Dietary genistein supplementation alters mRNA expression profile and alternative splicing signature in the thymus of chicks with lipopolysaccharide challenge

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ABSTRACT Genistein is abundant in the soybean products, which exerts prominent effects on immune function. Little information is available about the effect of dietary genistein on thymic transcriptome, especially when suffering from lipopolysaccharide challenge. In this study, 180 one-day-old male broilers were randomly allocated to 3 groups: nonchallenged chicks given a basal diet (**CON**), and lipopolysaccharide-challenged chicks fed a basal diet (**LPS**), or lipopolysaccharide-challenged chicks fed a basal diet supplemented with 40 mg/kg genistein (**GEN**). Lipopolysaccharide injection induced thymocyte apoptosis and inflammatory reactions in the chicks. The results showed dietary genistein significantly reduced the percentage of CD3+ T lymphocytes by 10.04% and CD4+/CD8+ T lymphocyte ratio by 21.88% in the peripheral blood induced by lipopolysaccharide injection ($P < 0.05$). In addition, genistein significantly reduced the thymus index by 50% and apoptotic index by 12.34% induced by LPS challenge ($P < 0.05$). Transcriptomic analysis identified 1,926 DEGs (1,014 upregulated and 912 downregulated, $P < 0.05$) between GEN and LPS groups, which altered the mRNA expression profile and signaling pathways (Toll-like receptor, and NOD-like receptor signaling pathway) in the thymus. Furthermore, 5 splicing (AS) isoforms of the Drosophila Disabled-2 (**DAB2**) gene were detected, which were significantly upregulated in the GEN group compared with that in the LPS group. In summary, dietary genistein supplementation altered the RNA expression profile and AS signatures in the thymus, and alleviated immune response against lipopolysaccharide challenge.

Key words: genistein, lipopolysaccharide, transcriptome, thymus, chicken

2022 Poultry Science 101:101561
https://doi.org/10.1016/j.psj.2021.101561

INTRODUCTION

Salmonellosis is a common disease in poultry and human, one serotype of which with high isolation rate is Salmonella Typhimurium (***STm***) (Li et al., 2013). *STm* can not only infect human with immunosuppression, but also cause fever and gastroenteritis through poultry products (Okoro et al., 2012; Wigley, 2014). Lipopolysaccharide is the antigen marker of *STm*, which can activate MyD88 (myeloid differentiation factor 88)/TIRAP (Toll-interleukin 1 receptor [**TIR**] domain-containing adapter protein)/**NF-κB** (nuclear factor kappa-B) signaling pathway and downstream proinflammatory factors (Fukata and Abreu, 2005). Furthermore, lipopolysaccharide exposure negatively affects the thymus by inducing thymocyte death (Huang et al., 2016). Actually, *STm* infection causes immune and gut disease in broilers, which causes the growth performance losses (Kollanoor-Johny et al., 2012). Intraperitoneal injection of lipopolysaccharide has been used to establish the inflammation model for exploring the therapeutic schedule of *STm* infection in many anim al experiments (Männel, 2007; Lv et al., 2020). The immune-potentiating function of soy-derived extracts might explain their use within prophylaxis or the therapy of different diseases. Even though numerous studies identified soybean isoflavones with immune enhancing properties, poor evidence exists for the transcript profile of the thymus, especially which of non-mammalian species. Genistein (4Y, 5, 7-dihydroxyysoflavone), a type of isoflavone, is abundant in soybean products. It can enhance protein synthesis, and improve the growth performance of broilers, which provides a reference for developing new feed additives (Lyou et al., 2002; Rasouli and Jahanian, 2015). Interestingly, adding genistein (20, 40 mg/

Received June 28, 2021.
Accepted September 26, 2021.

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kg) to the diet can also improve the antibody titer of Newcastle disease and infectious Bronchovirus in broilers (Alipour et al., 2012). Furthermore, genistein can inhibit the apoptosis process induced by toxic substances, and alleviate DNA damage in the lymphocyte (Subbiah and Raghunathan, 2008; Sonaa et al., 2013). Our previous study indicated that genistein (40, 400 mg/kg) could downregulate the expressions of tumor necrosis factor-α (**TNF-α**), interleukin-8 (**IL-8**), interleukin-1β (**IL-1β**), and NF-κB in the spleen of hens with fatty liver syndrome, and alleviate inflammatory cell infiltration (Lv et al., 2018). Our resent research revealed that dietary genistein could promote the growth performance of chicks under intestinal injury induced by intraperitoneal injection of lipopolysaccharide (Lv et al., 2020). The thymus is the important central immune organ, in which T lymphocyte differentiates and matures. Salmonella can ignore the protective function of the blood-thymus barrier, and invade the thymus tissue, which stimulates the inflammatory reaction (Bailey et al., 2005). Therefore, it is of great importance to clarify the effects of dietary genistein supplementation on the immune response of chicks that have undergone lipopolysaccharide challenge, which can bring huge economic benefits to poultry production.

Transcriptome-level gene expression provides effective evaluation of host responses to external stimuli, which allows identifying the pathways that act to alleviate the adverse effects. AS is the crucial event after transcription for eukaryotic cells, involving in the gene regulation (Moon et al., 2015; Juan-Mateu et al., 2016). Furthermore, the effect of 5 splicing (AS) on lipopolysaccharide/TLR4 signaling pathway has been emphasized in the field of immunity (Yanagisawa et al., 2003). Therefore, we hypothesized that genistein could relieve immune response, and alter the mRNA expression profile as well as AS signature in the chick thymus. The objective of this study was to clarify the immune response of chicks toward dietary genistein supplementation, and to characterize its effects on the thymic transcriptome, when suffering from lipopolysaccharide challenge using RNA-Seq technology.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of Animal Welfare Committee of China Agricultural University (Beijing, China, permit number CAU/20191683-C2).

Birds and Experimental Design

One hundred eighty 1-day-old male ROSS 308 broiler chickens with similar initial body weight (49.2 ± 1.1 g) were randomly allocated to 3 groups, with 4 replicates of 15 birds each. The current experiment lasted for 21 d (from 1 to 21 d of age). The treatments were as follows: nonchallenged chicks given a basal diet (**CON**, genistein monomer concentration = 0.72 ± 0.24 mg/kg), and lipopolysaccharide-challenged chicks fed a basal diet (**LPS**), or lipopolysaccharide-challenged chicks fed a basal diet supplemented with 40 mg/kg genistein (**GEN**, genistein monomer concentration = 41.2 ± 3.3 mg/kg). genistein monomer with 99.8% purity was purchased from Kai Meng. Bio. Limited (Xi'an, China). Lipopolysaccharide is from Escherichia coli (L2880, Sigma Aldrich Inc., St. Louis, MO). The basal diet was formulated based on the nutrient requirements given by the National Research Council (NRC 1994, Supplementary data 1). The additional level of genistein in the current experiment is according to our previous studies (Lv et al., 2019a). All broilers were housed in wired cages in a temperature- and light-controlled room with on a 23 h/d-lighting program. Birds were vaccinated using combined Newcastle disease virus (**NDV**) and infectious bronchitis virus on d 7 through intranasal and intraocular administration. All broilers had ad libitum access to the diet and water.

Lipopolysaccharide was dissolved in 0.9% sterile saline solution. At 7: 00 am of 17, 19, and 21 d, the LPS and GEN groups received an intraperitoneal injection of lipopolysaccharide solution at a dose of 1 mg/kg after 10 h of feed deprivation, whereas the CON group received sterile saline injection. The dosage and injection of lipopolysaccharide was referred to available findings (Chen et al., 2018).

Sample Collection

Three hours after injection of lipopolysaccharide at 21 d of age, 2 broilers in each replicate with body weights close to the average (SD ≤ 38 g) were selected. Blood sample was collected from the wing vein into vacuum blood collection tubes with heparin sodium to detect lymphocyte percentages. Then, the chickens were sacrified by intravenous injection of pentobarbital sodium (30 mg/kg body weight) and jugular exsanguination. The third pairs of thymuses were cut off from both the right and left sides of the distal neck. The left one was promptly fixed in 4% paraformaldehyde for histological analyses. The right one was immediately frozen in liquid nitrogen, and stored at −80°C for RNA extraction. Meanwhile, the spleen, thymus, and bursa of Fabricius was collected and weighed, respectively. The immune organ index (organ weight/body weight) was calculated.

