Beta-glucan enhanced immune response to Newcastle disease vaccine and changed mRNA expression of spleen in chickens

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ABSTRACT The present study was performed to investigate the effect of oral administration of β-glucan (**G70**), a product obtained from the cell wall of yeast, on Newcastle disease virus (**NDV**)-specific hemagglutination inhibition (**HI**) titers, lymphocyte proliferation, and the role of T lymphocyte subpopulations in chickens treated with live NDV vaccine. In addition, the influence of β-glucan on splenic gene expression was investigated by transcriptome sequencing. The results revealed that the supplementation of β-glucan boosted the titer of serum NDV HI increased the NDV stimulation index of lymphocytes in peripheral blood and intestinal tract, and promoted the differentiation of T lymphocytes into $CD4^+$ T cells. The RNA sequencing (**RNA-seq**) analysis demonstrated that G70 upregulated the mRNA expressions related to G-protein coupled receptor and MHC class I polypeptide, and downregulated the mRNA expressions related to cathelicidin and beta-defensin. The immunomodulatory effect of G70 might function through mitogen-activated protein kinase signaling pathway. To sum up, G70 could boost the immunological efficacy of live NDV vaccine in chickens and could be applied as a potential adjuvant candidate in the poultry industry.

Key words: Newcastle disease vaccine, beta-glucan, adjuvant, black-bone chicken, RNA-seq

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INTRODUCTION

Yeast cell wall polysaccharides are directly originated from the cell wall of *Saccharomyces cerevisiae* and are widely utilized as growth promoters, antimicrobial agents, or immunomodulators in poultry to improve production and health (Schiavone et al., 2017; Hasted et al., 2021). Previously, a product called PW220, which contains yeast cell wall polysaccharides, was demonstrated to boost the immune response provoked by Newcastle disease virus (**NDV**) and to alter the microbial community of the cecum of chickens by oral administration (Bi et al., 2020). Yeast cell wall polysaccharides are primarily made up of β-glucan and mannan-oligosaccharides (Wang et al., 2018). In the present study, β-glucan was investigated for its effect on the immune response to NDV vaccine in chickens.

The spleen is a pivotal organ for initiating the immune response. A recent study indicated that PW220, which is a yeast cell wall product, upregulated the mRNA expression of TGF-β, IL-6, TLR5, GATA-3, and T-bet in the spleen of chickens (Bi et al., 2022). However, the specific mechanism needs to be elucidated. RNA sequencing (**RNA-seq**) is one of the high throughput technologies that can be applied to assay quantitative gene expression and provide analysis of different expression profiles at the level of the entire transcriptome (Zhao et al., 2018). With the advantages of high sensitivity and low-cost, RNA-seq has been broadly employed in livestock and poultry study (Teixeira et al., 2019; Yuan et al., 2020). Thus, in this study, the effect of administrating β-glucan on NDV-specific hemagglutination inhibition (**HI**) titers, proliferation of lymphocytes and T-lymphocyte subpopulations in chickens orally vaccinated with NDV vaccine were assessed. Furthermore, transcriptome analysis was used to evaluate the effect of β-glucan on gene expression and underlying signal transduction pathways in spleen of chickens.

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MATERIALS AND METHODS

Animals

One-day-old commercial black-bone chickens (male) were acquired from Guizhou Yushun Poultry Co., Ltd. (Anshun, China). The chickens were divided into wire cages permitting free access to feed and water. In the first 7 d, the room temperature was maintained at 33°C to 35°Cand then gradually decreased 1°C for every 2 d until 27°C. All chickens were processed in accordance with the standards established by the Southwest University Animal Care and Use Committee (Ethics Certificate Number: IAC-2021-0057). All experimental birds were euthanized with CO_2 at the end of the study.

Vaccine

Live vaccine of NDV (Strain La Sota) were provided form Qingdao YEBIO Bioengineering Co., Ltd. (Qingdao, China).

Reagents

Yeast cell wall product (**YP**) G70 was obtained from yeast cell (*Saccharomyces cerevisiae*) wall, which contains β-glucan (≥70%) (AngelG70, QB/T4572, Angel Yeast, Yichang, China). Both antigen and positive control sera for measuring NDV-specific HI titers were purchased from Qingdao Regen Diagnostics Development Center (Qingdao, China). Anti-chicken CD3-APC (C2818-T9580), CD4-FITC (D0117-WA78E), and CD8-PE (L2413-TH49T) in rat were offered by Nanjing Zeweil Biological Technology Co., Ltd. (Nanjing, China).

Experimental Design

Forty-eight black-bone chickens at 5 days old were randomly assigned into 3 groups (Table 1) and were given either the basal diet (Table 2) or the basal diet with 0.1% G70 (β-glucan, 0.7 g/kg) supplementation for the duration of the present research. Group H (G70 + Vaccine) and C (Vaccine) were orally immunized with NDV vaccine on 14 and 28 d, respectively. Group S (Sailne) was treated with an equal volume of saline. Blood samples were harvested via wing venipuncture at the age of 5, 7, 14, 21, 28, 35, and 42 d to test the HI titer. Seven days after the booster vaccination, 8 chickens in each column were randomly picked and sacrificed by asphyxiation using CO_2. Lymphocytes were separated from both peripheral blood and jejunum in order to perform lymphocyte proliferation and flow cytometry analysis. In the case of jejunal sampling, the duodenal suspensory ligament was the starting point of the jejunum and 5 cm long section was collected from the beginning point of the jejunum. The spleen was rapidly frozen in liquid nitrogen and then stored at -80℃ for RT-qPCR and sequence profiling.

