± 0.26	1.40 ± 0.20	1.19 ± 0.26	0.805	0.177	0.382

[a]The data are presented as the means ± standard deviation.
[b]The "−" means without nutrients injection, while "+" means with nutrients injection.

and GSSG/GSH ($P < 0.01$) ratio from jejunum on the day of hatching. In addition, the GPX levels on days 7 ($P < 0.01$) and 28 ($P < 0.01$) post-hatching was increased by Met injection. However, DS injection had no significant effects on the jejunum antioxidant parameters (Table 11).

Jejunum nutrients transport gene expression

Effects of *in ovo* injection of Met and/or DS on jejunum nutrients transport gene expression were shown in Table 12.

TABLE 6 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on glucose 6-phosphatase (G-6-Pase) activity of geese during the early-life post-hatching[a,b].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
G-6-Pase activity							
Day of hatching	0.17 ± 0.01	0.14 ± 0.01	0.15 ± 0.02	0.13 ± 0.01	0.085	<0.001	0.237
Day 7 post-hatching	0.03 ± 0.003	0.03 ± 0.004	0.03 ± 0.01	0.03 ± 0.01	0.990	0.498	0.467
Day 28 post-hatching	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.881	0.758	0.657

[a]The data are presented as the means ± standard deviation.
[b]The "−" means without nutrients injection, while "+" means with nutrients injection.

TABLE 7 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on the expression of myogenic factor-5 (Myf-5) and myostatin (MSTN) from breast muscle of geese during the early-life post-hatching[a,b].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
Myf-5 expression abundance in breast muscle							
Day of hatching	1.00 ± 0.19	1.19 ± 0.17	1.50 ± 0.17	1.61 ± 0.16	<0.001	0.048	0.553
Day 7 post-hatching	2.53 ± 0.27	2.55 ± 0.32	3.08 ± 0.24	3.03 ± 0.55	0.003	0.946	0.815
Day 28 post-hatching	4.61 ± 0.33	4.58 ± 0.73	5.87 ± 0.86	5.62 ± 0.64	<0.001	0.608	0.688
MSTN expression abundance in breast muscle							
Day of hatching	1.00 ± 0.22	1.11 ± 0.16	0.73 ± 0.08	0.64 ± 0.07	<0.001	0.692	0.066
Day 7 post-hatching	4.40 ± 0.62	4.39 ± 1.17	3.12 ± 0.63	2.92 ± 0.37	<0.001	0.737	0.768
Day 28 post-hatching	1.36 ± 0.17	1.40 ± 0.20	0.83 ± 0.11	0.77 ± 0.10	<0.001	0.894	0.430

[a]The data are presented as the means ± standard deviation.
[b]The "−" means without nutrients injection, while "+" means with nutrients injection.

TABLE 8 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on small intestine parameters of geese during the early-life post-hatching[a,b].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
Relative weight of small intestine, %							
Day of hatching	2.54 ± 0.12	2.37 ± 0.15	2.61 ± 0.23	2.64 ± 0.17	0.027	0.320	0.190
Day 7 post-hatching	7.69 ± 0.62	7.87 ± 0.29	7.93 ± 0.46	8.08 ± 0.34	0.234	0.373	0.929
Day 28 post-hatching	5.04 ± 0.41	5.21 ± 0.32	5.36 ± 0.33	5.34 ± 0.38	0.154	0.629	0.534
Length of small intestine, cm							
Day of hatching	48.48 ± 1.65	47.93 ± 0.61	49.25 ± 1.07	48.65 ± 1.82	0.201	0.317	0.965
Day 7 post-hatching	97.95 ± 1.60	97.72 ± 0.55	99.65 ± 2.99	101.40 ± 3.55	0.015	0.461	0.337
Day 28 post-hatching	154.17 ± 14.57	160.42 ± 16.29	156.33 ± 12.42	165.08 ± 11.15	0.550	0.196	0.826

[a]The data are presented as the means ± standard deviation.
[b]The "−" means without nutrients injection, while "+" means with nutrients injection.

The Met injection upregulated the expression of GLUT-2 on the day of hatching ($P < 0.05$) and day 28 post-hatching ($P < 0.05$). DS injection upregulate the SGLT-1 expression on the day of hatching ($P < 0.01$) and day 7 post-hatching ($P < 0.01$), GLUT-2 expression on the day of hatching ($P < 0.01$), day 7 post-hatching ($P < 0.01$), and day 28 post-hatching ($P < 0.01$), and SI expression on the day of hatching ($P < 0.01$), day 7 post-hatching ($P < 0.01$), and day 28 post-hatching ($P < 0.01$). Additionally, the expression of GLUT-2 was synergistically upregulated by *in ovo* injection of Met plus DS on day 28 post-hatching ($P < 0.01$).

TABLE 9 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on jejunum parameters of geese during the early-life post-hatching[1,2].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
Villus height of jejunum, μm							
Day of hatching	138.56 ± 7.82[c]	219.82 ± 9.26[a]	194.89 ± 18.55[b]	221.72 ± 10.09[a]	<0.001	<0.001	<0.001
Day 7 post-hatching	471.42 ± 52.42	664.73 ± 23.02	556.17 ± 44.75	710.61 ± 56.49	0.002	<0.001	0.313
Day 28 post-hatching	1,030.49 ± 64.42	1056.30 ± 164.03	1173.26 ± 43.94	1190.12 ± 93.48	0.003	0.615	0.916
Villus width of jejunum, μm							
Day of hatching	38.84 ± 5.75[b]	35.82 ± 5.21[b]	36.59 ± 2.46[b]	46.84 ± 5.15[a]	0.037	0.081	0.003
Day 7 post-hatching	101.64 ± 13.01	108.22 ± 15.03	100.67 ± 12.57	108.94 ± 9.17	0.981	0.165	0.871
Day 28 post-hatching	192.77 ± 16.54	195.01 ± 10.27	208.45 ± 15.19	200.52 ± 13.92	0.082	0.628	0.390
Villus surface area of jejunum, $\mu m^2 \times 10^3$							
Day of hatching	5.38 ± 0.85	7.91 ± 1.45	7.17 ± 1.11	10.35 ± 0.85	<0.001	<0.001	0.471
Day 7 post-hatching	47.48 ± 3.66	72.22 ± 12.69	55.54 ± 2.61	77.75 ± 11.59	0.076	<0.001	0.731
Day 28 post-hatching	198.24 ± 15.37	207.40 ± 43.92	245.13 ± 26.59	237.66 ± 8.88	0.002	0.940	0.462

[1]The data are presented as the means ± standard deviation.
[2]The "−" means without nutrients injection, while "+" means with nutrients injection.
[a,b,c]Different superscripts within a row indicate a significant difference ($p < 0.05$).

Discussion

In the present study, the birth weight of goslings was improved by feeding DS into the embryo. Similarly, Foye et al. (23) noted that *in ovo* injection of carbohydrates (20% dextrin and 3% maltose dissolved in 0.9% saline) improved the growth performance of birds, manifested in high body weight. Indeed, the delivery of carbohydrates into the embryo has been reported to increase the level of available energy, thus avoiding the reduction of body weight induced by the lack of energy (24). We also observed the increase in liver, breast muscle, and total glycogen contents induced by DS delivery on the day of hatching, which indicated an increase in the level of available energy in the embryo. Therefore, we concluded that *in ovo* injection of DS positively affects the birth weight of goslings, which was attributed to the enhancement of energy storage.

The breast muscle is the largest tissue in the body of poultry, and it plays an important role in metabolic activity because of its relatively large size and glycogen storage. The development of skeletal muscle is a complex process, which comprises the differentiation of myoblast from the mesodermal precursor cells to mature myotubes, thus differentiating to form myofibers during embryogenesis (25–27). Early feeding has been reported to be a suitable strategy to promote the maturation of myofiber, which leads to myofibers with larger diameters (1). Uni et al. (28) injected the DS (25 g/L maltose, 25 g/L sucrose, 200 g/L dextrin, and 1 g/L β-hydroxy-β-methylbutyrate dissolved in 5 g/L NaCl) into the amnion of a chicken egg and found a higher growth of breast muscle. Dong et al. (29) noted that *in ovo* feeding of DS (25 g/L maltose and 25 g/L sucrose dissolved in 7.5 g/L saline) had stimulating effects on the development of breast muscle in pigeons. In this study, we also observed that *in ovo* injection of DS increased the relative weight of breast muscle and diameter of myofiber in geese. Additionally, the relative weight of breast muscle and diameter of myofiber were also improved in Met injection. However, studies on the effects of *in ovo* feeding of Met on the breast muscle parameters were still limited. Mechanisms in Met administration that promote the development of breast muscle may be related to sarcoplasmic hypertrophy (30, 31). Sarcoplasmic hypertrophy would lead to the increase of myofiber cross-sectional area (32). According to Sahebi-Ala et al. (33), Met as a donor of the sulfur group was beneficial to enhancing the diameter of myofiber, resulting in the development of muscle. Therefore, we concluded that *in ovo* injection of Met or DS had positive effects on the development of breast muscle. However, either Met or DS, the improvement effect of nutrient delivery on breast muscle parameters was only observed in the early-life post-hatching of gosling, while not observed on day 28 post-hatching, which indicated that the injection of DS or Met have short-term effect on the improvement of breast muscle.

Additionally, the growth and development of muscle are usually regulated by specific genes and transcription factors which include Myf-5 and MSTN. Myf-5 is a myogenic regulatory factor and its expression is beneficial to the development of muscle fiber (34, 35). MSTN also known as a growth factor and differentiation factor-8 is mainly expressed in skeletal muscle cells and acts as an essential negative regulator in the growth of skeletal muscle in poultry (36, 37). The downregulation of MSTN expression is contributed to the hypertrophy of myofiber and the development of breast muscle (38). In this study, DS injection upregulated the expression of Myf-5 on

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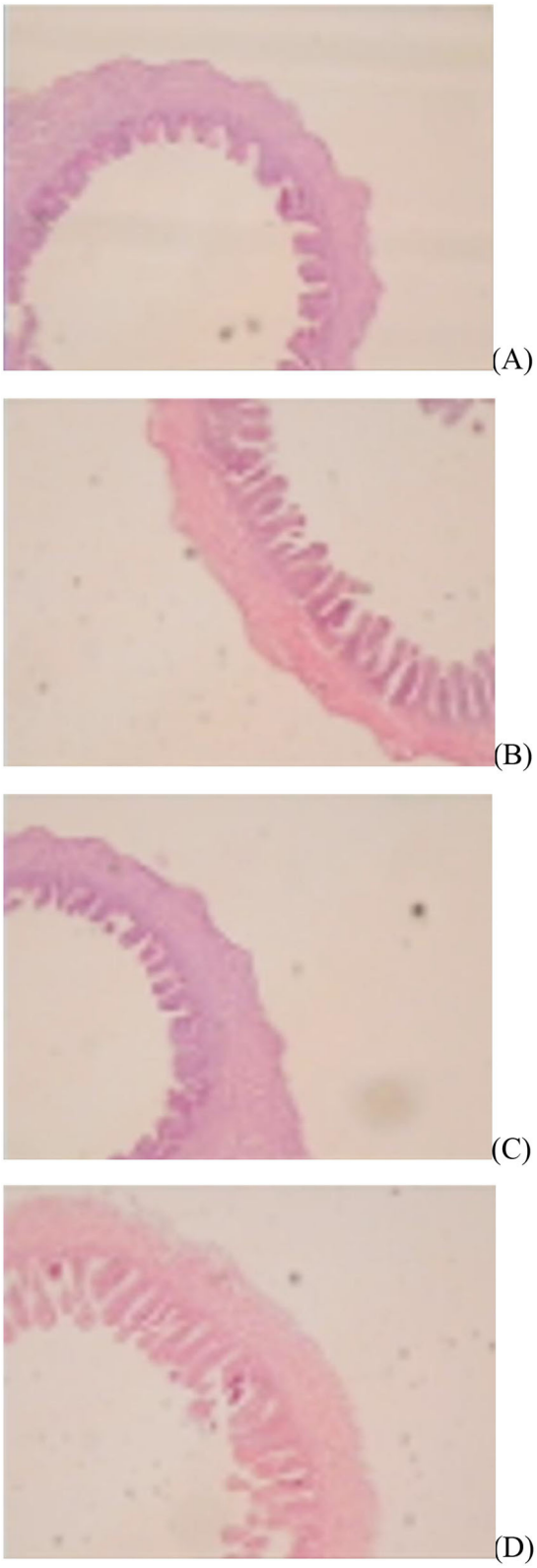

FIGURE 1
Slice of the jejunum in gosling treated by *in ovo* injection of methionine (Met) and/or disaccharide (DS) on day of hatching. **(A)** Non-injection group; **(B)** DS injection group; **(C)** Met injection group and **(D)** DS plus Met injection group.