Peripheral Blood Lymphocyte Classification

Peripheral blood lymphocyte (**PBLs**) were isolated using Ficoll density centrifugation. Briefly, heparinized blood was diluted with Hank's balanced salt solution (Aladdin Co., Ltd., Beijing, China). After centrifugation for 30 min at 3,000 *g* (20°C), the PBLs at the plasma-Ficoll interface were collected. PBLs were washed three times with cold RPMI-1640 medium (Aladdin Co., Ltd., Beijing, China) by centrifugation at 1,800 × g for 10 min (4°C). Cell counts and viability were evaluated

using trypan blue staining. Lymphocytes were then mixed with CD3 (SPRD), CD4 (FITC), and CD8 (RPE) antibodies (Southern Biotechnology Associates, Birmingham, AL). Then, cells were held in a water bath for 30 min (37°C), washed twice with Hanks solution and fixed with 3% paraformaldehyde. The lymphocyte subsets were measured by multichannel flow cytometry (Beckman Coulter, Inc., Brea, CA). The results are expressed as percentages (%).

Histological Structure Analyses of the Thymus

After fixation in 4% PFA for 24 h, the thymus was soaked through a graded series of ethanol and xylene, embedded in paraffin, and sectioned at 5 μm with a Lecia RM2235 microtome (Leica Biosystems Inc., Buffalo Grove, IL). The sections were deparaffinized with xylene and rehydrated through a graded ethanol, and stained with hematoxylin and eosin (**HE**). The images were acquired using an Olympus simon-01 microscope (Olympus Optical Co., Ltd., Beijing, China).

Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay of the Thymus

Thymic apoptosis was determined using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (**TUNEL**) assay with a TUNEL BrightRed Apoptosis Detection Kit (Vazyme Biotech, Nanjing, China). First, the paraffin sections of thymus were deparaffinised, rehydrated and then incubated with Proteinase K (20μg/mL) at room temperature for 20 min. Second, the sections were incubated with the TdT enzyme buffer containing double distilled H_2O, Equilibration Buffer, BrightRed Labeling Mix and Recombinant TdT Enzyme at 37°C for 60 min in the dark. Finally, the sections were stained with 40, 6-diamidino-2-phenylindole staining solution (Beyotime Biotechnology, Shanghai, China) for 5 min in the dark conditions. The negative control was performed as above, but without incubation of the TdT enzyme buffer to ensure that no nonspecific reaction appeared in the experiment. The fluorescence images of TUNEL were acquired through a LSM 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). The numbers of apoptotic cells (Red color) and total cells (blue color) were counted using the Image-Pro Plus software 6.0 (Media Cybernetics). The apoptotic index was defined as the ratio of apoptotic cells to total cells.

Process of High-Throughput RNA Sequencing and Quantitative Polymerase Chain Reaction

Total RNA for sequencing were purified from 20 mg of thymus samples from 9 chickens (3 replicates in each group) using the RNeasy Fibrous Tissue Mini messenger RNA (**mRNA**) extraction kit (Qiagen, Hilden, Germany). The concentration and purity of total RNA were determined using a UV/Vis spectrophotometer (ACTGene, NJ,) at 260 nm. RNA integrity was evaluated through a microfluidic assay using a Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, CA). Only high-quality RNA extracts (RNA integrity number [RIN] $\geq$ 8) were used to pool equal amounts of each RNA sample within single group. Complementary DNA (**cDNA**) libraries for RNA sequencing (**RNA-Seq**) were constructed using a TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA). RNA-Seq analysis was performed to identify transcriptional changes using a MiSeq instrument (Illumina) with paired end libraries (CapitalBio, http://cn.capitalbio.com/). Three pools of RNA from each treatment group were analyzed independently for library synthesis and sequencing. The quality of raw reads was assessed using FastQC (Version 0.10.1). Adapters, low-quality reads (Q < 20) at the 3′end, reads with fuzzy N bases, ribosomal RNA (**rRNA**), reads shorter than 20 nt were trimmed with the FASTX clipper (Version 0.0.13). Paired-end clean reads were mapped to the chicken genome sequence (Gallus_gallus-5.0, version 81, Ensembl) with TopHat v2.0.9 (Chen et al., 2018). The gene expression levels in each sample were estimated according to fragments per kilo-base of exon per million fragments mapped (**FPKMs**) and assessed with Cufflinks v2.1.1 (Trapnell et al., 2012). Transcripts with a $P < 0.05$ were considered differentially expressed.

The results of RNA sequencing were validated through qRT-PCR. qRT-PCR was performed with the LightCycler 480 real-time PCR system and SYBR Green PCR Master Mix (TaKaRa Biotechnology, Dalian, China). Total RNA was isolated from the thymic tissues using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). cDNA was generated using the PrimeScript RT reagent kit with cDNA eraser (Takara). The specific quantitative primers for 7 transcripts are listed in Supplementary data 2. Quantitative PCR assays were carried out using a commercial PCR kit (Takara), containing SYBR-Green (a dye with green excitation wavelength that binds to all dsDNA double helix groove regions). The reaction volume of 20 μL mixture contained 10 μL SYBR1Premix Ex Taq (Tli RNaseH Plus), 0.4 μL ROX Reference Dye, 0.4 μL of each forward and reverse primer, 6.8 μL dilution and 2 μL cDNA template. The conditions were 95°C for 2 min followed by 40 cycles (95°C for 20 s, 60°C for 30 s, and 68°C for 30 s). Each experiment was performed in triplicate. Target gene expression was quantified using the 2-△△CT method and normalized to the expression of GAPDH.

Bioinformatics Analysis

Differential expressed analysis of mRNA was carried out by DESeq2 software package between the LPS vs. CON groups and GEN vs. LPS. Low expressed mRNAs

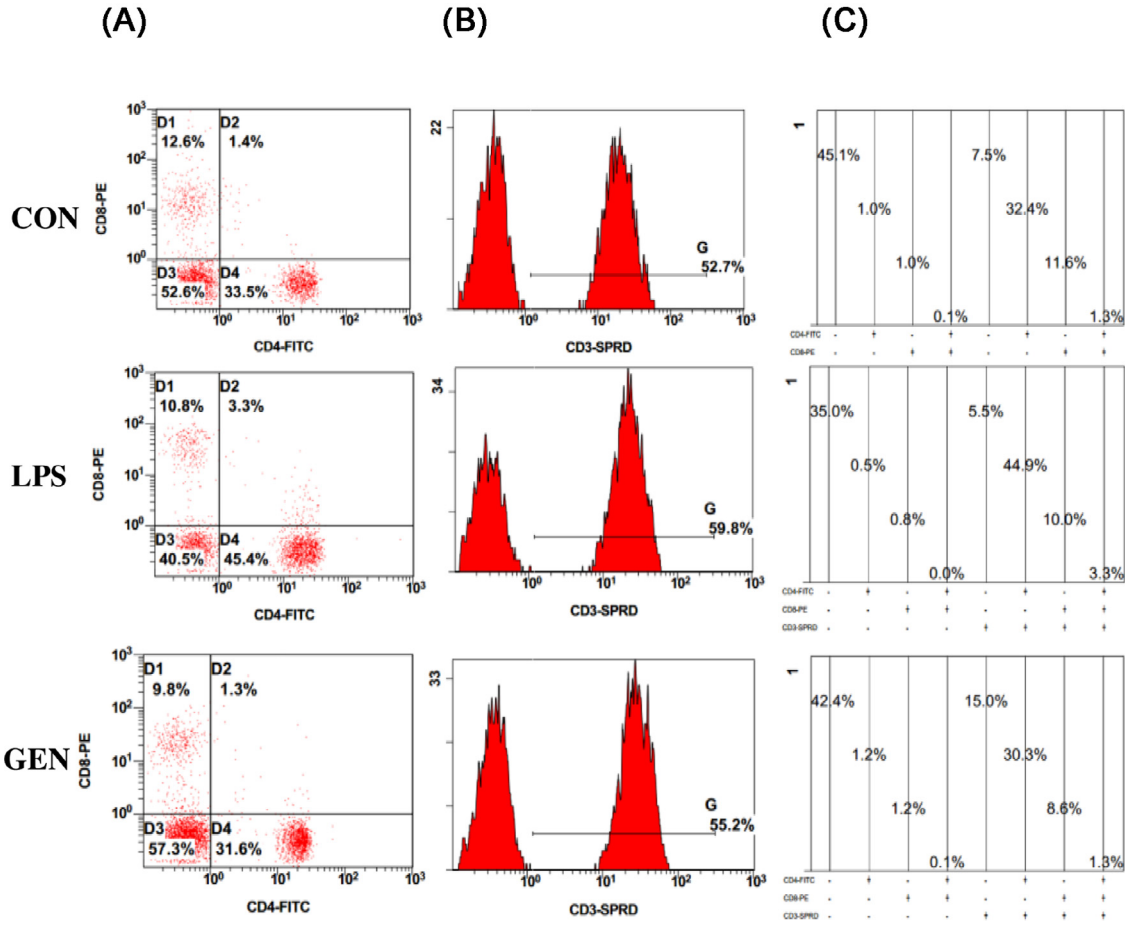

Figure 1. Effects of genistein on the classification of peripheral blood T lymphocyte by flow cytometer analysis. (A) Square dot graph of two parameters (FL1 INT LOG/FL2 INT LOG), (B) linear gate analysis of CD3+ T cells in peripheral blood T lymphocyte (FL1 INT LOG), (C) Prism analysis of CD3+, CD4+ and CD8+ T lymphocyte percentage. Data are presented as mean value ± SD (n = 8). Values without the same mark (A, B) represent statistically significant differences ($P < 0.05$). Abbreviations: CON, nonchallenge control; LPS, lipopolysaccharide-challenged group; GEN, lipopolysaccharide-challenged group fed diet supplemented with 40 mg/kg genistein.

were filtered out before differential analysis. Further analysis was conducted only using genes that demonstrated P-value < 0.05 by t tests. The databases for annotation, visualization, and integrated discovery, including PANTHER (http://www.pantherdb. org/) and OMICSBEAN (http://www.omicsbean.cn) were used to perform gene function enrichment analyses based on Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**).