Table 1. Experimental design.

Group	N	G70	Vaccination
H	16	0.1%	+
C	16	0	+
S	16	0	-

Table 2. [1]Composition and nutrient content of experimental broiler basal diets.

	1−21 d	22−42 d
Corn	55.34	60.53
Bran	2.70	2.70
Vegetable oil	3.17	3.83
Fish meal	5.01	4.90
Soybean meal	19.00	12.00
Hemp cake	4.50	5.20
Cottonseed meal	3.20	4.10
Rapeseed meal	2.70	3.06
Lysine	0.10	0.10
Methionine	0.12	0.02
$CaHPO_4$	3.13	2.46
Stone meal(crude)	0.15	0.33
NaCl	0.27	0.26
Choline chloride	0.12	0.02
Premix	0.50	0.5
Total	100	100
[2]Nutrient content		
Crude protein	20.00	18.90
Methionine	0.48	0.38
Metabolic energy, MJ/kg	12.11	12.54
Lysine	1.09	0.94
Calcium	1.00	0.90
Total phosphorus	0.45	0.41
Methionine + cystine	0.83	0.68

[1]The premix provided the following per kg diet: Fe 80 mg (as ferrous sulfate), Zn 40 mg (as zinc sulfate), Cu 8 mg (as copper sulfate), Mn 60 mg (as manganese sulfate), I 0.35 mg (as calcium iodate), Se 0.15 mg (as sodium selenite), vitamin A 2,700 IU, vitamin D_3 400 IU, vitamin E 10 IU, vitamin K 0.5 mg, thiamin 1.8 mg, riboflavin 7.2 mg (halved in later stages), pantothenic acid 10 mg, niacin 27 mg, pyridoxine 3 mg, biotin 0.15 mg, folic acid 0.55 mg, vitamin B12 0.009 mg.

[2]Values were calculated from data provided by Feed Database in China (2013).

HI Study

The assay for NDV-specific HI titers in the serum was performed following the previous description (Ball et al., 2019). In brief, serum was diluted with phosphate buffered saline (**PBS**) from 1:2 to 1:1,024 in the V-bottom 96-well microtiter plate. Then, 25 μL of NDV dilution was added per well and incubated at 37°C for 30 min. After that, 25 μL of 1% rooster erythrocyte suspension was added to each well and incubated at 37°C for 30 min. All samples were tested twice and both positive and negative controls were included on each plate. HI titers were based on the greatest dilution that resulted in a complete inhibition of hemagglutination. The average HI titer and standard error (**SE**) were measured per group.

Isolation of Lymphocytes From Peripheral Blood

Lymphocytes were isolated and collected from peripheral blood lymphocytes using the Lymphocyte Separation Medium Kit (Tianjin Haoyang Biological

Manufacture Co. Ltd., Tianjin, China). Then, lymphocytes were stored in suspension with RPMI 1640 medium (Solarbio, Beijing, China) including 5% fetal calf serum and 25 mM HEPES (pH 7.0).

Isolation of Lymphocytes From Jejunum

The assignment process was carried out in accordance with the previous description, and with slight modifications (Yuan et al., 2020). In short, use 70 μm cell filter to split intestine into 4 mL of cold PBS. Following that, suspension of sample cells was centrifuged at 4,500 rpm for 12 min with removal of the supernatant. Then, resuspend the gut cells onto a full medium (Solarbio Co., Beijing, China) consisting of 5% fetal bovine serum (**FBS**) in RPMI 1640 (Sijiqing Co., Hangzhou, China). In the end, using the chicken lymphocyte isolation kit (Tianjin Haoyang Biological Manufacture Co. Ltd.) to separate lymphocytes. the intestine tissue was split into 4 mL of cold PBS using a 70 μm cell filter. Next, the suspension of sample cells was centrifuged at 4,500 rpm for 12 min and the supernatant was discarded. After that, the intestinal cells were resuspended into RPMI 1640 (Solarbio Co.) containing 5% FBS (Sijiqing Co.). Finally, lymphocytes were separated using the chicken lymphocyte isolation kit (Tianjin Haoyang Biological Manufacture Co. Ltd.).

Proliferation of Lymphocytes

Cells were seeded in 96-well plates (5×10^5 per well), stimulated by the antigen of inactivated NDV for 4 hemagglutination unit or equal volume of saline. Each sample was tested in 3 replicates. The cells and antigen were incubated for 44 h at 37°C in 5% CO_2 and then 50 μL methyl thiazolyl tetrazolium (**MTT**) (2 mg/mL) was added to each well and incubated for another 4 h. The samples were centrifuged at 1,200 × g for 8 min at indoor temperature. Afterward, the supernatant was cautiously removed and 100 μL of DMSO was added to each well. The plates were shaken for 8 min to fully solubilize the crystals. Finally, the average optical density (**OD**) was recorded at 570 nm. The stimulation index (**SI**) was obtained using the equation: the OD values of stimulated wells/ OD values of unstimulated wells (Cui et al., 2020).