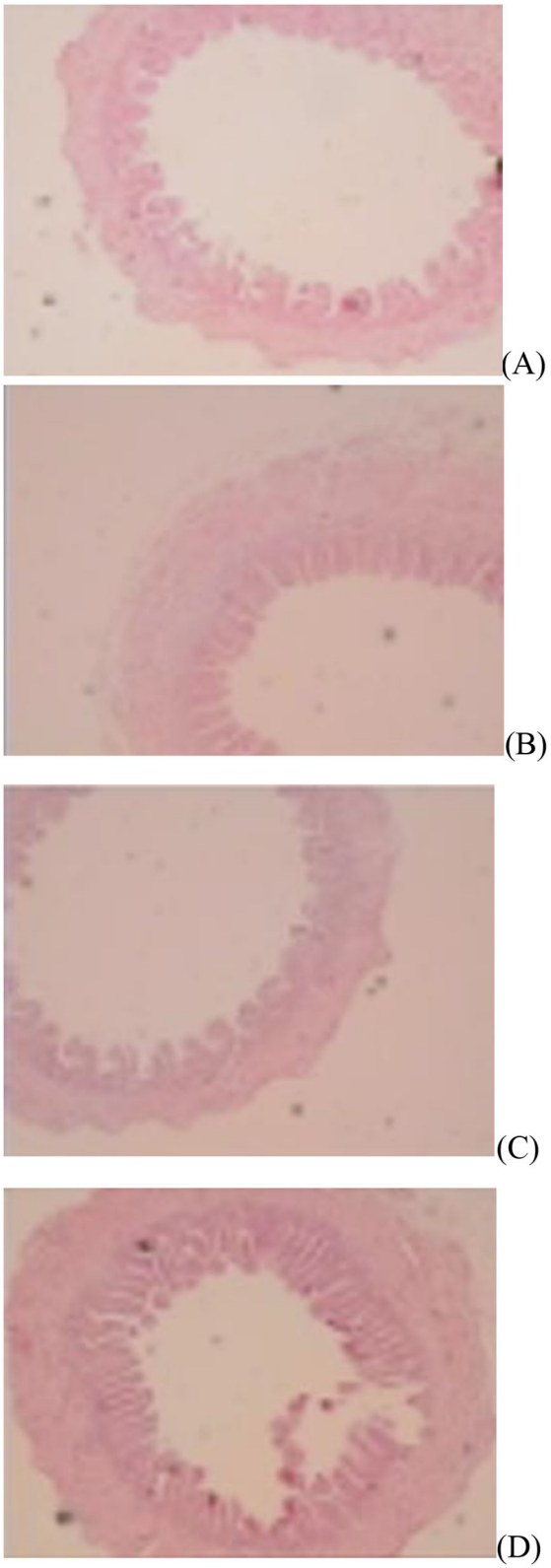

FIGURE 2
Slice of the jejunum in gosling treated by *in ovo* injection of methionine (Met) and/or disaccharide (DS) on day 7 post-hatching. **(A)** Non-injection group; **(B)** DS injection group; **(C)** Met injection group and **(D)** DS plus Met injection group.

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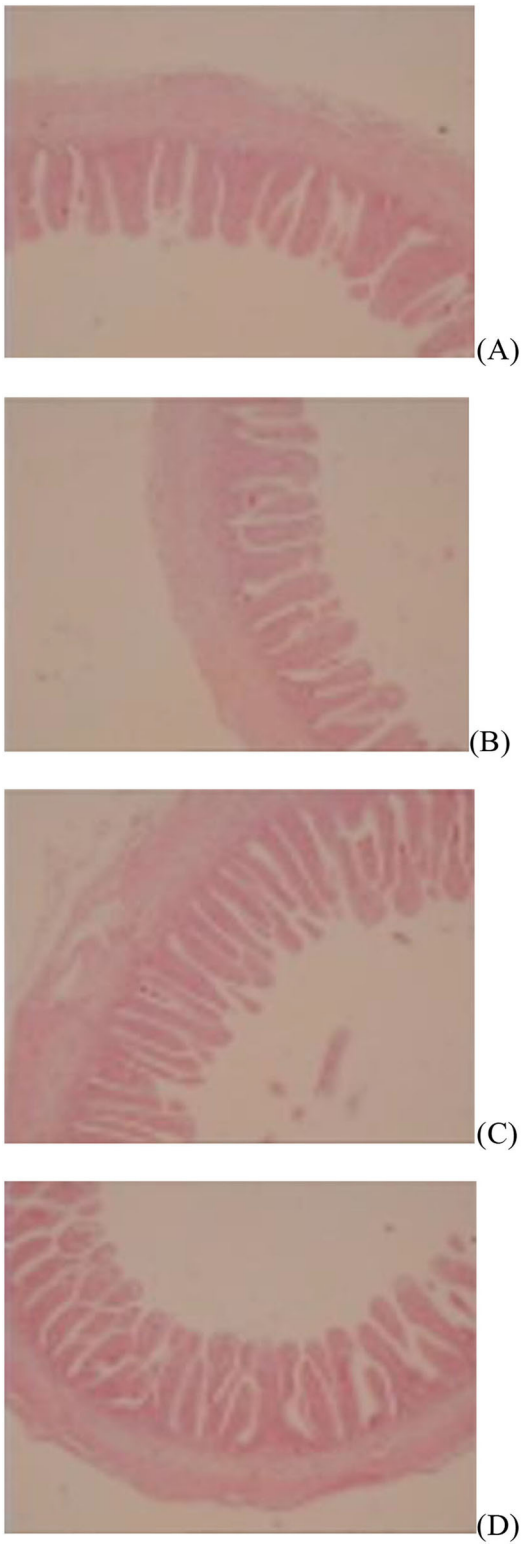

FIGURE 3
Slice of the jejunum in gosling treated by *in ovo* injection of methionine (Met) and/or disaccharide (DS) on day 28 post-hatching. **(A)** Non-injection group; **(B)** DS injection group; **(C)** Met injection group and **(D)** DS plus Met injection group.

the day of hatching, which was matched to the improvement of breast muscle parameters. Additionally, the improvement effects of Met administration on the upregulation of Myf-5 expression and the downregulation of MSTN expression were observed in each measured timepoint. However, higher breast muscle development did not last until day 28 post-hatching. An investigation of the development situation of other muscle parts is needed. As described by Wang et al. (39), the muscle growth-related genes have different expression schedules in different tissues (legs and breast muscle). In brief, we concluded that *in ovo* delivery of DS or Met positively affects the development of breast muscle by regulating the expression of breast muscle growth-related genes during the early-life post-hatching of goslings.

On the other hand, *in ovo* injection technique has been reported to support muscle growth *via* enhancing the liver and muscle glycogen reserves during post-hatching periods (1). Insufficient glycogen reserves will force the embryo to mobilize more muscle proteins toward gluconeogenesis, thus restricting the development of breast muscle (28). A sufficient glycogen store reduces the need for glucose synthesis *via* gluconeogenesis from muscle protein (3), resulting in the development of breast muscle (28). It has been reported that *in ovo* injection of carbohydrates improved embryo energy reserves status (10). Uni et al. (28) reported that *in ovo* injection of DS (25 g/L maltose, 25 g/L sucrose, 200 g/L dextrin, and 1 g/L β-hydroxy-β-methylbutyrate dissolved in 5 g/L NaCl) increased liver and muscular glycogen reserves in chicks. Foye et al. (23) noted that DS (20% dextrin and 3% maltose dissolved in 0.9% saline) injection increased the liver and muscle glycogen levels in turkey. In the present study, we also observed that DS injection increased the liver and muscle glycogen contents, which indicated an enhancement in glycogen store. Additionally, glycogen reserves have been reported to be negatively correlated with G-6-Pase activity (40). G-6-Pase is a membrane-bound enzyme, which is located on the internal membrane of the endoplasmic reticulum. It plays a key role in the terminal step of gluconeogenesis and glycogenolysis, and it can catalyze the conversion of glucose-6-phosphate to glucose, thus releasing glucose from the cell (41, 42). In the present study, DS injection inhibited the G-6-Pase activity. However, Met injection had no significant effects on the glycogen reserves and G-6-Pase activity. We concluded that the promotion effect of DS injection on the development of breast muscle was partially attributed to the increase of glycogen reserves induced by reducing G-6-Pase activity. However, it is worth noticing that the enhancement of glycogen reserves and the reduction of G-6-Pase activity induced by DS administration synchronously occurred, and only presented in the early life of goslings, which indicated the important role of G-6-Pase in regulating the glycogen reserves in the early life and the effect is temporary.

As reported, the increased growth of "demand" tissue such as skeletal muscle should be supported by the development

TABLE 10 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on jejunum digestive enzymes parameters of geese during the early-life post-hatching[a,b].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
Maltase activity in jejunum, μmol·min^{-1}·g^{-1} tissue							
Day of hatching	3.38 ± 0.34	4.51 ± 0.66	3.52 ± 0.49	4.44 ± 0.89	0.898	0.001	0.694
Day 7 post-hatching	7.00 ± 0.14	7.66 ± 0.69	7.57 ± 0.65	7.65 ± 0.39	0.202	0.093	0.184
Day 28 post-hatching	5.17 ± 0.66	5.29 ± 0.61	5.69 ± 0.71	5.76 ± 0.73	0.087	0.724	0.939
Sucrase activity in jejunum, μmol·min^{-1}·g^{-1} tissue							
Day of hatching	1.06 ± 0.16	1.80 ± 0.16	1.09 ± 0.22	1.64 ± 0.39	0.554	<0.001	0.380
Day 7 post-hatching	3.29 ± 0.26^{c}	4.24 ± 0.24^{ab}	4.41 ± 0.08^{a}	4.16 ± 0.07^{b}	<0.001	<0.001	<0.001
Day 28 post-hatching	4.26 ± 0.18	4.59 ± 0.52	4.39 ± 1.23	4.85 ± 0.43	0.520	0.186	0.829
Na^+/K^+ATPase activity in jejunum, U·min^{-1}·g^{-1} tissue							
Day of hatching	5.71 ± 0.20	6.71 ± 0.84	6.58 ± 1.19	7.38 ± 0.98	0.046	0.021	0.773
Day 7 post-hatching	21.84 ± 0.72	20.97 ± 1.17	20.68 ± 2.91	20.10 ± 1.41	0.173	0.324	0.843
Day 28 post-hatching	22.27 ± 0.56	22.90 ± 1.77	22.07 ± 1.90	23.17 ± 1.03	0.959	0.153	0.697
Alkaline phosphatase activity in jejunum, μmol·min^{-1}·g^{-1} tissue							
Day of hatching	1.15 ± 0.10^{d}	1.39 ± 0.16^{c}	1.64 ± 0.10^{b}	2.74 ± 0.27^{a}	<0.001	<0.001	<0.001
Day 7 post-hatching	8.44 ± 0.75	10.38 ± 1.85	9.80 ± 1.31	12.75 ± 1.84	0.007	0.001	0.425
Day 28 post-hatching	11.96 ± 2.39	11.68 ± 2.35	9.99 ± 0.65	12.36 ± 1.32	0.404	0.178	0.092

[1]The data are presented as the means ± standard deviation.
[2]The "−" means without nutrients injection, while "+" means with nutrients injection.
[a,b,c,d]Different superscripts within a row indicate a significant difference ($p < 0.05$).

in structure and function of "support" tissue such as gastrointestinal tract (43). Early growth and development of the gastrointestinal tract are critical to optimizing the growth of poultry. The greatest nutrient digestion and absorption happens in the small intestine of poultry, especially the jejunum (44). In the small intestine, the epithelium is thrown into long folds, the villi, which serves to increase the surface area for enzyme secretion and nutrient absorption. Histomorphology is one of the most commonly used parameters to evaluate the status of the gut. Generally, the length and relative weight of the small intestine, as well as the surface area, width, and height of villi from the small intestine, are considered important factors that reflect the development of the small intestine. It has been reported that *in ovo* feeding of maltose promoted the development of the jejunum villi in poultry (45). Chen et al. (46) noted that DS (25 g/L maltose and 25 g/L sucrose dissolved in 4.5 g/L NaCl) injected into the amnion of duck embryo improved the length and surface area of jejunal villi. In this study, we also observed that *in ovo* injection of DS increased jejunal villus height and surface area. Additionally, Met also has been reported to be crucial for the maintenance of gut integrity and function (47). Mohammadrezaei et al. (48) reported that *in ovo* injection of Met enhanced the nutrients absorption ability of chicks by altering the height and width of villi from the small intestine. Chen et al. (49) noted that Met injection increased the relative weight of the duodenum, jejunum, and ileum, as well as the villus height in the small intestine of chicks. Similarly, in the present study, DS injection provisionally improved the development of jejunal villi but did not affect the small intestine parameters. However, Met administration has a long-term effect on the improvement of jejunal villi development and a short-term effect on the improvement of small intestine parameters. Therefore, we concluded that *in ovo* delivery of Met or DS promoted the development of jejunal villi and small intestine in the early life, which was beneficial to support the absorption of the nutrients.