Identification of Alternative Splicing

We used rMATS to classify AS events into 5 types. The expressions of 2 isoform (exon inclusion isoform and exon skipping isoform) were divided by their effective length to get the corrected value. Finally, we conducted differential analysis of AS between the 2 groups. The threshold value for AS events screening with significant difference is false discovery rate (**FDR**) < 0.05.

Statistical Analysis

Comparison of gene expression levels from RNA-Seq was performed using the t test. Multigroup comparisons of the means were carried out by one-way analysis of variance (**ANOVA**) tests with post hoc contrasts by Student−Newman−Keuls tests, which were presented as mean ± SD or mean ± SEM (for gene expression). Differences among different treatments were analyzed by Duncan's multiple comparisons. Significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Classification of PBLs

CD4+ T lymphocytes produce lymphokines, which can enhance the cytotoxic activity of CD8+ T cells. Meanwhile, a high CD4+/CD8+ T lymphocytes ratio is the universal prognostic indicator for immune system activation (Sheu et al., 1999). In the current study, lipopolysaccharide injection increased the number of CD3+ T lymphocytes and the ratio of CD4+/CD8+ T lymphocytes in the PBLs of chickens (Figure 1 $P < 0.05$), indicating that an inflammatory response was induced inflammatory reaction. Similar to the present result, it is reported that lipopolysaccharide challenge could increase the ratio of CD4+/CD8+ T lymphocytes in the PBL (Yang et al., 2008). Genistein is the potential plant

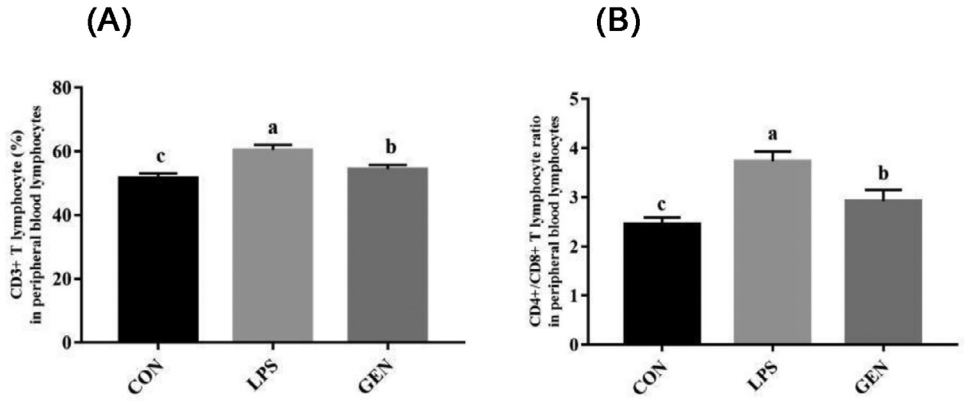

Figure 2. Effects of genistein on the classification of peripheral blood T lymphocyte by flow cytometer analysis. (A) Statistical analysis of CD3+ T lymphocyte. (B) Statistical analysis of CD4+/CD8+ T lymphocyte ratio. Data are presented as mean value ± SD (n = 8). Values without the same mark (A, B) represent statistically significant differences ($P < 0.05$). Abbreviations: CON, nonchallenge control; LPS, lipopolysaccharide-challenged group; GEN, lipopolysaccharide-challenged group fed diet supplemented with 40 mg/kg genistein.

extract for improving immunity of animals. It is reported that dietary inclusion of genistein increased lymphocytes and subsequently reduced heterophil to lymphocyte ratio in the PBLs of chick (Rasouli and Jahanian, 2015). In the present experiment, adding genistein into the diet reduced the ratio of 10.04% CD3 + T lymphocytes and 21.88% CD4+/CD8+ T lymphocytes in the PBLs of chicks challenged with lipopolysaccharide (Figures 2A and 2B, $P < 0.05$). Previous research reveals that genistein can inhibit inflammatory response induced by lipopolysaccharide, and reduce the production of nitric oxide and IL-6 (Choi et al., 2016). Furthermore, It can decrease the production of T and B lymphocytes by inhibiting topoisomerase (Wei et al., 2012). Therefore, dietary genistein supplementation can alleviate the stimulation of lipopolysaccharide on PBLs.

Immune Organ Indexes and Thymus Histological Observation

Lipopolysaccharide can be recognized by the host, inducing the aggregation of immune cells and resulting in an inflammatory response (Lu et al., 2008). Furthermore, lipopolysaccharide injection has profound effects on the immune response of CD4+ T lymphocytes (Martin and Leibovich, 2005). Th17 cells are an important subgroup of CD4+ T lymphocytes, which can secrete IL-17 and effectively induce inflammatory reaction and tissue injury (Korn et al., 2009). In the current experiment, lipopolysaccharide injection significantly increased the organ indexes of 50.0% thymus and 36.3% spleen (Table 1, $P < 0.05$). The swelling of immune organs reflected that lipopolysaccharide challenge induced inflammatory reaction in the thymus, which mainly be due to the increased production of lymphocytes. In comparison with *STm*, lipopolysaccharide macromolecules can invade the tissues more quickly, and induce an immune response. In the current experiment, the chick thymus still kept clear boundary structure between the region of medulla and cortex after lipopolysaccharide challenge. Similarly, the structure of thymus is also intact after *STm* infection in the SuiZhi mouse model (Sung et al., 2014). The pathogenicity of *STm* and its lipopolysaccharide is relatively weak towards poultry at 1 mg/kg treating dose. The weak stimulus of lipopolysaccharide has not induced severe inflammatory response in the thymus, which might not change the histological structure. Accordingly, there was no difference in the histological structure between the LPS and GEN groups (Figure 3).

Table 1. Effects of dietary genistein on the immune organ indexes.

Treatment	Thymus index (%)	Fabricius Bursal index (%)	Spleen index (%)
CON	0.32 ± 0.03^{b}	0.17 ± 0.01	0.11 ± 0.01^{b}
LPS	0.48 ± 0.02^{a}	0.17 ± 0.02	0.16 ± 0.01^{a}
GEN	0.35 ± 0.04^{b}	0.14 ± 0.02	0.12 ± 0.01^{b}
P-value	0.003	0.290	0.017

Abbreviations: CON, nonchallenge control; LPS, lipopolysaccharide-challenged group; GEN, lipopolysaccharide-challenged group fed diet supplemented with 40 mg/kg genistein.
The data were expressed as the mean ± SD (n = 8).
[ab]Represents significant difference ($P < 0.05$).