Flow Cytometry Analysis of T-Lymphocyte Subpopulations

Lymphoid cell suspensions were isolated and washed twice with PBS. The cell concentration was modified to 10^6/mL and then kept on the ice. Each sample was stained with 2 μL of rat anti-chicken CD3-APC, CD4-FITC and rat anti-chicken CD8-PE under light-proof conditions for 30 min, followed by 2 washes with PBS. The cells were assayed through flow cytometry analysis on Flow cytometer (BD Bioscience, San Jose, CA).

RT-qPCR Analysis

The total RNA was derived from the spleen using TRIzol reagent (Takara, Shiga, Japan) according to the manufacturer's guidelines and then converted into cDNA using PrimeScript RT Master Mix (Takara, Dalian, China) on a T100 thermal cycler (Bio-Rad, Hercules, CA). The chicken beta-actin was employed as an internal control gene. RT-qPCR of selected genes was carried out on a Multiple Real-Time PCR System (AB, Carlsbad, CA) with the SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara). The relative quantitative method ($2^{-\triangle\triangle CT}$) was performed to assess the quantitative variation (Bi et al., 2022). The primer sequences were listed in Table 3.

RNA Extraction

Each of the 3 spleen samples from the Group H (G70 +Vaccine) and group C (Vaccine) was used for the RNA-seq with TRIzol reagent (Takara). After that, the RNA was tested for contamination and degradation with 1% agarose gel and the RNA purity was analyzed using a NanoPhotometer spectrophotometer (IMPLEN, Westlake Village, CA). The concentration of RNA was determined employing the Qubit RNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA). The RNA integrity was measured using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). The results demonstrated that the RNA was complete and without DNA contamination.

Transcriptome Analysis

Transcriptome sequencing, sequences assembly, and data analysis were provided by Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). The main procedures for transcriptome were listed as follows: 1) Purification of mRNA from total RNA was performed using poly-T oligo-attached magnetic beads. Fragmentation was carried out with divalent cations in NEBNext First Strand Synthesis Reaction Buffer (5X) at a high temperature. 2) First-strand cDNA was synthesized using random hexamer primer and Moloney Murine Leukemia Virus (M-MuLV) Reverse. Second-strand cDNA synthesis was then synthesized using DNA Polymerase I and RNase H. 3) Remaining overhangs were transformed into blunt ends through the exonuclease/polymerase activities. A hairpin loop structure NEB Next Adaptor was then ligated to prepare for hybridization after adenylation of the 3' end of the DNA fragments. 4) About 250 to 300 bp cDNA fragments were obtained and the library were purified using the Beckman Coulter's AMPure XP system (Beckman Coulter, Beverly, MA). Then 3 μL NEBUSER Enzyme (Thermo Fisher, Hillsboro, OR) was applied with size-selected, adaptor-ligated cDNA at 37°C for 15 min, and followed by 5 min at 95°C. PCR

Table 3. Sequences of primers for RT-qPCR.

Gene	Primer sequence (5'-3')	Product size (bp)
β-actin	5′-GAGAAATTGTGCGTGACATCA-3′ 5′-CCTGAACCTCTCATTGCCA-3′	134
TGF-β	5′-TTCCAACACCAGGTCCTACTCCAG-3′ 5′-AAGCAGACAGGTCCAGCAATAACAG-3′	88
IL-6	5′-GAAATCCCTCCTCGCCAATCTGAAG-3′ 5′-GCCCTCACGGTCTTCTCCATAAAC-3′	108
TLR-3	5′-GCAAGCTATTGAGCAAAGTCGAGAC-3′ 5′-GCCAGTTCAAGATGCAGCAAGATC-3′	128
TLR-4	5′-CATCCCAACCCAACCACAGTAGC-3′ 5′-CCACTGAGCAGCACCAATGAGTAG-3′	119
TLR-5	5′-ACTCCCTTCCTTCCCACATCTGAC-3′ 5′-TGTGTTGCTACTATTGCCGTGTGAG-3′	87
IFN-γ	5′-ACGACACCATCCTGGACACC-3′ 5′-TTTGGCGTTGGCTGTCGTTC-3′	129
RGS16	5′-TTCCCGCTTCTACCTCTGAGTCTG-3′ 5′-GGACCTCTGTATGCCGTTCACTTC-3′	138
PAX-3	5′-AGATGGAGGAAGCAGGCAGGAG-3′ 5′-ATGGAGGTGGGCTGATAGGATGG-3′	134
CATH-3	5′-GCTCCCTGCACAACCTCAACTTC-3′ 5′-AGCCCGTCCTCCTTGAACTCG-3′	97
AvBD-7	5′-TGTTGCAGGTCAGCCCTTCATTC-3′ 5′-CCTTCGACAGATCCCTGGAAAGC-3′	85
LYG2	5′-GCTGGGGTGACCGTGGAAATG-3′ 5′-TCTCGTGCCCTGCCTGATATGG-3′	107
AvBD-6	5′-CTGTCTGTCCTCTTTGTGGTGCTC-3′ 5′-GTCCACTGCCACATGATCCAACC-3′	148
P38	5′-GCATCCATCTTCGTCGTCAT-3′ 5′-TCATCTACAGCAACCCAGAGG-3′	121
JNK	5′-ATTACGCCTTCTGCCTTGTG-3′ 5′-AAAGCGCTGCATAAATGCTT-3′	111
ERK	5′-TGGTACAGGGCTCCTGAAAT-3′ 5′-GGAAAGATGGGTCTGTTGGA-3′	113
DUSP5	5′-GGAGAGGAGCGATGTGGAGAGG-3′ 5′-GGAAGCATGATAGGCACTACCAAGG-3′	136
DUSP4	5′-AACCAAGACCCTGAGCAGCATTTC-3′ 5′-TGCCAAGGTAGAGGAAGGGAAGG-3′	131
FAS	5′-CTCTTCCACCTGCTCCTCATCATTG-3′ 5′-TCCCCTCTCCACAGGTAATTTCTCG-3′	145
CACNA2	5′-AGGACCTCATAACATTGGCACGAAC-3′ 5′-CTGACGAGGATTGTTGGCTTCTACG-3′	107