A mature intestine is often accompanied by abundant digestive enzyme secretion (46). The digestive enzyme plays an important role in the digestion of nutrients into smaller nutrient molecules to facilitate the absorption by the host. It has been reported that a high disaccharidase activity presented in the small intestine ensures rapid carbohydrate digestion, and it breaks down the DS into glucose (50). The alkaline phosphatase involves in digestive processes such as the absorption of glucose (51). Chen et al. (46) reported that DS (25 g/L maltose and 25 g/L sucrose dissolved in 4.5 g/L NaCl) injection increased jejunal sucrase activity in ducks. Similar to the above study, *in ovo* delivery of Met or DS increased the activities of digestive enzymes in the jejunum; however, this improvement effect was temporary. Further investigation is needed to evaluate the effects of *in ovo* Met or DS delivery on the digestive enzyme activities. We concluded that *in ovo* administration of Met or DS had

TABLE 11 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on jejunum antioxidant parameters of geese during the early-life post-hatching[a,b].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
GSSG, μmol·g^{-1} tissue							
Day of hatching	7.16 ± 0.63	6.95 ± 0.60	6.31 ± 0.24	6.30 ± 0.25	0.001	0.585	0.618
Day 7 post-hatching	10.86 ± 0.87	10.89 ± 0.88	11.15 ± 0.76	10.95 ± 0.28	0.576	0.776	0.706
Day 28 post-hatching	4.93 ± 0.59	4.98 ± 0.35	4.89 ± 0.51	4.75 ± 0.36	0.481	0.816	0.620
GSH, μmol·g^{-1} tissue							
Day of hatching	181.23 ± 14.01	179.92 ± 14.72	223.61 ± 19.68	221.67 ± 25.75	<0.001	0.837	0.968
Day 7 post-hatching	265.83 ± 24.37	262.56 ± 25.93	278.78 ± 20.61	285.01 ± 13.86	0.059	0.869	0.597
Day 28 post-hatching	161.04 ± 18.83	159.63 ± 11.97	162.52 ± 12.63	164.19 ± 10.23	0.598	0.981	0.786
GSSG/GSH							
Day of hatching	0.040 ± 0.01	0.039 ± 0.01	0.028 ± 0.002	0.029 ± 0.003	<0.001	0.891	0.774
Day 7 post-hatching	0.041 ± 0.01	0.042 ± 0.001	0.040 ± 0.003	0.038 ± 0.002	0.155	0.658	0.512
Day 28 post-hatching	0.031 ± 0.01	0.031 ± 0.004	0.030 ± 0.001	0.029 ± 0.002	0.256	0.829	0.624
GPX, U·g^{-1} tissue × 10^3							
Day of hatching	10.90 ± 0.46	11.20 ± 0.90	13.25 ± 1.23	12.83 ± 1.27	<0.001	0.877	0.395
Day 7 post-hatching	7.32 ± 0.85	7.38 ± 0.79	8.54 ± 0.79	8.54 ± 0.89	0.002	0.924	0.924
Day 28 post-hatching	7.28 ± 0.67	7.34 ± 0.83	8.72 ± 0.92	8.18 ± 0.44	0.001	0.427	0.328

GSH, glutathione; GPX, glutathione peroxidase; GSSG, glutathione disulfide.
[a]The data are presented as the means ± standard deviation.
[b]The "−" means without nutrients injection, while "+" means with nutrients injection.

TABLE 12 Effects of *in ovo* injection of methionine (Met) and disaccharide (DS) on jejunum nutrients transport gene expression of geese during the early-life post-hatching[1,2].

Met	−		+		*P*-value		
DS	−	+	−	+	Met	DS	Met × DS
SGLT-1 expression abundance in jejunum							
Day of hatching	1.00 ± 0.13	1.80 ± 0.19	1.06 ± 0.08	1.86 ± 0.12	0.248	<0.001	0.978
Day 7 post-hatching	5.82 ± 0.74	9.83 ± 1.09	5.45 ± 0.77	10.31 ± 0.99	0.881	<0.001	0.267
Day 28 post-hatching	5.23 ± 0.92	5.50 ± 0.47	5.13 ± 0.72	5.49 ± 0.46	0.832	0.262	0.871
GLUT-2 expression abundance in jejunum							
Day of hatching	1.00 ± 0.14	1.44 ± 0.10	1.14 ± 0.12	1.56 ± 0.20	0.037	<0.001	0.861
Day 7 post-hatching	5.20 ± 0.64	8.05 ± 0.79	5.65 ± 0.62	8.09 ± 0.84	0.436	<0.001	0.497
Day 28 post-hatching	1.37 ± 0.20^{c}	3.66 ± 0.32^{b}	1.40 ± 0.29^{c}	4.62 ± 0.82^{a}	0.019	<0.001	0.025
SI expression abundance in jejunum							
Day of hatching	1.00 ± 0.15	1.58 ± 0.19	1.14 ± 0.08	1.57 ± 0.13	0.281	<0.001	0.208
Day 7 post-hatching	3.44 ± 0.35	5.35 ± 0.65	3.06 ± 0.24	5.46 ± 1.26	0.654	<0.001	0.424
Day 28 post-hatching	1.02 ± 0.09	1.91 ± 0.23	1.08 ± 0.14	2.07 ± 0.12	0.101	<0.001	0.454

SGLT-1, sodium/glucose cotransporter protein-1; GLUT-2. glucose transporter-2; SI, sucrase-isomaltase.
[1]The data are presented as the means ± standard deviation.
[2]The "−" means without nutrients injection, while "+" means with nutrients injection.
[a,b,c]Different superscripts within a row indicate a significant difference ($p < 0.05$).

positive effects on the activities of digestive enzymes in jejunum during the early life of goslings.

The nutrient absorption in the intestine is relying on the Na^+-dependent kinetics (52). Sodium transportation is achieved by the enterocyte's basolateral Na^+/K^+ATPase (52). Na^+/K^+ATPase pumps out sodium to cells whereas it pumps potassium into cells, which is beneficial to maintaining resting potential and regulating nutrient transportation (53–55). In

this study, we observed that *in ovo* injection of Met or DS improved the Na^+/K^+ATPase activity in the jejunum in early life, with no long-term effect, which indicated that *in ovo* delivery strategies used in this study had positive effects on the nutrient transportation in the small intestine during the early life.

Moreover, the transport mediators expressed in the apical and basal membrane of enterocytes also play a key role in nutrient absorption. Nutrients are transported into enterocytes by special transporters. The expression of the nutrient transport gene could be used as an indicator of the growth and absorptive capacity of the small intestine (56). Because very small amounts of carbohydrates are presented in the intestine during the late term of incubation, the increase in SI expression at the apical membrane allows for the degradation of carbohydrates into glucose (57). Maintaining high expression levels of SI in the small intestine provides a sufficient supply of substrates for the nutrient transporters such as SGLT-1 and GLUT-2 (57). Glucose is the key fuel and metabolic substrate that could be absorbed *via* SGLT-1, which is located on the apically of the intestinal epithelium, and transported into the blood *via* GLUT-2, which is expressed on the basolateral membrane (58, 59). In this study, *in ovo* injection of Met long-term upregulated the expression of GLUT-2, while DS injection short-term upregulated the expression of SGLT-1, long-term upregulated the expression of GLUT-2, and downregulated the expression of SI. Similarly, Dong et al. (29) reported that the enhancement of the jejunal SGLT-1 and GLUT-2 expression was found during post-hatching periods in pigeon embryos that received carbohydrate solution (2.5% maltose and 2.5% sucrose dissolved in 0.75% saline) into the amniotic fluid. On the other hand, SGLT-1 has been reported to be coupled to the action of the Na^+/K^+ATPase to mediate glucose transportation (59, 60). GLUT-2 has been reported to be combined with G-6-Pase acted in concert to control the release of glucose from the liver (61). In this study, the increase of Na^+/K^+ATPase activity was observed by *in ovo* injection of DS or Met, moreover, *in ovo* injection of DS decreased G-6-Pase activity. Therefore, we concluded that *in ovo* injection of DS and/or Met improved nutrient absorption by regulating the nutrient transport gene expression and digestive enzyme activities.

It has been reported that the expression of GLUT-2 was inhibited by the stress response in poultry (62). However, this inhibition effect could be reversed by alleviating oxidative stress (63). Ibrahim et al. (64) reported that feeding broiler chicks with antioxidant herbal extract improved the antioxidant situation of birds, thus upregulating the expression of GLUT-2. The decrease of GSSG and the increase of GSH and GPX concentrations are characteristic of the alleviation of oxidative stress (65). Met is known to decrease oxidative stress and has antioxidant properties (66). Its *in ovo* delivery has been reported to improve the antioxidative capacity in the embryo (66). In this study, Met injection short-term increased GSH concentration and decreased GSSG and GSSG/GSH levels from jejunum, as well as long-term increased jejunal GPX concentration. Therefore, we concluded that *in ovo* delivery of Met is beneficial to improve the antioxidative situation of jejunum, thus improving the GLUT-2 expression and further improving intestinal health. Abolfathi et al. (67) reported that feeding broiler chicks with antioxidant herbal extract improved intestinal morphology through increasing intestinal antioxidant capacity. Long et al. (68) noted that dietary supplementation of antioxidant substances improved intestinal antioxidant capacity, thus increasing intestinal digestive enzyme activities. Therefore, we considered that the improvement of jejunum antioxidant parameters by Met injection also benefited the improvement of intestine morphology and digestive enzyme activities.

Conclusion

Our results demonstrated that DS injection increased glycogen reserves and regulated muscle growth-related gene expression, thus improving breast muscle parameters and birth weight during the early-life post-hatching. The short-term effect on the improvement of digestive enzyme activities, nutrient transport enzyme activities, and jejunal villus parameters, as well as the long-term effect on the regulation of nutrient transport-related gene expression, were observed to deliver DS into the embryo, which contributed to promoting the nutrient absorption in post-hatching.

In ovo injection of Met continuously regulated the breast muscle growth-related gene expression, thus improving breast muscle parameters. The temporary improvement effect of Met delivery on the digestive enzymes activities, nutrient transport enzymes activities, small intestine parameters, and nutrient transport-related gene expression, as well as the continuous improvement effect on the jejunal villus parameters and jejunal antioxidative situation, were beneficial to support the nutrient absorption after incubation.

Moreover, *in ovo* feeding of DS plus Met mixture synergistically improved the diameter of myofiber, jejunal villus height and width, jejunal sucrase, and alkaline phosphatase activities in early-life post-hatching, but continuously upregulated the expression of jejunal GLUT-2. Therefore, we concluded that *in ovo* delivery of DS plus Met is a suitable strategy to improve the nutrient absorption and breast muscle development of goslings post-hatching.