Quality Control of RNA-Seq Data and Identification of Transcripts Expressed in the Chick Thymus

In this study, we established nine cDNA libraries from the thymus of chicks in the CON, LPS, and GEN groups, with three replicates each. A RNA-Seq quality control summary, including base content along reads, error rate distribution along reads, classification of raw data, percent of genome regions was shown in Supplementary data 3 a. We concluded that the quality of RNA-Seq data was reliable and qualified. RNA-Seq generated 61,703,504 to 76,856,286 raw reads for each library, with an average of 206,694,640, 217,102,652, and 202,603,216 paired-end reads for the CON, LPS and GEN groups, respectively. Low-quality

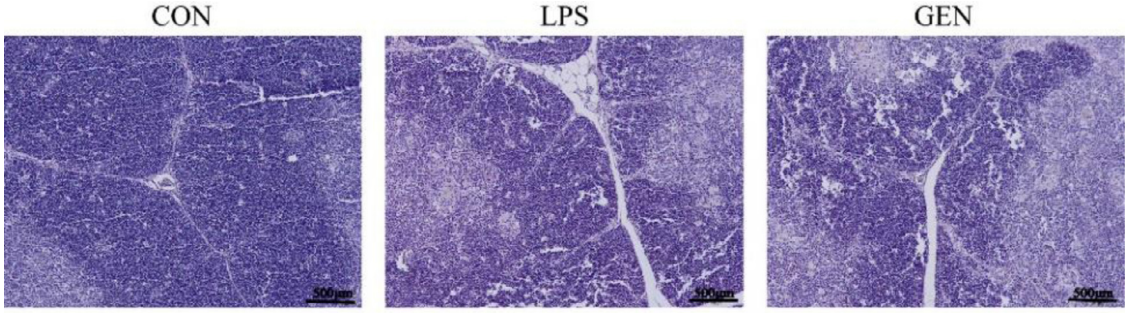

Figure 3. Histological observation of chick thymus by he staining. Scale bars = 500 μm. Abbreviations: CON, nonchallenge control; LPS, lipopolysaccharide-challenged group; GEN, lipopolysaccharide-challenged group fed diet supplemented with 40 mg/kg genistein.

reads were filtered out, and the average numbers of clean reads were 202,745,746, 213,289,282, and 198,754,292 for the CON, LPS, and GEN groups, respectively. The clean reads were used for all further analyses. After assembly, a total of 25,017 mRNAs were obtained from the 3 groups. The average mapping rates were 87.24, 89.22, and 88.81% for the CON, LPS and GEN groups, respectively (Supplementary data 3 b). The mapped reads of different regions of the genome are displayed in Supplementary data 3 c. The top 10 most abundantly expressed genes among the 3 groups, ranked by absolute abundance, were *RPLP1, COX1, FAU, RPS6, ENSGALG00000015617, COX3, ATP6, ENSGALG00000041611, RPS19, RPS19* and *COII* (Supplementary data 3).

Real-Time PCR Validation of Differential Gene Expression

To confirm the accuracy of the RNA-Seq data, we randomly selected 7 genes. The expression levels of the selected genes were quantified using qRT-PCR, and the results were consistent with the findings obtained by RNA-Seq. As is shown in Figure 5, the gene expression abundance of *CXCL12, NFKBIE, CCR2, TNFSF8, COX1, COII, DAB2* detected by RNA-Seq and RT-PCR were consistent. The results suggested that RNA-Seq reliably identified differentially expressed mRNAs in the thymic transcriptome of chicks.

Identification of Differentially Expressed Genes and Venn Analysis

We identified 712 differentially expressed genes (**DEGs**) (367 upregulated and 345 downregulated) between the LPS vs. CON group with a |fold change| >1 ($P \leq 0.05$, Figure 6A). There are 1,926 DEGs (1,014 upregulated and 912 downregulated) in the GEN group compared with the LPS group (Figure 6B). The expression abundance and fold change of DEGs are shown in Supplementary data 4. Cluster analysis was carried out on DEGs to gather genes with similar expression

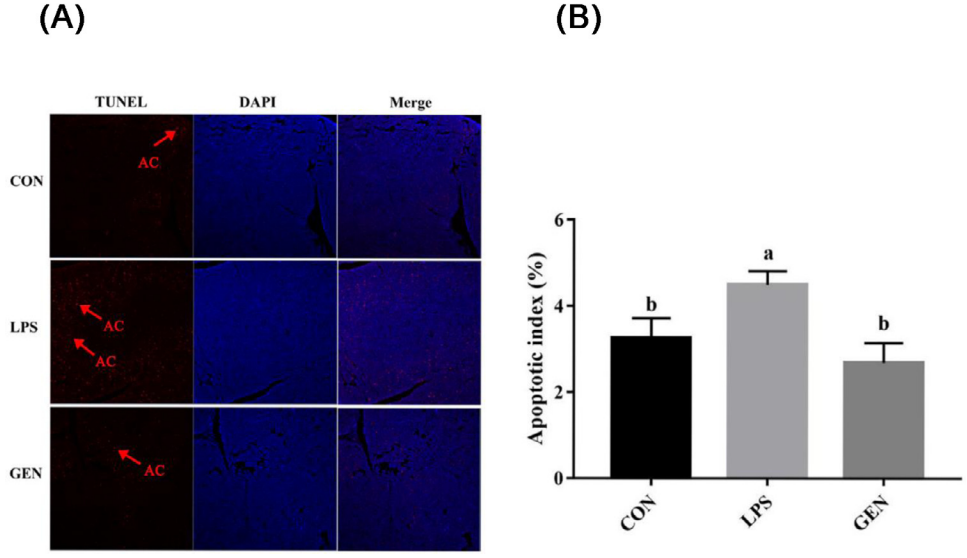

Figure 4. Thymus histological observation and tunel assay. (A) Tunel assay of thymus sections at 21 d of age by immunofluorescence. The blue color represents the total cells in the thymus, and the red color represents the apoptosis cells. (B) Apoptotic index of thymus at 21 d of age. AC, apoptosis cells. Data are presented as mean value ± SD (n = 8). Values without the same mark (A, B) represent statistically significant differences ($P < 0.05$). Scale bars = 200 μm. Abbreviations: CON, nonchallenge control; LPS, lipopolysaccharide-challenged group; GEN, Lipopolysaccharide-challenged group fed diet supplemented with 40 mg/kg genistein.

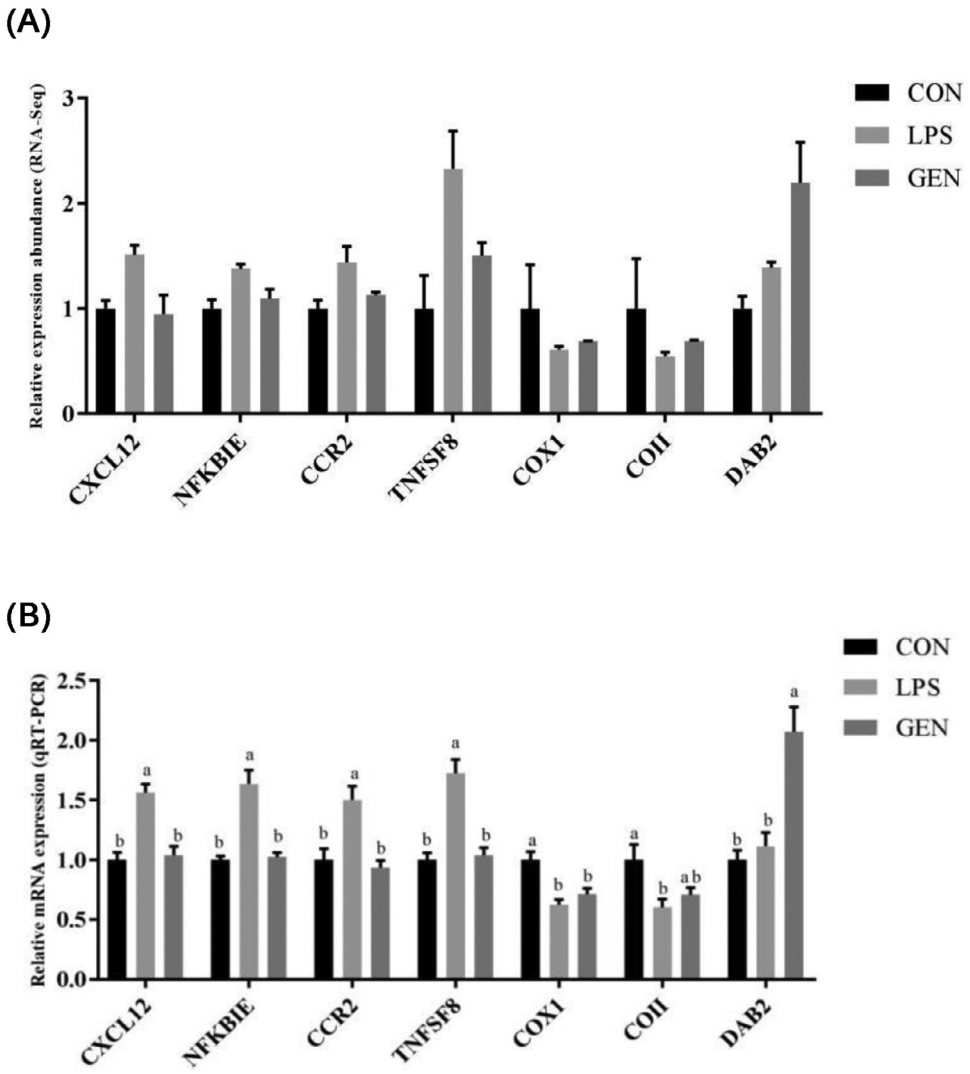

Figure 5. (A) The relative gene expression abundance from RNA-Seq data as determined by using Cufdiff software (n = 3). (B) The relative mRNA expressions of random selected genes in the chick thymus as determined by using qRT-PCR. Data are presented as mean value ± SEM (n = 8). Values without the same mark (A−C) represent statistically significant differences ($P < 0.05$). Abbreviations: CXCL12, motif chemokine 12; CCR2, C-C chemokine receptor type 2; COX1, cytochrome c oxidase subunit 1; COII, cytochrome c oxidase subunit 2; CON, nonchallenge control; DAB2, disabled homolog 2; GEN, lipopolysaccharide- challenged group fed diet supplemented with 40 mg/kg genistein; LPS, lipopolysaccharide- challenged group; NFKBIE, NF-kappa-B inhibitor epsilon; TNFSF8, tumor necrosis factor ligand superfamily member 8.