amplification was carried out with Phusion High-Fidelity DNA polymerase. In the end, PCR products were purified (AMPure XP system) and library quality was estimated using the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA). Then, the sequencing was performed on an Illumina Novaseq platform and 150 bp paired-end reads were generated. Reference genome and gene model annotation files were downloaded from genome website (ftp://ftp.ensembl.org/pub/release-98/fasta/gallus_gallus/ and ftp://ftp.ensembl.org/pub/re-lease-98/gtf/gallus_gallus/). An index of the reference genome was established using HISAT2 (v2.0.5) and paired-end clean reads were aligned to the reference genome. The number of reads mapped to each gene and the Fragments Per Kilobase per Million (**FPKM**) for each gene based on the length of the gene and the number of reads mapped to the gene were calculated using Feature Counts v1.5.0-p3. Differential expression between group H (G70 + Vaccine) and group C (Vaccine) was derived using DESeq2 R package (1.16.1). Genes with $P < 0.05$ and |log2 (fold change)| > 1 were defined as differential expression genes (**DEGs**).

Real-Time Quantitative PCR Validation

Two up-regulated DEGs and 4 down-regulated DEGs in the comparison of group H (G70 + Vaccine) vs. Group C (Vaccine) were chosen to confirm the transcriptome sequencing results by RT-qPCR. The RNA was converted to cDNA using PrimeScript RT Master Mix (Takara) on the T100 thermal cycler (Bio-Rad). The primer sequences were listed in Table 3. Chicken β-actin was used as the internal control gene. RT-qPCR of selected genes was carried out on a Multiple Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with the SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara). A relative quantitative method ($2^{-\triangle\triangle CT}$) was applied to assess the variation in quantification. All samples were performed in triplicate for analysis.

Statistical Analysis

One-way ANOVA with Duncan post hoc test was used for multiple comparisons between groups using SPSS software (version 21.0, SPSS Inc., Chicago, IL). Data are expressed as the mean ± SE. A value of $P < 0.05$ was regarded statistically significant.

RESULTS

Effect of β-Glucan on Serum Antibody Titers

As shown in Figure 1, NDV-specific HI titers decreased in all groups prior to vaccination and increased in H (G70+Vaccine) and C (Vaccine) groups after immunization. Interestingly, higher HI titers were observed in group H (G70+Vaccine) at 21 ($P > 0.05$), 28 ($P < 0.05$), 35 ($P > 0.05$), and 42 ($P > 0.05$) d of age than that in group C (Vaccine).

Effect of β-Glucan on Lymphocyte Proliferation

The effect of G70 on the proliferation of peripheral blood lymphocytes was depicted in Figure 2A. SI in blood lymphocytes was enhanced in birds supplemented with G70 (G70 + Vaccine) at 7 d ($P < 0.05$) post the booster immunization, when compared with the Vaccine group. Furthermore, SI in jejunal lymphocytes was significantly increased at 7 d ($P < 0.05$) post booster immunization when compared with the Vaccine group (Figure 2B).

Effect of β-Glucan on the Ratio of CD4 + / CD8 + T Cell in Peripheral Blood

Figures 3A and 3B indicated the gating of lymphocytes and CD3 + cells and Figures 3C to 3E showed the ratio of CD4 + /CD8 + T cell in the G70, vaccine and

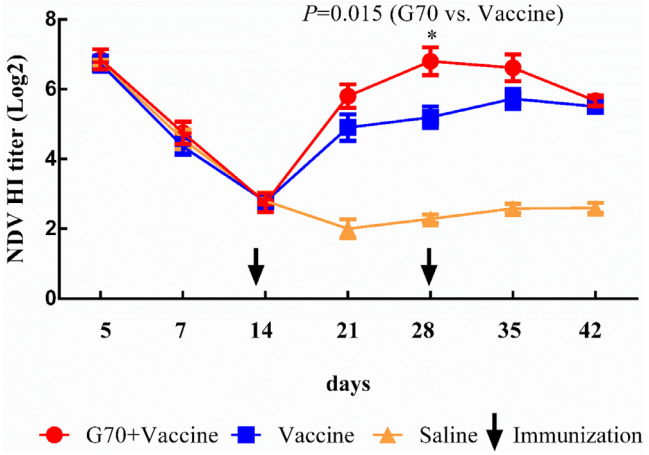

Figure 1. Effect of oral administration of β-glucan on serum antibody titers to NDV vaccine. Data are expressed as mean ± SE.

saline groups, respectively. As shown in Figure 3F, there was no remarkable difference between the Vaccine group and the Saline group following booster immunization ($P > 0.05$), whereas the proportion of CD4 + /CD8 + T cell was obviously higher in G70 (G70 + Vaccine) group than that in Vaccine group ($P < 0.05$).