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 Animal Care and Use Committee of Jilin Agricultural University (Changchun, China).

Author contributions

DD: writing—original draft, investigation, and writing—review and editing. HZ: formal analysis and investigation. YL: conceptualization and methodology. DL: supervision and writing—review and editing. All authors contributed to the article and approved the submitted version.

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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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 September 2022
DOI 10.3389/fvets.2022.948069

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 19 May 2022
ACCEPTED 22 August 2022
PUBLISHED 16 September 2022

CITATION
Bhanja SK, Rath PK, Goel A, Mehra M, Dhara SK, Paswan VK, Attia YA, Alqhtani AH, Ali ABA and Shehata AM (2022) *In ovo* nano-silver and nutrient supplementation improves immunity and resistance against Newcastle disease virus challenge in broiler chickens. *Front. Vet. Sci.* 9:948069. doi: 10.3389/fvets.2022.948069

In ovo nano-silver and nutrient supplementation improves immunity and resistance against Newcastle disease virus challenge in broiler chickens

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Silver nanoparticles (AgNPs) interact with the microbes and host immune system to protect against diseases. Fertile broiler eggs ($n = 900$) were allotted to six groups: un-injected control, sham (sterile water), AgNPs (50 μg), AgNPs+Amino acids (Methionine-10 mg + Arginine-25 mg), AgNPs+Vitamins (Vit B1-72μg + Vit B6-140μg), and AgNPs+Trace Elements (Zn-80 μg and Se-0.3 μg) and incubated for 18 days. On 18th embryonic day, 0.6 ml test solution was injected at the broad end of egg using 25 mm needle and transferred to hatcher. Post-hatch, half of the chicks from each group were vaccinated with Newcastle disease (ND) vaccine, and the other half were kept as unvaccinated unit and reared for 42 d with standard management practices. Hatchability, 1st and 42nd d body weight, feed intake, and feed conversion ratio were similar between treatment groups in both vaccinated and unvaccinated units. The relative weight of bursa Fabricius and thymus was similar, but spleen weight was higher ($P \leq 0.05$) in AgNPs, AgNPs+Vits, and AgNPs+TEs chicks than control group. Cellular immune response (against mitogen phytohemagglutinin-P) was higher ($P \leq 0.05$) in AgNPs+TEs chicks, whereas HA titer against sheep red blood cells antigen, serum IgG, IgM, and HI titer against ND vaccine was apparently higher in AgNPs+Vits group chicks than control. No clinical symptoms were observed in the vaccinated groups except for a few control birds 6 days postchallenge (PC). Three days PC, unvaccinated birds show depression, off feed, greenish diarrhea, and nasal discharge and the control group started dying. The highest cumulative infection (CI) was observed in sham (79.17%) and un-injected control (75%), but lowest in AgNPs+AAs birds (58.33%) on 3rd dpi. The CI reached 100% on 5th dpi in control groups and AgNPs, and 91.67% and 93.75% in AgNPs+TEs

and AgNPs+AAs group, respectively. The AgNPs+TEs and AgNPs+AAs group birds lived for more than 90 h compared to 75 h in control groups and also had higher IL-6 and IL-2 gene expressions at 24 h PC. It was concluded that 50 μg/egg AgNPs with vitamins (B1 and B6) and trace elements (Zn and Se) improved performance, but AgNPs with trace elements and amino acids enhanced immune response and resistance against vND virus challenge in broilers.

KEYWORDS

silver nanoparticles, *in ovo* feeding, amino acids, vitamins and minerals, immunity, ND virus challenge, gene expression, broiler chickens

Introduction

Modern broilers have tremendous improvement in the growth performance, but exhibit weaker immunity (1, 2). Loss of immune homeostasis is attributed to heavy vaccinations, early medications, transport stress, housing environment, climatic conditions, nutrition, and diseases (3–7). Thus, to achieve higher and effective poultry production, a balance between nutrition and immune status of broiler chickens would play a very significant role.

The nanomaterials have become the contemporary area of interest, for their application in the fields of animal nutrition, health, production, and many other such factors. Due to their higher surface area-to-volume ratio, these particles act better than their bulky counterparts (8–11). Recent studies reported that administration of silver nanoparticles (AgNPs) improves late embryonic development and metabolism (12–15), influencing the expression of VEGFA and FGF2 gene in the breast muscle of broilers (16), immune response, and enhancing expression of immune-related genes (17). However, the impacts of intervention with nanoparticles are yet to be fully assessed on post-hatch growth, nutrient–gene interaction, immune-competence, and disease resistance in chicken. Moreover, silver nanoparticles interact with virus, bacteria, and immune system as well. Development of B and T lymphocytes initiates during embryogenesis in the bursa of Fabricius and thymus, respectively, and matures in the spleen until post-hatch (18). The cell produced in these organs differentiates into Th1 type (cellular immunity) and Th2 type (humoral immunity) and thus imparting immunity against the different pathogens. Goel et al. (17) reported that *in ovo* injection of AgNPs enhanced the *in vivo* immune response to phytohemagglutinin type-P (PHA-P), the cell-mediated immunity and sheep red blood cells (SRBC), and the humoral immunity in comparison with control group. Saki and Salary (19) have also reported about the enhanced cell-mediated immunity in terms of mean skin thickness sensitive to phytohemagglutinin, in chicks treated with AgNPS. Similarly, the application of 1% AgNP cream (<50 nm particle size) in rats inhibited contact allergic dermatitis and modulated cytokine excretion *in vitro* and *in vivo* (20). AgNP administration (0.5 mg/Kg body weight) had no significant effect on growth performance but showed a lower plasma cholesterol, triglycerides, and antioxidant capacity but better malondialdehyde and glutathione peroxidase than that of control rabbits (21).

Early post-hatch or embryonic (*In ovo)* feeding has been helpful in improving the nutritional status of the hatchlings. During the early period of embryonic growth, there is higher utilization of amino acids like Lys, Arg, Gly, and Pro. *In ovo* fed amino acids also spare the maternal antibodies from being utilized as protein source. Earlier studies revealed that *in ovo* feeding of Met and Arg had higher percent hatchability, body weight, and cell-mediated immune response (22, 23). *In ovo* administration of combined amino acids Lys+Met+Cys and Thr+Gly+Ser improved both cellular and humoral immunity (24). Thiamin (vitamin B1) helps in carbohydrate metabolism and is required for growth, development, and function of cells. Pyridoxine (vitamin B6) plays an important role in protein synthesis needed for an immune response. *In ovo* feeding of vitamin B1 improved growth and immunity, while vitamin B6 modulates immunity in broiler chickens (25). Zinc (Zn) and selenium (Se) play a vital role in various biological processes as a component of many enzymes and are also essential for growth, antioxidant defense mechanism (26), cell-mediated immunity, and higher expression of growth and immune genes (27–29). AgNPs have mainly been studied for their antimicrobial potential against bacteria, but have also proven to be active against several types of viruses, including human immunodeficiency virus, hepatitis B virus, herpes simplex virus, respiratory syncytial virus, and monkey pox virus (30–32). Silver's mode of action is presumed to be dependent on its positive ions, which strongly inhibit bacterial growth through suppression of respiratory enzymes and electron transport components and interference with DNA functions (33). Silver has also been found to be non-toxic to humans at very small concentrations. The microorganisms are unlikely to develop

resistance against Ag as compared to antibiotics, as Ag attacks a broad range of targets in the microbes. The overuse of antibiotics in the standard management practices of modern broiler production systems has resulted in an increased number of antibiotic-resistant bacteria, posing imminent threat to human health. Hence, alternative health-promoting agents are looked for enhancing growth and immunity in chickens (10, 11, 34).

Though AgNPs are used in various fields, until now very few studies have been undertaken to determine the immunological effects of AgNPs when delivered to broiler chickens especially before hatch *via in ovo* feeding. Therefore, this study was planned to demonstrate how AgNPs alone or in a combination with other critical nutrients can act as an inducer of innate or adaptive immunity in broiler chickens.

Materials and methods

Ethical permission

All the experimental procedures on birds were carried out according to the recommendations and approval of the ICAR-Central Avian Research Institute, Izatnagar, India's Institute Animal Ethics Committee vides approval no. CARI/CPCSEA/2016/8 dated 23.08.2016 for the Purpose of Control and Supervision of Experiments on Animals in India.

Experimental design

In a completely randomized block design, 900 fertile eggs (58.0 ± 2.0 g) collected from 35th-week-old breeder hens in a 4-day period were allotted to six groups. The eggs were incubated in a force draft incubator at standard incubation temperature of 37.5^0C and relative humidity of 60.0 % for 18 d.

Silver nanoparticles

The AgNPs solution aXonnite (100 mg/l deionized water, 99.9999% purity) was obtained from Nano-Tech Poland Ltd (Warsaw, Poland). The average particle size was 3.5 nm, which accounts for more than 80% of particles. The average surface area of the particles was 2.83 10^{-13} cm^2. The pH of the solution was in the range of 7.1 to 8.1, and the redox potential was 0.1 mV.

Treatment groups and *in ovo* injection

The six treatment groups were as follows: 1-Un-injected control, 2-Sham control (Sterile water), 3-AgNPs (50 μg), 4-AgNPs+Amino acids: Methionine-10 mg and Arginine 25 mg (AgNPs+AAs), 5-AgNPs+Vitamins: Thiamine-72 μg and Pyridoxine-140 μg (AgNPs+Vits) and 6-AgNPs+Trace elements: Zinc 80 μg and Selenium 0.3 μg (AgNPs+TEs). The concentration of critical nutrients like amino acids, trace elements, and vitamins was calculated at 5, 50, and 100%, respectively, of the National Research Council (35) recommendation for a broiler chick's consumption during first 4 d (22). Separate nutrient solutions were prepared by dissolving amino acids, vitamins, and trace elements in sterile water, and then, these nutrient solutions (0.1 ml) were mixed to 0.5 ml AgNPs solution containing 50 μg of Ag. On the 18th day, about 0.6 ml of the treatment solutions was injected *in ovo*, to each egg of treatment groups (except un-inj. Control) using a 24 gauge needle at the broader end of the egg, following the procedure described by Bhanja et al. (36).

Housing and management

After hatch, all the chicks were vaccinated against Marek's disease, wing banded, and weighed. The chicks hatched from the different treatment groups were further distributed into two separate units (treatment-wise) where 50% of the chicks were vaccinated with Newcastle disease (ND) vaccine (F1 strain) on 4th d post-hatch and other 50% of the chicks (who were tested negative for antibody against ND, in serum) were kept unvaccinated. The ND-vaccinated birds were again vaccinated with inactivated ND vaccine strains on 24th d post-hatch. These two sets of chicks (vaccinated and unvaccinated) were housed in two different sheds but provided similar managemental practices. The unvaccinated unit was reared under utmost care to provide strict isolation and attended by a separate worker. The chicks were housed in 4-tier battery brooder cages. They were kept in a well-lit and ventilated open-sided house. The birds were provided with standard diets and management till 42 d of age. Food and water were provided *ad lib*.

Performance parameters

Different pre-hatch parameters like pre-incubated egg weight, embryonic mortality, and percent hatchability were recorded. During post-hatch, body weight of individual birds and feed consumption by a group of eight birds (replicate wise) in each pen were recorded for 42 d to estimate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

Lymphoid weight and immune response

The relative development of the lymphoid organs was recorded at 42nd days post-hatch in vaccinated group. Six birds from each treatment (one bird from each replicate) were fasted for 8 h prior to being sacrificed. The weights of the bursa of

Fabricius, thymus, and spleen were recorded and expressed as g kg−1 body weight. On 22nd d post-hatch, one ml of sheep RBC (1% v/v) suspension was injected intravenously to 12 birds from each treatment (two birds/replicate) from vaccinated and unvaccinated (data not presented) groups to study the primary antibody response against SRBC antigen. On 7 d post-immunization, 2 ml blood was collected from the wing vein and allowed to clot for serum separation. The antibody titer against SRBC was determined by hemagglutination (HA) method (37). The reciprocal of highest dilution showing clear agglutination was selected as end point of titer, and the value was expressed as log 2. *In vivo* response to PHA-P was evaluated by injecting about 0.1 ml PHA-P (1 mg/ml of PBS) intra-dermally into left foot web of 12 birds/treatment from the vaccinated and unvaccinated (data not presented) groups on 28th d post-hatch. Right foot web of the same birds received 0.1 ml sterile PBS and thus served as control. The skin thickness of foot webs (Right and Left) of the birds was measured by a micrometer at 0 and 24 h after injection of mitogen. Foot web index (FWI) was calculated by deducting the difference in thickness at 0 and 24 h of mitogen injected foot web with the difference in thickness of control foot web (38).