patterns. We used the hierarchical clustering analysis to cluster the genes according to the expression values. Genes with similar expression patterns are clustered together in the heat map. In comparison with the CON group, lipopolysaccharide injection altered the RNA expression profile in the thymus of the chick. In addition to immune regulation, genistein has more diverse regulatory effects on thymus than lipopolysaccharide. Researchers have suggested that genistein can act as the ligand to activate PPAR receptors, and participate in the oxidative metabolism of fatty acids (Shen et al., 2006). Moreover, it can inhibit tyrosine protein kinase and topoisomerase, which alter the related signal pathways directly (Akiyama et al., 1987). Therefore, the similarity between the LPS and CON groups was higher than that between the GEN and CON groups. However, genistein treatment reversed the altered gene expressions induced by lipopolysaccharide treatment in some hierarchical clustering levels (Figure 6C). These genes might enrich in the common metabolic pathways and signaling pathways. As shown in Figure 6D, the samples between groups were scattered, while samples within groups were clustered. Twenty seven genes were commonly differently expressed in the 3 groups. Dietary genistein treatment reversed the expressions of 133 genes, which differently expressed between LPS and CON groups.

Gene Ontology, KEGG, and PPI Analysis of DEGs

To better understand the network that regulates apoptosis process and immune function after lipopolysaccharide and genistein treatment, we conducted GO Biological Process, Cellular Component and Molecular

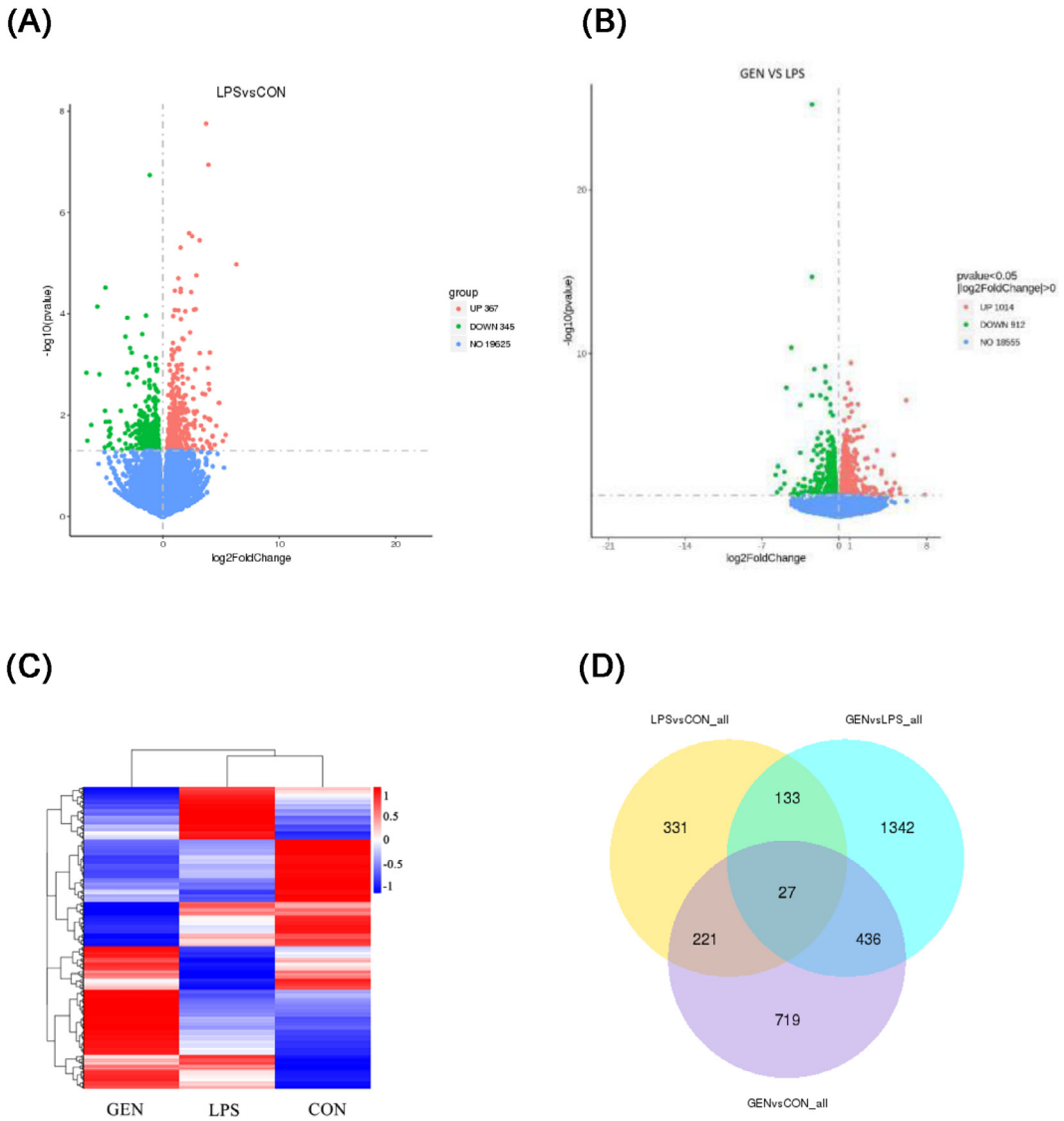

Figure 6. Cluster analysis of RNA-Seq data. (A) Scatter plot of DEGs (LPS vs. CON). (B) Scatter plot of DEGs (GEN vs. LPS). Red points represent upregulated genes with |Fold change| >1 and P_value < 0.05. Green points represent upregulated genes with |Fold change| <1 and P_value < 0.05. Blue points represent genes with no significant difference. (C) The hot map of hierarchical clustering analysis using genes expression values between the CON, LPS and GEN groups. Red color indicates high gene expression; blue color indicates low gene expression. (D) Venn diagram of differentially expressed genes among the CON, LPS and GEN groups. Abbreviations: CON, nonchallenge control; LPS, lipopolysaccharide-challenged group; GEN, lipopolysaccharide-challenged group fed diet supplemented with 40 mg/kg genistein.

Function categories using upregulated and downregulated genes between the LPS vs. CON groups (Supplementary data 5) and GEN vs. LPS groups (Supplementary data 6), respectively. RNA−Seq analysis suggested that the upregulated genes between the LPS vs. CON groups were enriched into cell adhesion, immune response, immune system process, response to stimulus, G-protein coupled receptor signaling pathway, Ras protein signaling pathway, scavenger receptor activity, and cytokine receptor binding (Figure 7A). Furthermore, KEGG pathways analysis suggested that lipopolysaccharide injection activated cytokine-cytokine receptor interaction, along with upregulated expressions of genes (*IL1R2, IL8, CXCL12, LIFR, IL1R1, TNFSF8, CSF1R, PRLR, IL-18, IFNG,* and *CCR2,* Figure 7E). In detail, the above DEGs and *TLR2-1, NFKBIE* were enriched into Salmonella infection, NOD-like receptor signaling pathway, Influenza A, and Toll-like receptor signaling pathway (Supplementary data 5). It is well known, IL-1 can activate CD4+ T lymphocytes, and induce neutrophils to release inflammatory factors. IL-1 and IFN-γ can enhance the inflammatory function of TNFα. IL8 can promote the inflammatory cells migrating to the area of inflammation, and induce Th1 cells to produce IL-2, IL-12, and IFN-γ (Del Prete et al., 1994). Stromal cell derived factor 21 (***CXCL12***), a type of chemokines, is also known as proinflammatory cytokines (Balabanian et al., 2005). Previous research indicated that lipopolysaccharide could activate transcriptional expressions of active protein 21 (*AP-1*), signal translator and activator of transcription 1 (***STAT1***) and NF-κB (Ansari et al., 2017). In the present study, the above DEGs and *NFKBIE* were enriched into the Salmonella infection, NOD-like receptor signaling pathway, and

Toll-like receptor signaling pathway, which was consistent with the previous study (Geddes et al., 2010). In addition, lipopolysaccharide injection affected P53 signaling pathway, and downregulated expression of *GTSE1, CDK1 and CCNB2* genes. These changed genes enhanced apoptosis process, and cell cycle arrest. Interestingly, the genes enriched into cytokine-cytokine receptor interaction, including *FGFR1, SNAI1, SSX2IP, BAIAP2*, and *TCF7L2*, also affected the cell growth and differentiation.