Related Gene Expression

As illustrated in Figure 4, significantly increased mRNA expression of TGF-β ($P < 0.05$), IL-6 ($P < 0.05$), and TLR5 ($P < 0.05$) was detected in spleen of chickens treated with G70 (G70 + Vaccine), as compared with the Vaccine group. No significant difference was found in the mRNA expression of IFN-γ ($P > 0.05$), TLR4 ($P > 0.05$), and TLR3 ($P > 0.05$) in spleen between the G70 (G70 + Vaccine) and Vaccine groups.

RNA Sequencing Data Analysis

Sequencing of RNA from 8 spleen libraries yielded 24.1 G of raw data as presented in Table S1 of the supplementary material. C_N means group Vaccine, H_N means group G70 + Vaccine. Library C_1, C_2, C_3, C_4, H_1, H_2, H_3, and H_4 respectively, consist of 45,591,492, 47,424,782, 46,193,492, 54,403,376, 47,118,476, 45,899,262, 45,841,884, and 45,504,438 original reads. Following aptamer, ambiguous sequences and low-quality sequences were removed, leaving 44,050,198, 45,449,884, 44,261,112, 52,097,846, 45,542,404, 44,566,290, 44,113,172, and 44,026,960 clean reads. More than 96% of the clean reads were among the original reads, and 8 libraries were matched using HISAT2 for sequencing reads against a reference database consisting of the Gallus genome. Further, the clean reads were mapped to this database in more than 95% of cases. Specific to the libraries from the C_1, C_2, C_3, C_4, H_1, H_2, H_3, and H_4 were 40,921,384 (92.9%), 41,302,100 (90.87%), 40,771,075 (92.11%), 46,905,784 (90.03%), 42,444,112 (93.2%), 40,213,444 (90.23%), 40,922,895 (92.77%), and 40,971,972 (93.06%) reads were assigned uniquely to the reference database, respectively.

Differentially Expressed Genes

As indicated in Figure 5A, a total of 198 differentially expressed genes, including 47 up-regulated genes and 151 down-regulated genes were identified. The DEGs were described in detail in supplementary material Table S2. Figure 5B indicated that DEGs have good reproducibility of treatments.

Analysis of Gene Ontology Classification and Kyoto Encyclopedia of Genes and Genomes Enrichment

Genes that are differentially expressed genes were classified into 3 main functional categories based on the Gene Ontology (**GO**) classification system: biological process, cellular component, and molecular function. The top 10 GO terms in the 3 categories were presented in Figure 6. There were a predominance of genes

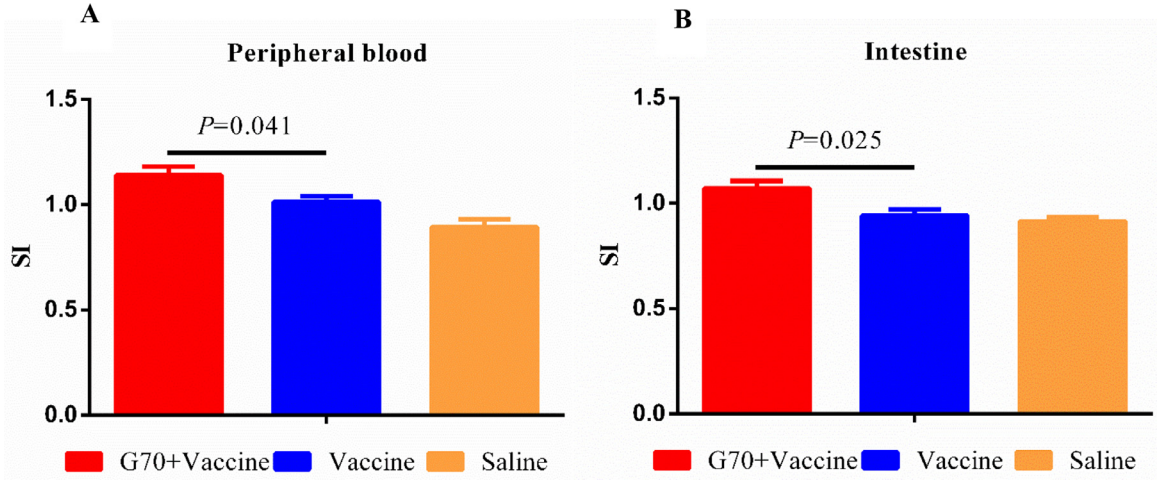

Figure 2. Effect of oral administration of β-glucan on lymphocyte stimulating index (SI). (A) Peripheral blood lymphocytes; (B) intestine lymphocytes. Data are expressed as mean ± SE.