The specific antibody response against ND vaccine antigen was studied in the vaccinated group on 7th, 14th, and 21st d post-hatch. The hemagglutination inhibition (HI) test was performed in the serum. The plates were read under bright light. The highest dilution, at which there was complete inhibition of hemagglutination, was the HI titer value. Mercapto-ethanol-resistant antibodies (MER or IgG) against SRBC were determined as per the method described by Martin et al. (39) with slight modification. Mercapto-ethanol sensitive antibodies (MES or Ig M) against SRBC were calculated as the reduction from total SRBC HA titer due to 2-ME resistant titer.

Newcastle disease virus challenge study

Eight birds each from vaccinated and unvaccinated groups of six treatments (a total of 96 birds) were selected based on their immune response to SRBC and PHA-P (one—low, two—moderate, and one—high responder bird from each treatment group) to eliminate individual variation for the challenge study. On 43rd d post-hatch, the birds were shifted to a challenge shed at IVRI, Izatnagar, India, to evaluate the response against virulent ND virus (vNDV). The Vndv-infected allantoic fluid was collected from Division of Pathology, IVRI, which was earlier recovered from a field outbreak. To get desired volume of virus, the infected fluid was propagated in 9th d old embryonated eggs as per the standard protocol (40). The presence of ND virus in the allantoic fluids was confirmed by typical lesions in the infected/dead embryos and HA tests. For calculating the required dose of NDV, a 10-fold serial dilution of the virus suspensions was carried out. The range of dilutions included at least two 10-fold dilutions above or below the dilution expected to contain the end point. At least five eggs were inoculated with each dilution. Separate needle and syringe for each dilution were used. The eggs were incubated for 4 days at 38°C. After 4 days, allantoic fluid was harvested from each egg and tested for the presence or absence of NDV. The application of the Reed and Muench mathematical technique was used to calculate the infectivity titer of the original suspension as 50 % embryo infectious or lethal dose (EID50/ELD50) as described below:

$$\text{Index} = \frac{(\%\ \text{infected at dilution immediately above 50\%}) - 50\%}{(\%\ \text{infected at dilution immediately above 50\%}) - (\%\ \text{infected at dilution immediately below 50\%})}$$

Recording of clinical signs and cumulative infection

Based on the EID50 /ELD50, eight birds from each treatment group were challenged with 0.1 ml (100 µl) of vNDV. Half of the dose was supplied as eye drop, and the remaining half was given intranasal, on the same side. The clinical symptoms and death time of the birds were recorded dpi (days postinoculation) and hour wise by the same observer six times a day. The extremely sick/terminally weak birds (showing severe neural symptoms with thick mucus in mouth) were euthanized and recorded as dead birds. A scoring pattern was followed as described by Oyebanji et al. (41) with little modifications. Based on the degree of sickness, the birds were classified into mild (Mi), moderate (Mo), severe(S), and dead/euthanized (D/E) categories on each dpi and granted with "clinical signs severity scores" of 10, 20, 30, and 40, respectively. The number of birds from each category was also expressed as percentage to the total number of live birds (n = L), at the end of previous dpi or in the beginning of that dpi. The "score obtained" was calculated as the sum of clinical signs severity scores of birds multiplied with number of birds in each category. Cumulative infection (CI), that is, the percentage of infection achieved until that dpi by the challenge virus, was estimated dpi wise and expressed as score obtained on that dpi / maximum possible score on that dpi ×100. The "maximum possible score" on that dpi was calculated as product of the clinical signs severity score for dead birds (40) and the total live birds on the beginning of that dpi. Post-mortem examination was carried out on dead chickens to determine the degree of disease severity. The vaccinated birds were killed on 2nd, 4th, and 6th dpi to observe the pathological changes.

Expression of immune genes

Before and following the vNDV challenge, blood samples were collected at periodic intervals (0/8/24 h). PBMC cells were

separated to study the expression pattern of IL2 and IL6 genes. Total mRNA from PBMC cells was isolated using TRIzol® reagent following manufacturer's instructions. The purified RNA samples (using the SV Total RNA Isolation System, Promega Corporation, Madison, WI, USA) were quantified in a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). About 2 μg of total RNA was reverse-transcribed, after which real-time PCR was performed with complementary DNA and gene-specific primer pairs (8, 10) in an IQ5 cycler (BioRad, Hercules, CA, USA). The samples were first denatured for 5 min at 95 °C and then amplified using 45 cycles of 30 s at 95 °C for denaturation, 30 s at specific annealing temperature, and 30 s at 72 °C (elongation), followed by quantification. A melting curve was applied to verify the specificity of the product. For each complementary DNA, the reaction was performed in triplicate. The quantification of gene expression (fold change with respect to control) was done in REST 2009 software using the CT values obtained in Rt PCR study.

Statistical analysis

Data were subjected to statistical analysis for one-way analysis of variance (ANOVA) using SPSS-16 software. Duncan's multiple range test was used for verifying significant difference among treatment means, and $P<0.05$ was considered to be statistically significant.

Results

Incubational parameters and post-hatch growth

There was no difference ($P \geq 0.05$) in the weight of fertile eggs used for *in ovo* injection, day old chick weight, chick weight to egg weight ratio, and percent hatchability (on fertile egg basis) between the treatment groups (Table 1). The 42nd d body weights, average daily feed intake (ADFI), average daily weight gain (ADG), and feed conversation ratio (FCR) during 0 to 42 d were also not different ($P \geq 0.05$) between the treatment groups of vaccinated birds (Table 2). The performance of unvaccinated birds (data not presented) for 42nd d body weights, ADFI, ADG, and FCR ranged between 1,372.0 and 1,479 g, 69.3 and 73.5 g, 31.65 and 34.25 g, and 2.11 and 2.22, respectively.

TABLE 1 Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on hatchability parameters of vaccinated birds.

Groups[a]	Egg weight (g)	Chick weight (g)	Chick weight to egg weight ratio (%)	Hatchability on fertile egg set basis (%)
Control	61.25	42.93	70.63	93.33
Sham control	60.28	43.30	71.96	91.67
AgNPs	60.53	43.13	71.25	90.20
AgNPs+AAs	60.10	44.05	73.37	90.91
AgNPs+Vits	60.90	43.93	72.16	93.85
AgNPs+TEs	60.4	44.28	73.33	92.86
SEM	0.36	0.29	0.46	ND
P-value	0.95	0.71	0.46	ND

[a]Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg).
Values have been analyzed by one-way ANOVA. ND, not determined as single hatch was taken.

Lymphoid organ and immune response in vaccinated birds

The relative weight of bursa and thymus was similar in the treatment groups; however, the spleen weight was higher ($P \leq 0.05$) in AgNPs+TEs group in comparison with control and sham control groups. Primary antibody titer against SRBC was similar between treatment groups of vaccinated birds; however, the foot web index was significantly higher in AgNPs+TEs group chicks than both the control groups (Table 3). Similarly, in the unvaccinated chicks (data not presented), HA titer against SRBC (log 2) and foot web index (mm) ranged from 8.0 to 9.5 and 0.39 to 0.60, respectively.

The 2-mercapto-ethanol-resistant (IgG) and sensitive (IgM) antibodies were similar ($P \geq 0.05$) in treatment groups; however, apparently higher values of IgG and IGM were seen in AgNPs+TEs and AgNPs+AAs group chicks, respectively. The specific antibody response against ND vaccine was studied in the vaccinated group on 7th, 14th, and 21st d post-hatch. The HI titer values at all the periods were similar ($P \geq 0.05$) between treatment groups; however, the AgNPs+Vits group chicks had apparently higher values at 7th and 14th d post-hatch (Table 4).

Clinical signs, cumulative infection in VNDV challenged birds

Birds appeared clinically normal, and no visible symptoms were recorded on 1st dpi in all the treatment groups. On 2nd dpi, clinical signs such as ruffled feathers, depression, loss of appetite, and periorbital edema were observed. Five birds (62.5%), each from control, sham control, AgNPs+AAs, and AgNPs+Vits, showed mild infection on 2nd dpi, and the cumulative infection (CI) was estimated as 15.62% for these groups. However, in the AgNPs (37.5%) and AgNPs+TEs (50%) group, three or more birds were mildly sick with CI

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TABLE 2 Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on performance of vaccinated birds.

Groups[a]	42nd d body weight (g)	ADFI, 0-42 d (g)	ADG, 0-42 d (g)	FCR, 0-42 d
Control	1,392.9	71.84	32.12	2.23
Sham control	1,351.3	69.50	31.17	2.24
AgNPs	1,347.0	65.74	31.03	2.12
AgNps+AAs	1,366.4	70.50	31.48	2.24
AgNps+Vits	1,465.3	71.94	33.88	2.12
AgNps+TEs	1,443.1	70.63	33.32	2.12
SEM	20.2	1.00	0.48	0.03
P-value	0.459	0.638	0.443	0.659

[a]Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg). Values have been analyzed by ANOVA.

TABLE 3 Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on the weight of immune organs in broiler chickens.

Groups[a]	Bursa (g/100 g BW)	Spleen (g/100 g BW)	Thymus (g/100 g BW)	HA titer against SRBC (log 2)	Foot web index, mm)
Control	0.132	0.171[a]	0.104	9.29	0.22[a]
Sham control	0.129	0.188[ab]	0.103	9.25	0.26[a]
AgNPs	0.131	0.203[bc]	0.108	9.43	0.41[ab]
AgNPs+AAs	0.133	0.195[abc]	0.109	9.13	0.28[a]
AgNPs+Vits	0.131	0.203[bc]	0.099	9.63	0.34[ab]
AgNPs+TEs	0.132	0.223[c]	0.113	10.50	0.55[b]
SEM	0.003	0.005	0.003	0.29	0.03
P-value	1.000	0.020	0.911	0.805	0.049

[a]Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg). Values have been analyzed by one-way ANOVA; [a,b,c] Means bearing different alphabets in a column differ significantly ($P < 0.05$).

of 9.37 and 12.5%, respectively. By the end of 2nd dpi, two birds from each treatment group (vaccinated and unvaccinated) were euthanized to observe the gross pathological lesions. On 3rd dpi, the sick birds from different treatment groups (unvaccinated) progressed to complete depression, off fed with passage of greenish watery diarrhea, sternal recumbency, and head downing with nasal/lacrimal discharge. Three birds (50%), each from sham control, AgNPs, AgNPs+Vits, and AgNPs+TEs groups, and two birds (33.33%), from control and AgNPs+AAs groups, expressed severe clinical symptoms. Two unvaccinated birds (33.33%) each from control and sham control groups died by the end of 3rd dpi. Rest of the live birds from unvaccinated groups showed moderate degree of sickness (Table 5). The highest CI was observed in sham control (79.17%) and control (75%) groups, while it was the lowest in the AgNPs+AAs (58.33) until 3rd dpi. The CI was same (62.5%) in rest of the treatment groups (Table 6).

On 4th dpi, progressive symptoms in the forms of leg paralysis, muscle twitching, diarrhea, and anorexia preceded to severe symptoms of open mouth breathing, drooling salivation, total off feed, complete paralysis, and death. In the 4th dpi, two birds in control, sham control group (50%), and AgNPs+AAs group (33.3%), three birds (50.0%) each from of the AgNPs and AgNPs+TEs groups, and four birds from AgNPs+Vits group (66.6%) were found dead. Rest of the birds which survived exhibited complete inappetence and paralysis, and they were grouped under "severely sick" category (Table 5). The CI was the highest in the AgNPs+Vits (91.67%), whereas the AgNPs+AAs group maintained a lowest CI (83.33) until 4th dpi. The CI of 87.5% was found in control, sham control, AgNPs, and AgNPs+TEs groups (Table 6). However, two birds, one each from AgNPs+AAs and AgNPs+TEs groups, survived the entire 5th dpi and were dead on 6th dpi. Hence, the CI reached to the mark of 100% in most of the treatment groups, except AgNPs+AAs (93.75%) and AgNPs+TEs (91.67%) groups until 5th dpi. These two groups, particularly the AgNPs+AAs, exhibited maximum resistance to the NDV and attained 100% CI on 6th dpi (Table 6).