Previous research revealed that dietary genistein (20, 40 mg/kg) supplementation could improve the immune function of broilers, along with the increase the antibody titer of Newcastle disease and infectious bronchitis (Alipour et al., 2012). In the current study, the downregulated genes between the GEN and LPS groups were also enriched into immune response, immune system process, which indicated that dietary genistein could affect the immune response induced by lipopolysaccharide injection (Figure 7C). KEGG analysis indicated that the DEGs between the GEN and LPS groups were enriched into Toll-like receptor (**TLR**) signaling pathway, NOD-like receptor (**NLR**) signaling pathway. It is reported that *Salmonella enteritidis* infection can activate NOD1, then up-regulate NF-κB in a RIP2 (receptor- interacting protein 2) -dependent manner (Tao et al., 2015). Meanwhile, TLRs can activate NF-κB signaling pathways through myeloid differentiation factors, inducing immune response (Knight et al., 2008). Similarly, previous study indicated that genistein could inhibit the degradation of IκB-α through tyrosine kinase, thereby inhibiting the secretion of NF-κB and IL-17 (Knight et al., 2008). What we concerned about the immune regulatory effects of genistein is the downregulated genes, including *TLR4, CCL5, CCL4, FOS, AvBD2 HSP90, AB1, CATHL2,* and *NFKBIB,* all of which were involved in the inflammatory reaction. In detail, the downregulated genes (*GDF9, CXCL12, CCL5, CCR7, TNFSF8 TGFB3, CCL4, CD40LG, CSF3R, IL5RA, GDF10, CXCR5*) between the GEN and LPS groups were enriched into the cytokine-cytokine receptor interaction. Similarly, previous study revealed that genistein could inhibit the inflammatory response and downregulate the expressions of inflammatory factors (Sung et al., 2014). Therefore, dietary geinistein supplementation can inhibit the inflammatory reaction in the thymus of chicks challenged with lipopolysaccharide via TLR and NLR signaling pathway.

Cytoscape bioinformatics analysis of potential protein interactions for all DEGs was performed using the software OmicsBean. The DEGs between the LPS and CON group were significantly related to the categories calcium signaling pathway, neuroactive ligand-receptor interaction, Salmonella infection, histidine metabolism, PPAR signaling pathway, primary bile acid biosynthesis, as well as phenylalanine, tyrosine, and tryptophan biosynthesis (Figure 7G) DEGs between the GEN and LPS group were clustered in the categories herpes simplex virus 1 infection, necroptosis, cell cycle, apoptosis, p53 signaling pathway, fanconi anemia pathway, phosphatidylinositol signaling system, and N-Glycan biosynthesis (Figure 7H).

TUNEL Assay

Abnormal cell death can disrupt the development of thymus cells. In particular, the proportion of apoptotic thymocyte in the mice increased significantly at 7 d after *STm* infection, along with the highest level of CD4 +CD8+ thymocytes (Ross et al., 2012). The TUNEL testing can indicate the cell death status through detecting DNA damage (Frankfurt et al., 1997). After stained with TUNEL assay, TUNEL-positive cells with the nuclei of red color represented the apoptotic cells. These apoptotic cells were mainly distributed at the cortex region of the thymus (Figure 4A). Chicks in the LPS group exhibited greater apoptotic percentages than the CON group ($P < 0.05$, Figure 4B). In comparison with the LPS group, the GEN group showed a lower apoptotic index of thymus and reduced 12.34% ($P < 0.05$, Figure 4B. GO clustering analysis indicated that the downregulated genes between the LPS vs. CON groups were enriched into molecular functions of DNA packing and DNA conformation. Previous study has suggested that lipopolysaccharide can activate TLR4 signaling pathway, and induce inflammatory cell infiltration, DNA damage and cell cycle arrest (Vadiveloo et al., 1996). Therefore, lipopolysaccharide might promote thymocyte apoptosis by inducing DNA damage. Furthermore, DNA damage can activate cell cycle checkpoint regulator, and inhibit the activity of cyclin (Zhou and Elledge, 2000). RNA-seq analysis indicated that the expressions of cyclin-dependent kinase (**CDK1**), Cyclin (**CCNB2**), G2 and S phase-expressed-1 (**GTSE1**) was significantly downregulated after lipopolysaccharide challenge. GTSE1 silencing was found to inhibit AKT phosphorylation and downregulated cell cycle-related protein (Guo et al., 2016). These changed genes enhanced the apoptosis process, and cell cycle arrest after lipopolysaccharide challenge.

Interestingly, the upregulated genes between the GEN and LPS groups were enriched into DNA repair, chromosome organization, which indicated that dietary genistein can alter the DNA packing adversely influenced by lipopolysaccharide challenge (Figure 5C). X-chromosome-linked inhibitor of apoptosis protein (**XIAP**) prevents TNF-mediated, receptor-interacting protein 3 (**RIPK3**)-dependent cell death, by controlling RIPK1 ubiquitylation and preventing inflammatory cell death (Jiao et al., 2020). In the current study, genistein treatment upregulated the gene expressions of XIAP and PIK3R1, and downregulated the gene expressions of IL-3R and CAPN11, which weakened the process of apoptosis. Similarly, previous research genistein partially inhibited amyloid-beta induced cell death, primarily apoptosis (Petry et al., 2020). Therefore, dietary genistein supplementation can inhibit the apoptosis in the chick thymus with lipopolysaccharide challenge (Table 1, $P < 0.05$).