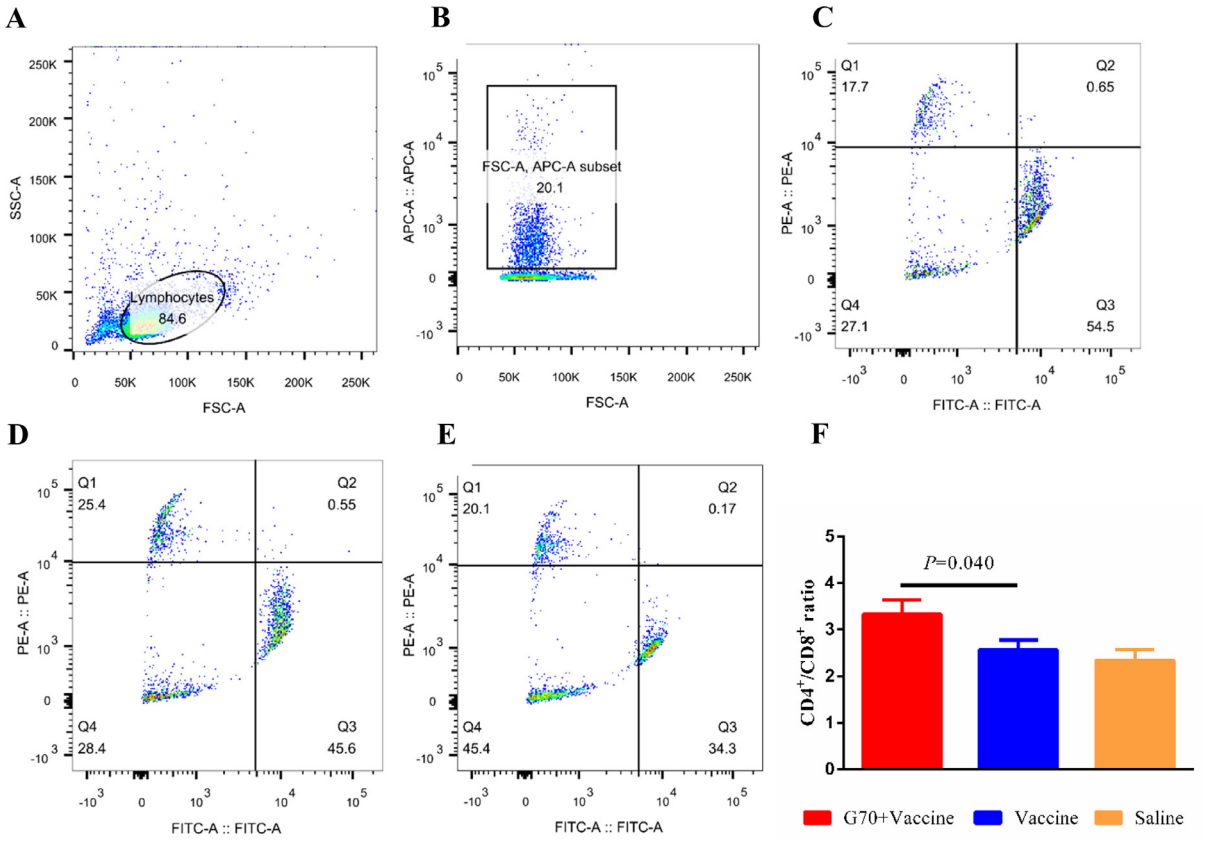

Figure 3. Effect of oral administration of β-glucan on $CD4^+$/ $CD8^+$ cell ratio of peripheral blood. (A) Gate on lymphocytes; (B) gate on CD3+ T cells; (C) fre- quencies of CD3+ CD4 + CD8 + T cells in G70+Vaccine group; (D) fre- quencies of CD3+ CD4 + CD8 + T cells in Vaccine group; (E) fre- quencies of CD3+ CD4 + CD8 + T cells in Saline group; (F) bardiagram representing $CD4^+/CD8^+$ ratio. Data are expressed as mean ± SE.

involved in "response to humoral immune," "chemokine response," "response to antimicrobial humoral," "defense response against bacterium," "immune response to antimicrobial humors mediated by antimicrobial peptides," "defense response against Gram-negative bacterium," and "defense response against Gram-positive bacterium" in the category of biological processes. In addition, in the category of molecular function, a remarkable ratio of the genes were relate to "C-C motif chemokine receptor (**CCR**) chemokine receptor binding," "chemokine receptor binding," and "lipopolysaccharide binding." Furthermore, "mitochondrial respiratory chain complex I," "NADH dehydrogenase complex," and "respiratory chain" were the most dominant enriched terms in the cell components category.

Kyoto Encyclopedia of Genes and Genomes pathway analysis was also performed on the differentially expressed genes. The results suggested that genes were mainly grouped into 7 pathways including "Neuroactive ligand-receptor interaction," "Gap junction," "mitogen-activated protein kinase (**MAPK**) signaling pathway," "ABC transporters," "Biosynthesis of amino acids," "Drug metabolism," and "Protein export."