TABLE 4 Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on the mercapto-ethanol-resistant (IgG) and sensitive (IgM) antibodies.

Groups[a]	IgG	IgM	HI titer against ND vaccine (Log2)		
Control	2.71	6.57	Day 7	Day 14	Day 21
			6.75	5.50	3.50
Sham control	3.00	6.14	6.25	5.25	3.25
AgNPs	2.86	6.00	6.75	5.75	4.00
AgNPs+AAs	2.43	7.43	6.25	5.00	2.50
AgNPs+Vits	3.29	7.00	7.75	6.75	3.25
AgNPs+TEs	3.43	6.00	6.25	5.50	2.75
SEM	0.17	0.27	0.22	0.25	0.17
P value	0.592	0.592	0.349	0.440	0.127

[a]Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg).
Values have been analyzed by one-way ANOVA.

TABLE 5 Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on classification of clinically ill birds on the basis of degree of severity (dpi wise) in treatment groups of unvaccinated birds.

Groups[a]	2nd dpi ($n = 8$)					3rd dpi ($n = 6$)					4th dpi ($n =$ L on 3rd dpi)				5th dpi ($n =$ L on 4th dpi)				6th dpi ($n =$ L on 5th dpi)	
	No	Mi	Mo	E	L	Mi	Mo	S	D	L	Mo	S	D	L	Mo	S	D	L	D	L
Control	3 (37.5)	5 (62.5)	0	2	6	0	2 (33.3)	2 (33.3)	2 (33.4)	4	0	2 (50)	2 (50)	2	0	0	2 (100)	0	0	0
Sham control	3 (37.5)	5 (62.5)	0	2	6	0	1 (16.7)	3 (50.0)	2 (33.3)	4	0	2 (50)	2 (50)	2	0	0	2 (100)	0	0	0
AgNPs	5 (62.5)	3 (37.5)	0	2	6	0	3 (50.0)	3 (50.0)	0	6	0	3 (50)	3 (50)	3	0	0	3 (100)	0	0	0
AgNPs+AAs	3 (37.5)	5 (62.5)	0	2	6	0	4 (66.7)	2 (33.3)	0	6	0	4 (66.6)	2 (33.3)	4	0	1 (25.0)	3 (75.0)	1	1 (100)	0
AgNPs+Vits	3 (37.5)	5 (62.5)	0	2	6	0	3 (50.0)	3 (50.0)	0	6	0	2 (33.3)	4 (66.7)	2	0	0	2 (100)	0	0	0
AgNPs+TEs	4 (50.0)	4 (50.0)	0	2	6	0	3 (50.0)	3 (50.0)	0	6	0	3 (50)	3 (50)	3	0	1 (33.3)	2 (66.7)	1	1 (100)	0

[a]Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg).
No, normal; Mi, mild; Mo, moderate; S, severe; D/E, dead/euthanized; L, remaining live birds at the end of that dpi; figures in the parenthesis are expressed as percentage to "n," that is, live birds at the beginning of same dpi.

Mean death time of challenged birds in unvaccinated group

The estimated mean death time was apparently higher ($P \leq 0.01$) in the birds of AgNPs+AAs and AgNPs+TEs groups than the controls and AgNPs+Vits (Figure 1).

Gross lesions in unvaccinated group birds

The gross lesions in different visceral organs appeared in a similar chronology in the birds irrespective of treatment groups. However, there was a variation in the degree of severity, and the birds were categorized accordingly. Variation in the macro-pathological lesions of the trachea, bursa, brain, intestine, cecal tonsils, proventriculus, and kidney was examined and scored accordingly. The scores for intestinal lesions were awarded based on the observed lesions from outside (redden patches), lesions in lumen (hemorrhages with bloody content), and cecal tonsils (hemorrhages). A score of 30, 20, 10, or 0 was granted for severe (+ + +), moderate (++), mild (+), or absence (-) of typical ND lesions. The observed gross pathological lesions in different treatment groups are classified dpi wise and are presented in Table 7. The average scores for gross pathological lesions in different treatment groups have been presented in Table 8. The control group continued to show higher lesion scores (110) consistently up to 4th dpi, and the pathological lesions were less severe in the AgNPs+AAs (105) and AgNPs+TEs (93.33) group. Interestingly, the nutrient-injected groups showed comparatively less severe lesions consistently on each dpi as compared to control group chicks.

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TABLE 6 Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on scoring of clinically ill birds on the basis of degree of severity (dpi wise) from each treatment groups of unvaccinated birds.

Groups[a]	2nd dpi			3rd dpi			4th dpi			5th dpi			6th dpi		
	Score obtained	Max. possible score	C.I (%)	Score obtained	Max. possible score	C.I (%)	Score obtained	Max. possible score	C.I (%)	Score obtained	Max. possible score	C.I (%)	Score obtained	Max. possible score	C.I (%)
Control	50	320	15.62	180	240	75.00	140	160	87.50	80	80	100	-	-	-
Sham control	50	320	15.62	190	240	79.17	140	160	87.50	80	80	100	-	-	-
AgNPs	30	320	9.37	150	240	62.50	210	240	87.50	120	120	100	-	-	-
AgNPs+AAs	50	320	15.62	140	240	58.33	200	240	83.33	150	160	93.75	40	40	100
AgNPs+Vits	50	320	15.62	150	240	62.50	220	240	91.67	80	80	100	-	-	-
AgNPs+TEs	40	320	12.50	150	240	62.50	210	240	87.50	110	120	91.67	40	40	100

[a]Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg).
Score obtained: the sum of clinical signs severity scores for mild (10), moderate (20), severe (30), and dead or euthanized (40) multiplied with number of birds in each category; maximum possible score: clinical signs severity score for dead birds (40) multiplied with the total live birds on the beginning of that dpi; CI: cumulative infection is the percentage of infection achieved on that dpi by the challenge virus as (score obtained/maximum possible score) ×100.

Clinical signs and gross pathology in the vaccinated groups

Eight vaccinated birds from each treatment groups were challenged in the same manner as described for the unvaccinated birds. No clinical symptoms were observed in the vaccinated groups, except few birds with mild cases of ruffled feathers and greenish diarrhea. None of the vaccinated birds were dead until the end of the experiment (6th dpi). The birds were fully active, and there was no evidence of sickness or inappetence. Two birds were euthanized (treatment-wise) on 2nd and 4th dpi, and the remaining four birds were euthanized on 6th dpi, where only moderate levels of intestinal hemorrhages were observed.

Relative expression of IL6 and IL-2 in the PBMC cells of unvaccinated challenged birds

There was no difference ($P \geq 0.05$) in the relative expression of IL-6 or IL-2 genes just before vND virus challenge in AgNps treated groups; however, the expression significantly downregulated ($P \leq 0.05$) in sham control group (Figures 2A,B). At 8 h post-vND virus challenge, IL-6 gene expression downregulated in AgNPs and AgNps+TEs group chicks as compared to un-injected chicks, but upregulation ($P \leq 0.05$) of IL-2 gene was seen in all AgNPs group chicks, especially in AgNps+Vits and AgNps+TEs group (Figures 3A,B). Similarly, at 24 h post-vND virus challenge, relative expression of both the genes (IL-6 and IL-2) was significantly ($P \leq 0.05$) upregulated in all AgNPs group chicks except that of IL-6 expression in AgNps+AAs group chicks (Figures 4A,B).

Discussion

Pre- and post-hatch performance in vaccinated birds

The chick weight increased to 2.6, 2.3, and 3.1% in the AgNPs+AAs, AgNPs+Vits, and AgNPs+TEs groups, respectively, in comparison with the control group. Similarly, the chick weight to egg weight ratio was higher (up to 3.87%) in the *in ovo*-injected groups as compared to the un-injected control. The present observation is in consistent with earlier reports showing that there was no effect of AgNPs on incubational and hatching parameters like embryo development, hatchability, and relative chick weight (17, 42–44). Similarly, different nutrients when injected *in ovo* into the yolk sac or extra embryonic membranes had no adverse effects on embryo, with a little influence on hatchability (17, 45).

In the present study, *in ovo* supplementation of AgNPs+Vits (B1: 72μg and B6:140 μg) and AgNPs+TEs (Zinc 80 μg

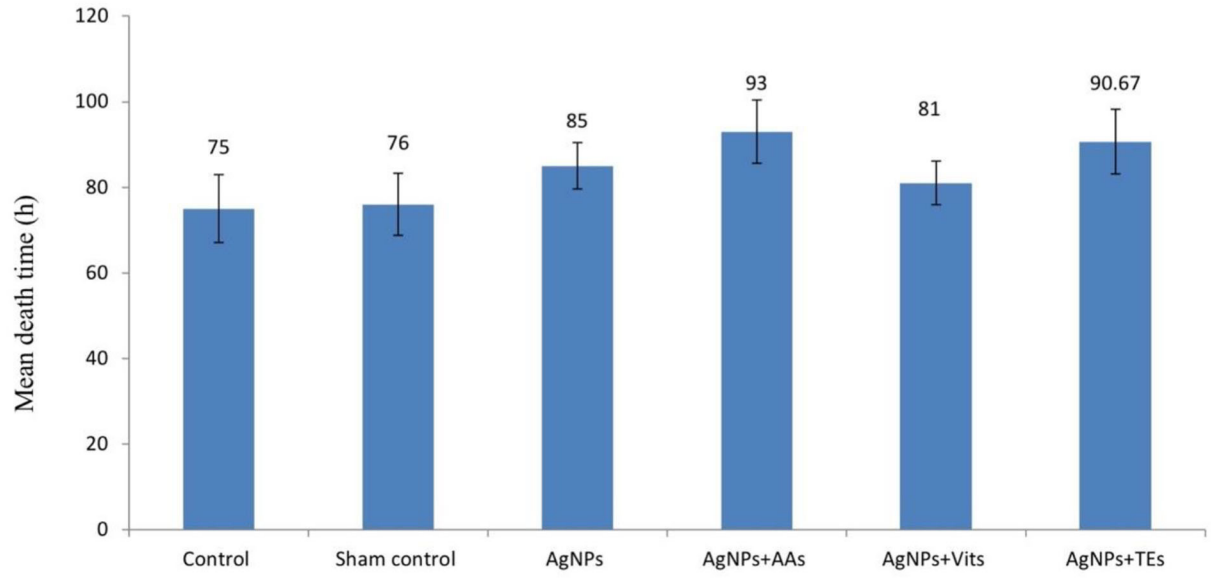

FIGURE 1
Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on mean death time (h) of virulent ND virus (vNDV) challenged birds in unvaccinated group. Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg).

TABLE 7 Chronological appearance of gross lesions in unvaccinated challenged birds.