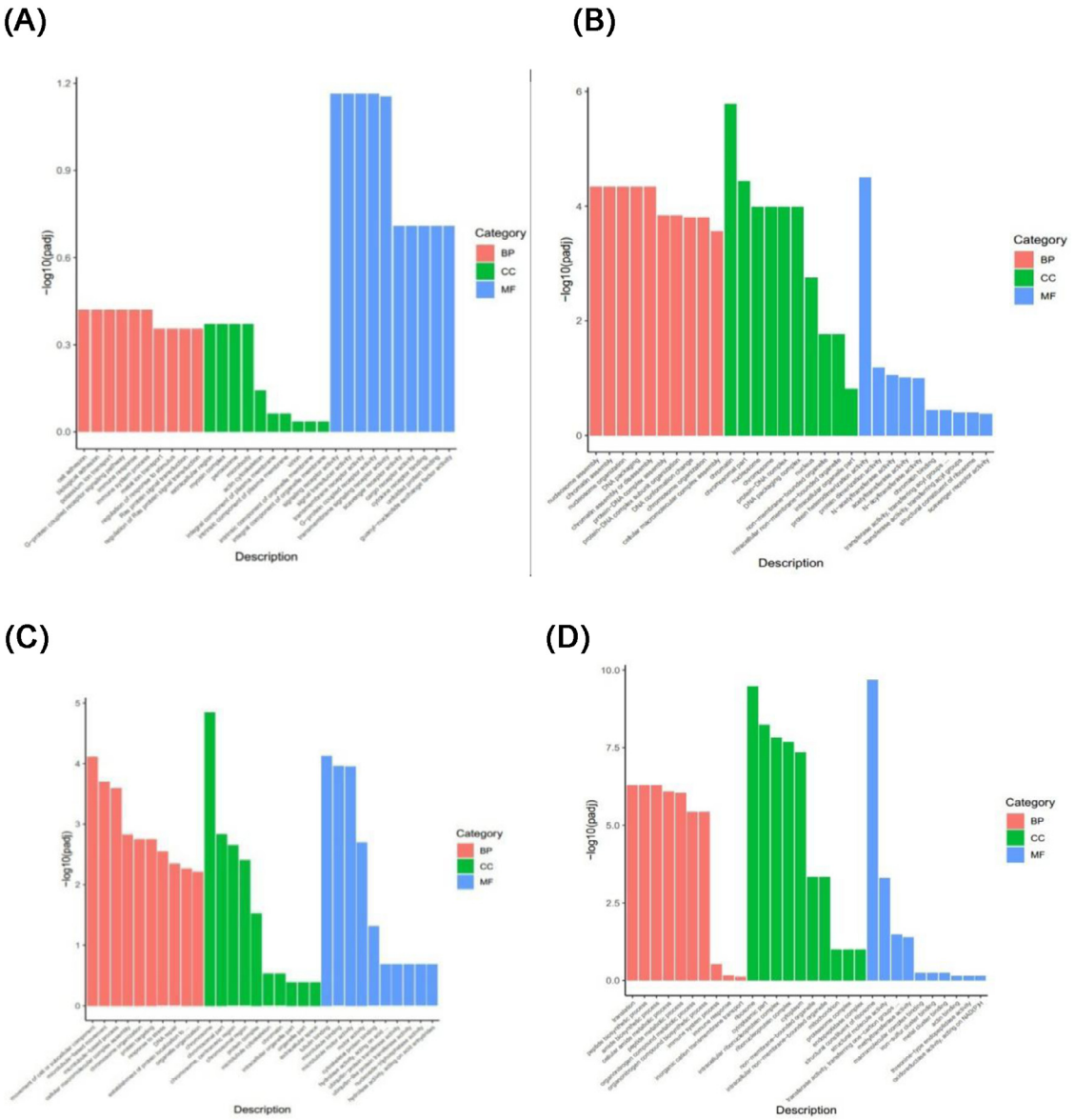

Figure 7. Bioinformatics analysis of RNA-Seq data. (A and B) The top 10-enriched items in each main category (biologic process, cell component, and molecular function) of the GO database at all levels using upregulated and downregulated DEGs between the LPS vs. CON groups. (C and D) The top 10-enriched items in each main category (biologic process, cell component, and molecular function) of the GO database at all levels using upregulated and downregulated DEGs between the GEN vs. LPS groups. (E and F) The top enriched KEGG items using DEGs of the LPS vs. CON groups and the GEN vs. LPS groups, respectively. (G and H) Protein−protein interaction analysis using DEGs of the LPS vs. CON groups and the GEN vs. LPS groups, respectively. Circular nodes represent genes/proteins; rectangles represent KEGG pathways or GO Biologic Process terms. The pathways are colored with a gradient from yellow to blue, in which yellow indicates a smaller *P* value, and blue indicates a larger *P* value. GO biologic processes are colored red. In the fold-change analysis, genes/proteins are colored red for upregulation or green for downregulation. Abbreviations: CON, nonchallenge control; LPS, lipopolysaccharide-challenged group; GEN, lipopolysaccharide-challenged group fed diet supplemented with 40 mg/kg genistein.

Identification of AS and Functional Annotation

AS of pre-mRNA plays an important role in regulating gene expression in eukaryotes. About 40 to 60% of human genes have AS isoforms, although some variants exist only in relatively low abundance (Wang et al., 2006). AS events run through all links in the lipopolysaccharide/TLR4 signaling pathway (Lee et al., 2020). New targets for negative regulation of inflammatory response have been found in some studies. For example, soluble TLR4 reversed attenuating effect of Chinese herbal Xiao-Qing-Long Tang on allergen induced nerve growth factor and thymic stromal lymphopoietin (Chang et al., 2013). Therefore, we used the genes with AS events to do functional clustering. We detected a total 23,712 AS in the chicks of both the LPS and CON groups. The analysis detected 5 different splice patterns of the LPS and CON groups in the chicken thymus transcriptome data, namely skipped exon (**SE**), alternative 5′splicing site (**A5SS**), alternative 3′splicing site (**A3SS**), retained intron (**RI**), and mutually exclusive exon and complex (**MEX**). Two of these AS events (SE and MEX) between the LPS and CON groups were the major

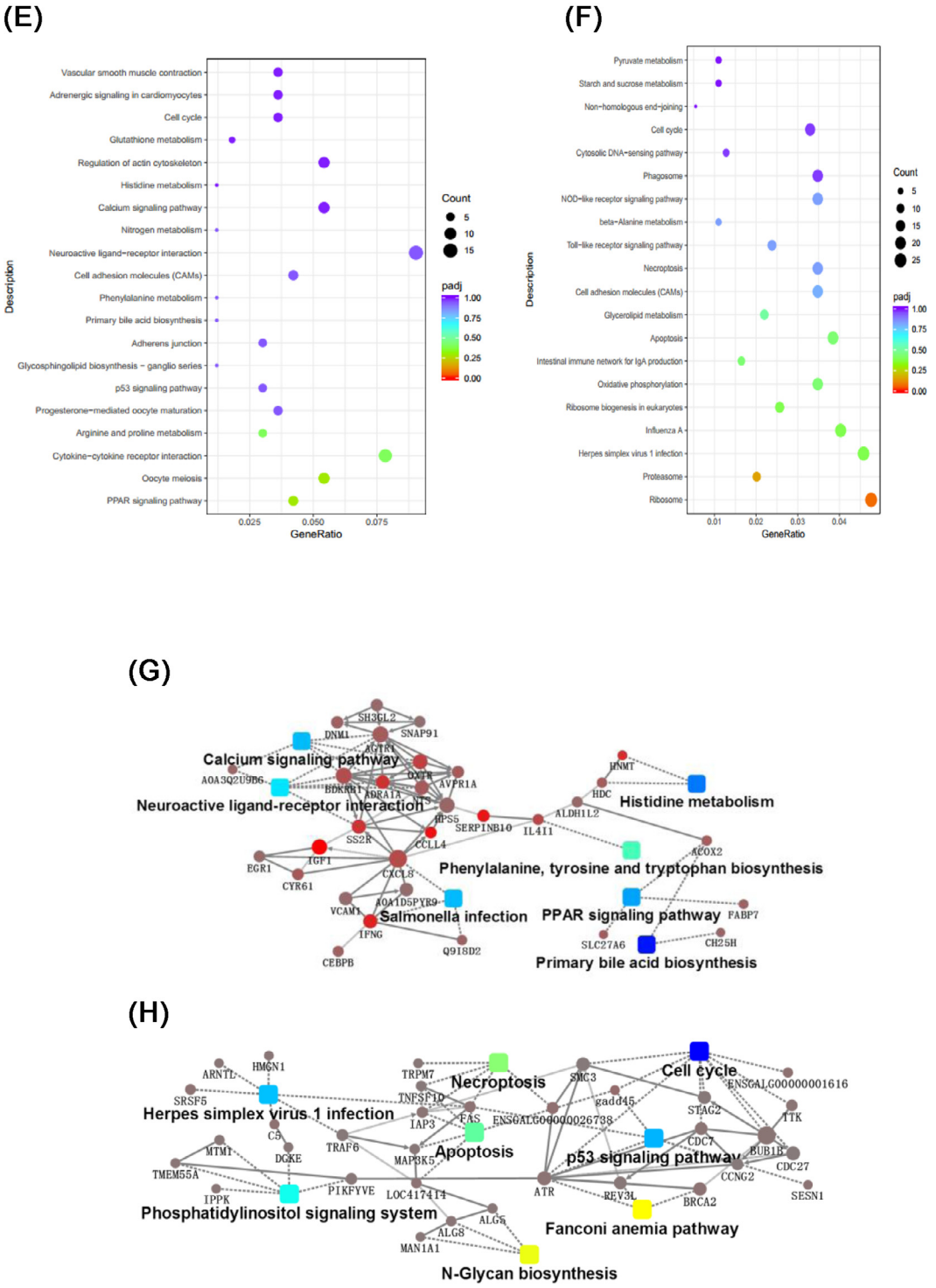

Figure 7 Continued.

patterns, representing 95.1% of the total AS events. A5SS, A3SS and RI retained intron accounted for only 4.9% of the total AS events (Supplementary data 7). The chromosomal position of each transcript was obtained by aligning the sequence to the chicken reference genome. A total of 1,210 differently expressed isoforms (611 downregulated and 599 upregulated; $P\_value < 0.05$) were detected in the LPS group compared with the CON group, 940 (77.7%) of which were annotated. The average number of alternative transcripts per chromosome was 697, and chromosomes 16 (95 transcripts) and 31 (47 transcripts) had the smallest numbers of alternative transcripts. There were 24,471 AS events in both GEN and LPS groups. A total of 1,088 DE isoforms (576 downregulated and 512 upregulated; $P\_value < 0.05$) were detected in the LPS group compared with CON group, 843 (77.4%) of which were annotated (Supplementary data 8).