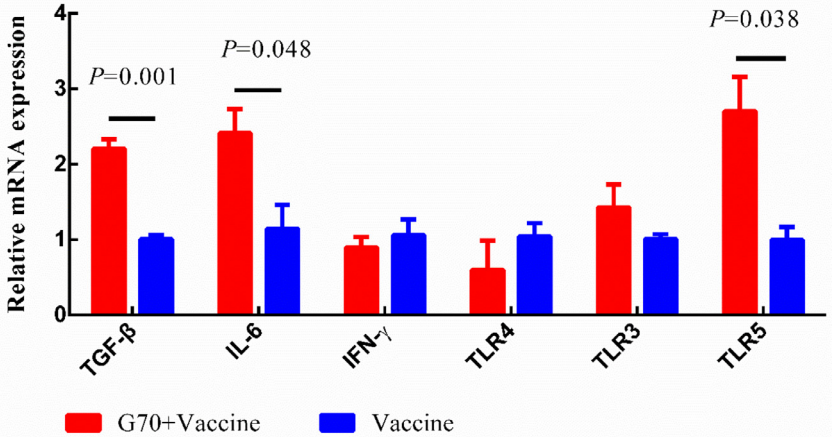

Figure 4. Effect of oral administration of β-glucan on mRNA expression in chicken spleen. Data are expressed as mean ± SE.

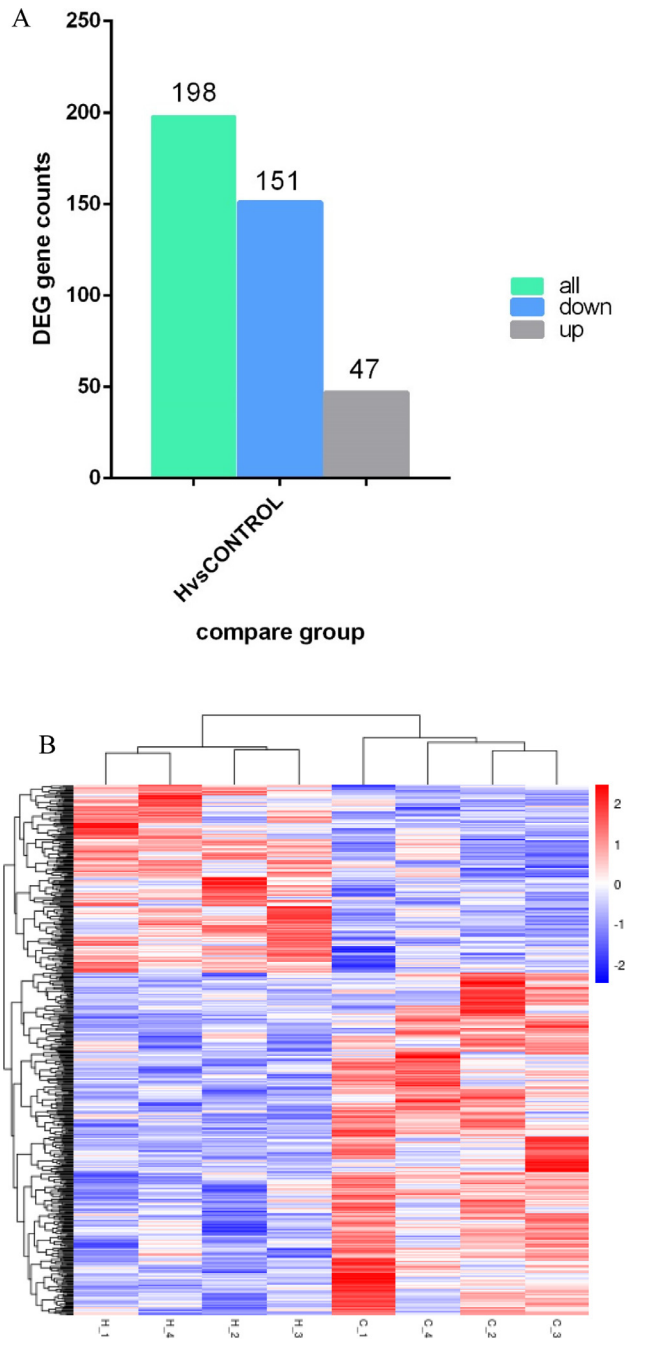

Figure 5. Summary of RNA-Seq data. (A) List of differentially expressed genes, (B) clustering map of the DEGs.

Verification of Genes Expressed Differentially by RT-qPCR

The 6 DEGs that were up- or down-regulated were confirmed by real-time quantitative PCR. The result suggested that the RT-qPCR data were generally in accordance with the RNA-seq data in general, indicating a reliable sequencing result (Figure 7).

Expression of mRNA of MAPK Pathway-Associated Genes

As shown in Figure 8, significantly decreased mRNA expression of *FAS* ($P < 0.05$) and *CACNA2* ($P < 0.05$) were detected in the spleen of chickens in G70 group (G70 + Vaccine), when compared with the Vaccine group. In addition, numerically increased mRNA expression of *DUSP5* and *DUSP4*, and numerically decreased mRNA expression of *p38, JNK*, and *ERK* were detected in G70 group (G70 + Vaccine), when compared with the Vaccine group ($P > 0.05$).