2nd dpi	Congestion in the lower eye lid and periorbital swelling in few birds. Mild to moderate degree of hemorrhages in intestine. Mild hemorrhages in tracheal lumen in few birds.
3rd dpi	Petechial hemorrhages and edema of lower eye lid, mild meningeal congestion, mottled spleen (multifocal and diffused white necrotic areas), mild to moderate edema and hemorrhages in intestine and cecal tonsils. Slight edematous bursa, congested kidneys and congested meningeal capillary was noticed.
4th dpi	Moderate to severe hemorrhages in the cerebellum and brainstem region of skull, moderate to severe lesion in lymphoid organs and aggregates, watery greenish content in intestine, mild pinpoint hemorrhages at the tip of proventricular glands, moderate to severe mottling of spleen, multiple foci of edema, necrosis and hemorrhages in intestine and cecal tonsils, mild congestion in tracheal lumen, edematous bursa, congested kidneys and meningeal capillary congestion was noticed
5th dpi	Mild lower eyelid congestion, several multifocal hemorrhages at the tip of the proventricular glands, enlarged whitish kidneys, severe congestion in meninges of brain, severe lesions in visceral organs, hemorrhagic edematous intestine with greenish/bloody intestinal content in lumen, severe hemorrhages in the cecal tonsil, severely mottled spleen and moderate to severe hemorrhages in tracheal lumen.
6th dpi	Severe hemorrhages at cranial and caudal parts of tracheal lumen, semi-solid greenish content in intestine, severe multifocal pinpoint hemorrhages at the tip of the proventricular gland.

and selenium 0.3 μg) had 3.60–5.20 % higher 42nd d body weight as compared to un-injected control birds. Vitamin B1 plays a crucial role as a cofactor (thiamine pyrophosphate) in the conversion of glucose to energy, whereas vitamin B6, as pyridoxal phosphate, has role in amino acid transformations for protein synthesis. Altogether these two vitamins had also contributed for efficient feed conversion in the birds, helping them gaining more body weight. Goel et al. (25) observed that *in ovo* vitamin B1, B2, or E treated chicks attended higher body weight at 42nd d with a difference of 50 to 80g (3.6–5.8%) compared to un-injected control group. The effect of vitamin injection (B1, B2, and E) was more prominent in younger birds (till 21st d) with an increment of 5.3 to 13.3% body weight over control group (22). Role of trace elements, especially Zn as growth enhancer, cannot be ruled out. Goel et al. (27) reported higher growth-related gene expressions (i.e., Somatotropin, IGF-I, IGF-II, and mucin gene) in *in ovo* Zn injected chicks. Zn is found in more than 200 enzymes (Zn metalloenzymes) which

TABLE 8 Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on the average scores of gross pathological lesions in different treatment groups (dpi wise).

Groups[a]	2nd dpi	3rd dpi	4th dpi	5th dpi	6th dpi
Control	20 ($n = 2$)	45 ($n = 2$)	120 ($n = 2$)	160 ($n = 2$)	*ND
Sham control	15 ($n = 2$)	60 ($n = 2$)	110 ($n = 2$)	140 ($n = 2$)	*ND
AgNPs	15 ($n = 2$)	*ND	106.67 ($n = 3$)	146.67 ($n = 3$)	*ND
AgNPs+AAs	10 ($n = 2$)	*ND	105 ($n = 2$)	146.67 ($n = 3$)	140 ($n = 1$)
AgNPs+Vits	15 ($n = 2$)	*ND	110 ($n = 4$)	135 ($n = 2$)	*ND
AgNPs+TEs	15 ($n = 2$)	*ND	93.33 ($n = 3$)	140 ($n = 2$)	150 ($n = 1$)

[a]Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg).

*ND, not determined as no death or no birds were available from these groups, n is the number of birds necropsied on that dpi those contributed to the average scores for gross lesions on that dpi.

perform catalytic or co-catalytic functions. Earlier reports have also shown that higher IGF-I gene expression correlated with early post-hatch growth rate and feed efficiency in broilers (46) and accelerated skeletal muscle development in quail embryos (47), though many researchers have recommended that AgNPs have an insignificant role in weight gain, feed intake, and FCR (8, 17, 19, 44, 48) but can act as carrier of critical nutrients (49). The hatchlings of today's improved broiler strains do not have an adequate energy store to meet the huge metabolic requirement and delay in transportation from hatchery to farms which further affects growth and immune system in these birds. *In ovo* feeding addresses these issues by providing nutrients to the young chicks through supplementation into the yolk sac or amnion of the developing embryo at later stage of incubation, thus complements the nutrient reserve of egg and post-hatch feed intake.

Immune organ development and immune response

In this study, spleen weight was significantly higher (up to 30.4%) in AgNPs+TEs group, injected with Zn and Se in combination with AgNPs, in comparison with control and other groups. A significant increase in spleen weight (18.71%) was also observed in both AgNPs and AgNPs+Vits group as compared to the control group. Earlier reports have also recorded higher spleen weight in AgNPs treated birds (17, 50). Conversely, significant reduction in the lymphoid weight was also observed with increasing concentration of AgNPs in the diet (19, 50). In another study by Bakyaraj et al. (22), the thymus weight was significantly higher in the trace element-injected chicks than both amino acid and vitamins injected chicks. Goel et al. (27) also observed that the spleen weight was numerically higher in the Se supplemented chicks at 21st day post-hatch. The higher spleen weight in AgNPs injected chicks might be due to increase anabolic activity and subsequently stimulation of growth and development of lymphoid organs, as the structure of AgNPs enables to sustain atomic oxygen inside its octahedral holes for better oxygen supply (51).

In vaccinated birds, the *in vivo* immune response to PHA-P was significantly higher in the AgNPs+TEs group, in comparison with the control groups. This result correlates the previous studies where *in ovo* supplementation of AgNPs significantly enhanced the *in vivo* immune response to SRBC and PHA-P (17, 19, 27). Bhanja et al. (15) had also documented that the expression of cytokines (Th1 and Th2) and TLR genes, which are crucial for innate and adaptive immunity in chickens, was upregulated in AgNPs injected chicks. Goel et al. (27) reported significant upregulation of immune genes in Zn or Se injected chicks in comparison with the un-injected control chicks. AgNPs can increase activity of cell's immunity by stimulating heat shock protein (HSP) synthesis, without pro-inflammatory pathway activation as evidenced from lack of influence of AgNPs on expression of NF-kB, which is a transcriptional factor involved in defense of the organism (52). Furthermore, the antimicrobial and anti-inflammatory properties and possibility to enrich cells with oxygen improve immune system function of the organism supplemented with AgNPs. Selenium is considered as an immunological enhancing agent to enhance or recover immune functions of the organism (53). Earlier studies have also reported that Se played an important role in cellular immunity by increasing mitogen response to PHA-P (54) and wing web reaction in birds (55).

No significant difference was observed for the serum concentrations of IgG and IgM among the groups. However, the titer for IgG was higher in the AgNPs+TEs and that for IgM was higher in the AgNPs+AAs. Pineda et al. (42) found no discernible effects of pre-hatch and post-hatch silver nanoparticle exposure on plasma concentrations of IgG and IgM. Higher levels of IgM and IgG were produced by the birds under nano-silver treatment, but difference was insignificant (19). IgG is the major antibody isotype in chickens which mediate anaphylactic reactions, a function similar to IgE in mammals (56). In birds, the Fc region of IgG mediates most biological effector functions like complement fixation, anaphylactic reactions, and opsonization, whereas the Fab region contains the antigen-binding sites (57). The apparently higher IgG titer in the AgNPs+TEs can be correlated with the total Ab production against SRBC antigen but not against the ND vaccine.

Response to VNDV challenge study

Eight birds from each treatment group (both vaccinated and unvaccinated) were infected with $10^{7.4}$ ELD_{50}/0.1 ml/bird of

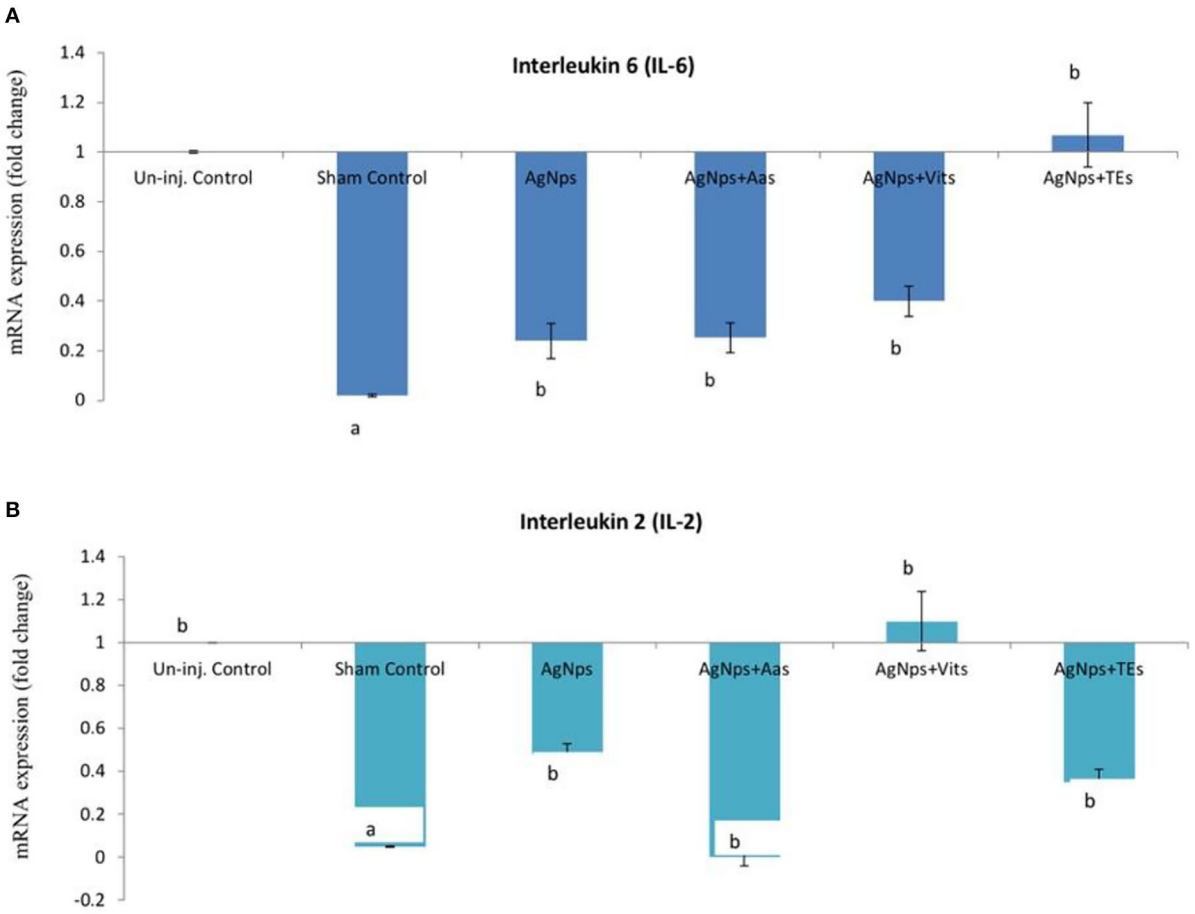

FIGURE 2
Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on the relative expression of IL-6 **(A)** and IL-2 **(B)** genes before virulent ND virus (vNDV) challenge. Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg). [a,b]Means bearing different alphabets in a histogram differ significantly ($P < 0.05$).

ND challenge virus on 45th d post-hatch. Only the unvaccinated birds showed clinical symptoms and gross lesions. Most of the unvaccinated birds were found dead within 5th d postinfection (dpi) except two birds, one each from AgNPs+AAs and AgNPs+TEs, who died on 6th dpi. However, no death was observed in the vaccinated groups.

Our finding is in line with the study by Taylor et al. (58), where well-vaccinated chickens were challenged with high doses of vNDV daily for 10 days. Though all sham-vaccinated birds died by the fourth day postchallenge, no morbidity or mortality was reported in the NDV vaccinated birds even up to 14 d postchallenge. They also observed that repeated challenge with high-dose of vNDV did not overcome the vaccine immunity. In another experiment by Desingu et al. (59), the ND virus-infected chickens exhibited 100% mortality with marked lesions in proventriculus, intestine, spleen, and bursa. The cumulative infection percentage (dpi wise), mean death time (in h), and the average score for gross lesions show that the unvaccinated birds from AgNPs+AAs and AgNPs+TEs endured maximum resistance to the challenge virus. The mean death time was lowest for control birds and increased up to 13.0–24.0% in the *in ovo*-injected groups. This ensures the effect of combined supplementation of AgNPs and other nutrients in general, and the colloidal nano-silver in particular on immunocompetence and antiviral response in broiler chickens. Other studies, corroborating our findings, show that the amount and specificity of humoral antibodies induced by different vaccines affect viral replication and clinical protection (41, 58, 60). Antimicrobial and antiviral activity of silver nanoparticles (30–32) is mediated through Ag+ ions, which inhibit bacterial growth by suppressing respiratory enzymes and electron transport components which interfere with DNA functions (33) or through interaction with virus surface proteins (30) and viral genome (DNA or RNA).