Three of these AS events (SE, RI, and MEX) between the GEN and LPS groups, were the major patterns, representing 97.9% of the total AS events. A5SS and A3SS accounted for only 2.1% of the total AS events. The

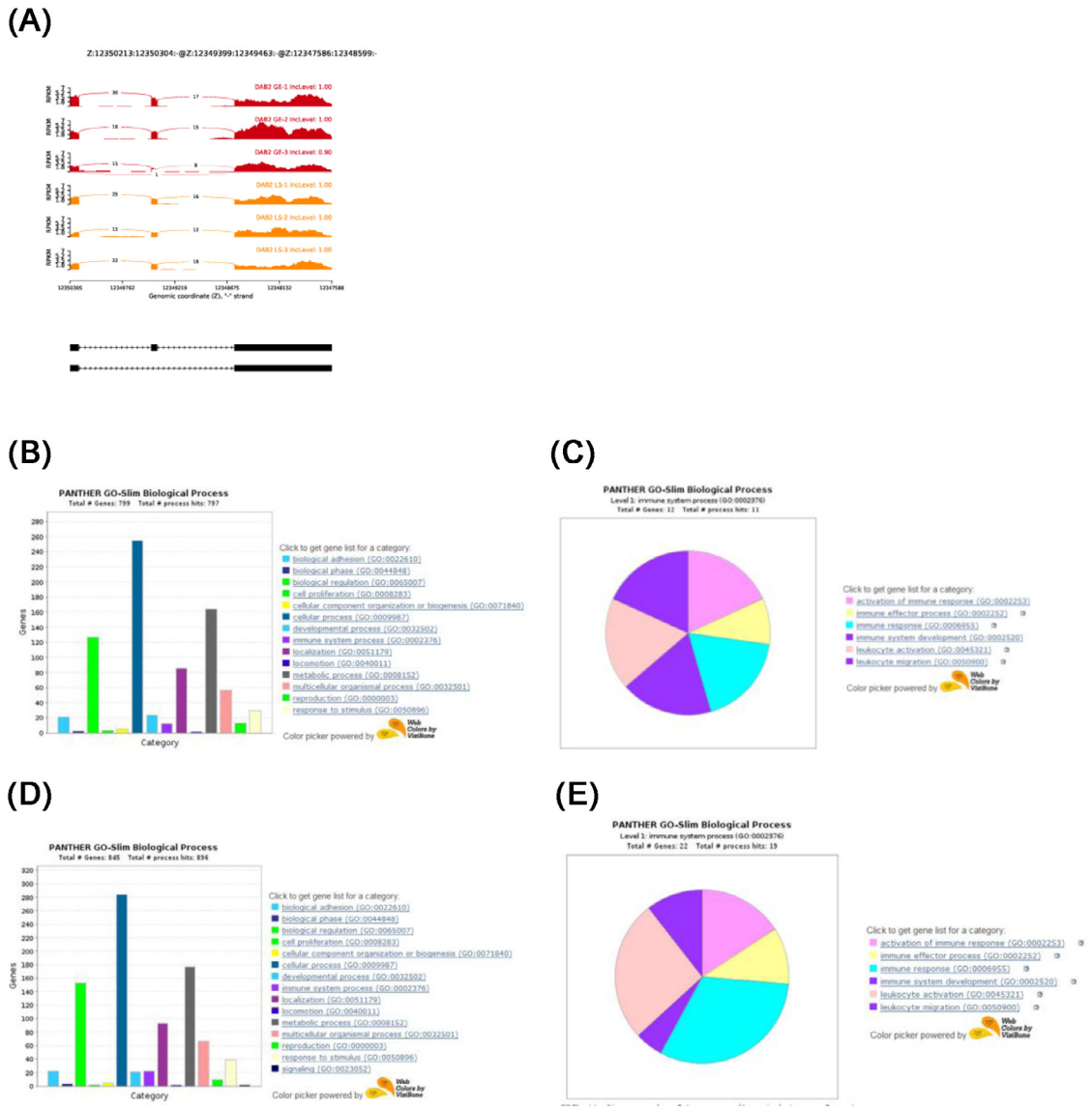

Figure 8. The enriched PANTHER GO-Slim using genes with AS. (A) PANTHER GO-Slim biological processes using genes with AS between LPS and CON groups at all levels. (B) PANTHER GO-Slim biological processes using genes with AS between LPS and CON groups at level 1: immune system process. (C) PANTHER GO-Slim biological processes using genes with AS between GEN and LPS groups. (D) PANTHER GO-Slim molecular functions using genes with AS between HGE and CON groups at level 1: immune system process. (E) The visual demonstration of DAB2 in the style of skipped exon. The chromosome coordinates and positive and negative chain information of the three exons with the alternative splicing event are titled. Reads for cross exon alignment are represented by arcs connecting the boundary of the exon junction. The thickness of the arc is directly proportional to the number of reads compared to the junction. Meanwhile, the number on the arc indicates the number of junction reads, and the gene and inclevel value of the alternative splicing event are marked on the top right.

average number of alternative transcripts per chromosome was 720, and chromosomes 16 (105 transcripts) and 31 (40 transcripts) had the smallest numbers of alternative transcripts. The 5 AS isoforms of the *DAB2* gene were detected in transcriptomic data, which were significantly upregulated in the GEN group compared with the LPS group (Figure 8A). Drosophila Disabled-2 (**Dab2**) homolog is a foxp3 target gene required for regulatory T cell function (Jain et al., 2009). It is involved in enhancing TGFβ (transforming growth factor-β) responses, which is exclusively expressed in FOXP3+ (forkhead box P3) regulatory T cells. Regulatory T cells lacking DAB2 are functionally impaired in vitro and in vivo (Jain et al., 2009; Ahmed et al., 2015). Accordingly, we found that genistein treatment significantly upregulated the target gene (*ROCK1*) of TGFβ responses. *ROCK1* mediates leukocyte recruitment and neointima formation following vascular injury (Noma et al., 2008). Although the induction mechanism of AS is still unknown, the thymic transcriptome reported here is conducive to finding new targets for positive regulation of inflammatory response after genistein treatment.

We used PANTHER online database to analyze the enriched biological processes using AS transcripts among the 3 groups. The 12 AS transcripts between the LPS and CON groups were enriched into immune system process (GO: 0002376, Figure 8B). It can be seen from Figure 8C, AS transcripts were involved in the activation of immune response (GO:0002253, *RBM14, TLR1B*); immune effector process (GO:0002252, *CD226*); immune response (GO:0006955, *CD226, BF2*); immune system development (GO:0002520, JAK3,

RUNX2); leukocyte activation (GO:0045321, *CD226, CD3E*); leukocyte migration (*VEGFA, LRCH1*). Meanwhile, the 14 AS transcripts (*KALRN, TLR21, CARMIL3, TNS1, OTUD4, CD226, DAB2, CD48, RUNX2, TLR7, CD3D, CD3E, DAB1,* and *BG1*) between the GEN and LPS groups were enriched into immune system process (GO: 0002376, Figure 8D). It can be seen from Figure 8E, DE transcripts were involved into the activation of immune response (GO:0002253, *TLR21, OTUD4, TLR7*), immune effector process (GO:0002252, *CD226, TLR7*), immune response (GO:0006955, *CD226, BTN1A1, BG1*), immune system development (GO:0002520, *RUNX2*), leukocyte activation (GO:0045321, *CD226, CD3D, CD3E*), leukocyte migration (GO:0050900, *VEGFA, LRCH1*). Although tissue specific differences should be considered when comparing chicken with mammalian systems, the current findings appear to be consistent with conservation of immune processes in chick livers after genistein treatment (Lv et al., 2019b). Therefore, dietary genistein exposure altered AS signatures in the thymus, and regulated the thymic immune reaction after lipopolysaccharide challenge.

CONCLUSIONS

Our research has evidenced a novel finding in chicks that dietary genistein could inhibit the increased percentage of CD3+ T lymphocytes, and CD4+/CD8+ T lymphocyte ratio in the peripheral blood induced by lipopolysaccharide injection. Furthermore, dietary genistein exposure altered the mRNA expression profile and AS signatures in the thymus, and regulated the immune response, along with the improvement of thymus index and apoptotic index after lipopolysaccharide challenge. It would help to understand the role of dietary genistein to the regulation of immune function and provide theoretical support for improving healthy production of poultry.

ACKNOWLEDGMENTS

The study was funded by National Key Research and Development Program of China（2021YFD1300004）

Author contributions: Z. Huang conducted the animal trial. S. Jin conducted the animal trial, performed the sample analyses and wrote the manuscript. Z. Lv contributed to the experimental design and preparation of the manuscript. All authors have read and approved the final manuscript.

DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary data 1: Supplementary Table 1. Diet composition and nutrient levels; Supplementary data 2: Primers used for quantitative real-time PCR analysis; Supplementary data 3: Quality control of RNA-Seq data; Supplementary data 4: mRNA expression abundance of genes in the CON, LPS, and GEN groups; Supplementary data 5:The enriched biological process, KEGG pathway and PPI using DEGs between the LPS and CON groups; Supplementary data 6: The enriched biological process, KEGG pathway and PPI using DEGs between the GEN and LPS groups; Supplementary data 7: Alternative splicing events between the LPS and CON groups; Supplementary data 8: Alternative splicing events between the GEN and LPS groups. RNA-Seq data have been deposited in a repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160489).

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