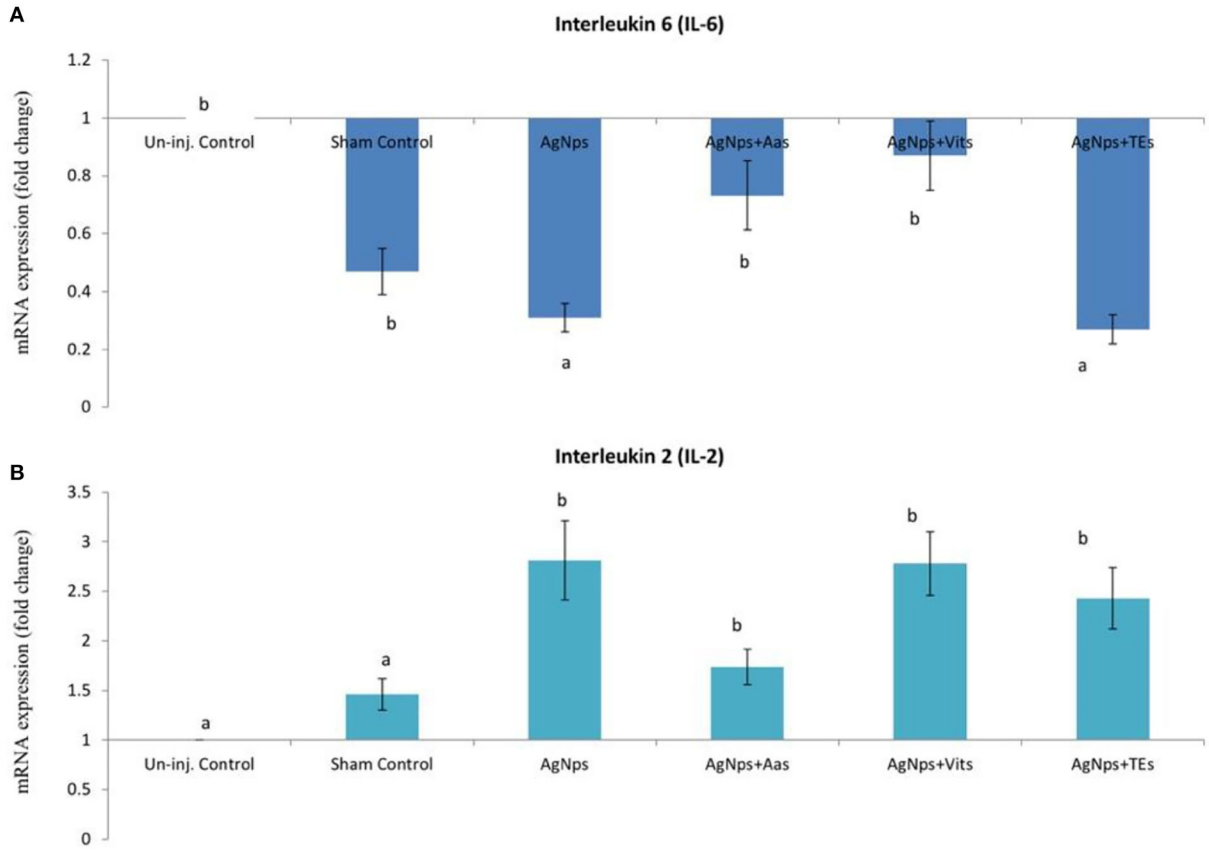

FIGURE 3
Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on the relative expression of IL-6 **(A)** and IL-2 **(B)** genes 8 h post-virulent ND virus (vNDV) challenge. Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg). [a,b]Means bearing different alphabets in a histogram differ significantly ($P < 0.05$).

Differential expression of IL-6 and IL-2 gene

Significantly higher expression of IL-6 and IL-2 gene was seen in AgNPs and their combination with AAs, Vits, and TEs after vND virus challenge. Bhanja et al. (15) also reported enhanced expression of humoral (IL-6) and cell-mediated (IL-12) immunity gene in either AgNPs or Cys+AgNPs treated embryos following activation by LPS. In addition, the expression of humoral and cellular immunity-related genes was also increased in *in ovo* trace element (Zn and Se) and AgNPs supplemented chicks (17, 27). In chicken, T lymphocyte cells are important for immune system which after activation differentiates into T helper cells (61), which produce CD4 or CD8 antigens during embryogenesis (62). These cells either act as a source for pro-inflammatory cytokines (IL-6) that induces final maturation of B cells into antibody-secreting plasma cells (63) causing the proliferation and differentiation of immunoglobulin or by increasing T lymphocyte proliferation through production of IL-2 and involved in activation of NK natural killer and T cytolytic cells (64). Zinc is an essential component of thymulin, a hormone from thymus, and is involved in maturation and differentiation of T cells (65). In this study, AgNPs +AAs and AgNPs+ TEs group chicks after vND virus challenge had significantly higher expression of IL-6 and IL-2 expression and also had better response against SRBC and PHA-P.

Conclusion

In ovo supplementation of AgNPs along with critical nutrients like amino acids, vitamins, and trace elements has shown promising result in improving post-hatch growth and immunity in broiler chickens, particularly that 50 μg/egg AgNPs in combination with vitamins (B1& B6) and trace

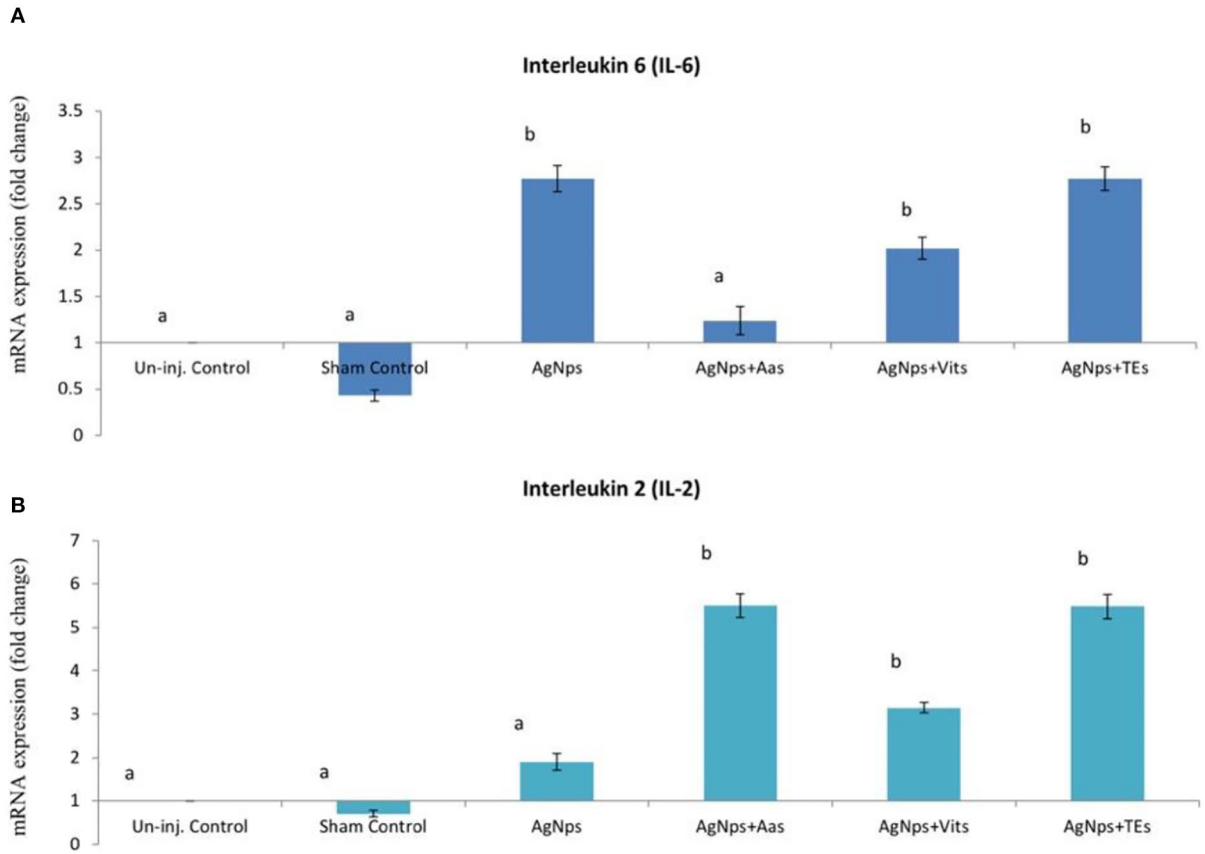

FIGURE 4
Effect of *in ovo* administration of silver nanoparticles (AgNPs) and critical nutrients on the relative expression of IL-6 **(A)** and IL-2 **(B)** genes 24 h post-virulent ND virus (vNDV) challenge. Control = un-injected, sham (sterile water), AgNPs = silver nanoparticles (50 μg), AgNPs+AAs = silver nanoparticles (50 μg) + amino acids (Methionine- 10 mg + Arginine-25 mg), AgNPs+Vits = silver nanoparticles (50 μg) + vitamins (Vit B1-72 μg + Vit B6-140 μg) and AgNPs+TEs = silver nanoparticles (50 μg) + minerals (Zn-80 μg and Se-0.3 μg). [a,b]Means bearing different alphabets in a histogram differ significantly ($P < 0.05$).

elements (Zn & Se) improved the production performances and 50 μg/egg AgNPs with trace elements and amino acids enhanced immune response and resistance against vND virus challenge in broiler chickens. The antiviral property of AgNPs was apparently evident in our detailed challenge study. Nonetheless, the AgNPs to be used in therapeutic or prophylactic treatment in chickens, additional research is needed to determine the exact mode of antiviral mechanism and the highest safe dose in broilers, to augment the resistance to lethal avian viruses without creating new risk to humans or the environments.

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

All the experimental procedures on birds were carried out according to the recommendations and approval of the ICAR-Central Avian Research Institute, Izatnagar, India's Institute Animal Ethics Committee vides approval no. CARI/CPCSEA/2016/8 dated 23.08.2016 for the Purpose of Control and Supervision of Experiments on Animals in India.

Author contributions

SB conceptualized the study, carried out biological study, analyzed data, and prepared the manuscript with input from AAlq, AAli, YA, VP, and AS. PR conducted the challenge experiment, data collection, and sample analysis. AG helped in sample collection, molecular, and gene expression study. MM helped in sample collection, sample analysis, and article writing. SD helped in molecular study, data analysis, and report writing.

AAlq, AAli, YA, VP, and AS participated in the discussion and editing of the manuscript.

Funding

This work was supported by Researchers Supporting Project number (RSP2022R439), King Saud University, Riyadh, Saudi Arabia.

Acknowledgments

We acknowledge the Director, ICAR-CARI, for the support and cooperation in providing experimental animals and all the necessary facilities.

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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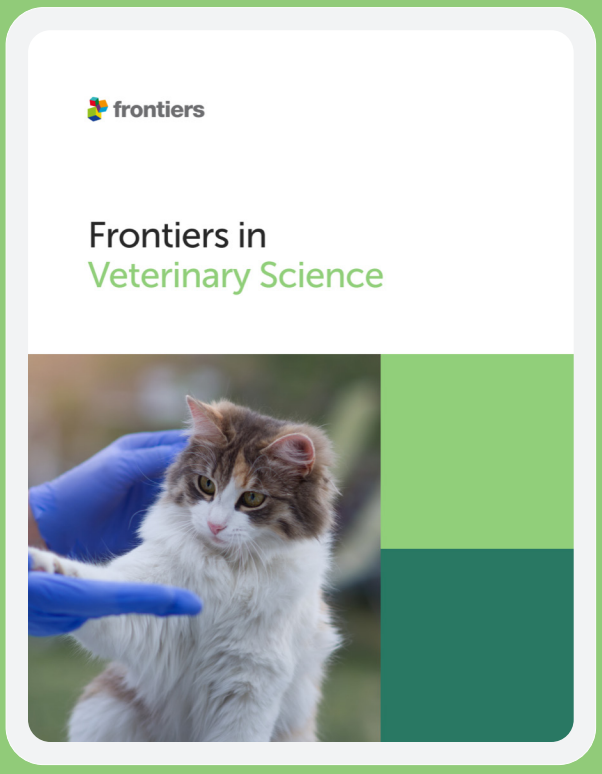

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