DISCUSSION

Live ND vaccine is widely inoculated in chicken farms and yet there are still sporadic outbreaks of Newcastle disease in immunized poultry flocks (Zhang et al., 2022). The optimal vaccines are defined to stimulate cellular and mucosal immunity as well as effective humoral immunity (Shan et al., 2019). Therefore, more and more attention was taken on adjuvants which could confer mucosal as well as cellular immune responses (Chen et al., 2014; Yu et al., 2015). Compared with the injection route, oral administration is an easier approach that reduces costs and induces fewer stress for the broiler chickens (Boyaka et al., 2003; Zhang et al., 2008). The results of this study showed that diet supplemented with β-glucan remarkably increased the level of NDV-specific HI titers in serum of chickens, which is consistent with previous reports (Horst et al., 2019). NDV-specific antibody is essential for the development of humoral immune response to protect chickens from NDV infection (Martinez et al., 2018; Li et al., 2020).

B lymphocytes produce antibody and T lymphocytes expand when in response to mitogen (Bohacova et al., 2021). In the present study, the stimulation index of chicken peripheral blood lymphocytes to NDV of the G70 group was obviously higher than that of the vaccine group, indicating that more T lymphocytes were activated. The predominant subpopulation of T lymphocytes are the CD4 + and CD8 + T cells (Cui et al., 2020). CD4 + T cells mainly produce cytokines and promote B cell maturation, whereas CD8 + T cells are associated with killing the target cells (Zhang et al., 2021b). In this research, the ratio of CD4 +/CD8 + T cells detected in the G70 group was clearly increasing, suggesting that G70 could activated more CD4 + T cells from peripheral blood. In addition, we observed significantly higher intestinal lymphocyte stimulation index in G70 group than that in Vaccine group, which confirmed that β-glucan could also stimulate intestinal lymphocyte. Our previous experiments confirmed that yeast cell wall extract PW220 significantly increased intestinal specific sIgA and IgA + cells (Bi et al., 2020). That is to say, both β-glucan G70 and PW220 are effective to promote intestinal mucosal immunity.

Improved immunity of animals by administration of β-glucan has been reported previously. Guo et al. (2003) confirmed that administration of β-glucan improved index of bursa and NDV-specific antibody in chickens. Levine et al. (2018) suggested that β-glucan supplementation can up-regulate intestinal MHC Ⅱ and increase the number of CD45 + cells to alleviate intestinal injury.

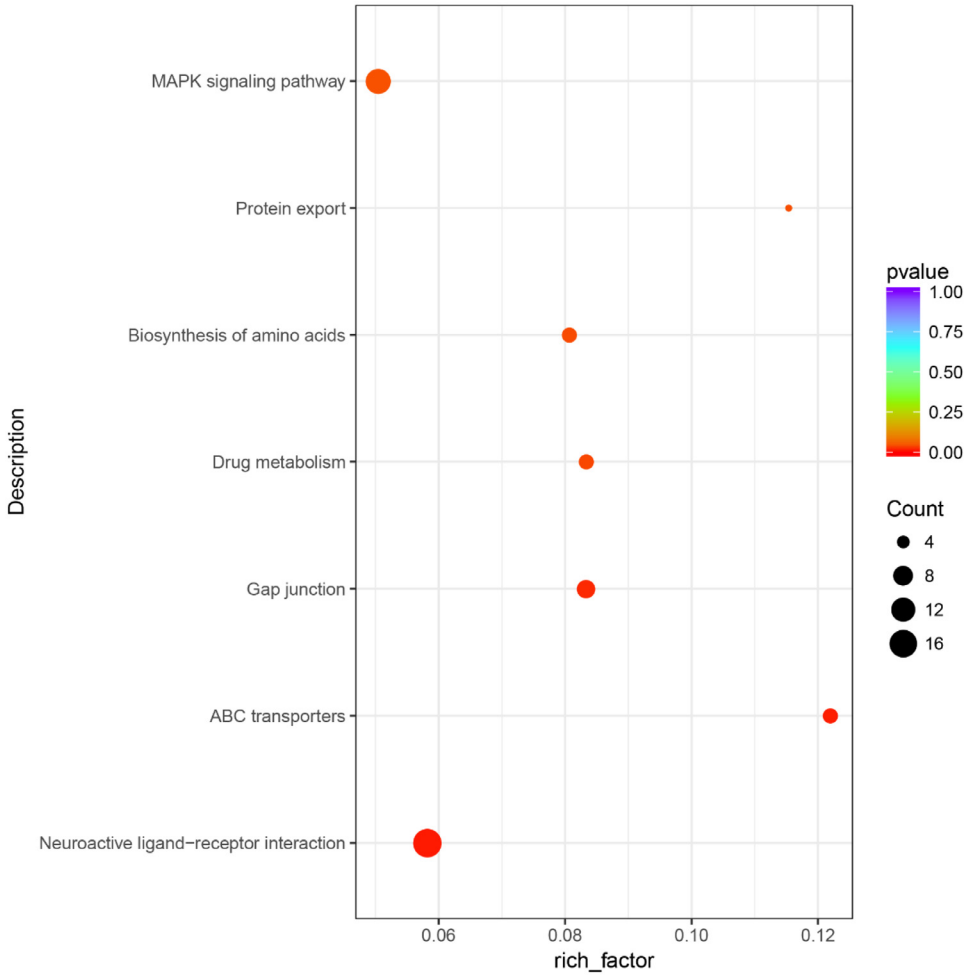

Figure 6. KEGG pathway analysis.

Li et al. (2005) revealed that dietary β-glucan could up-regulate mRNA expression levels of IL-8 and TGF-β in the intestinal tract of pigs. The mechanism on immunomodulatory effect of β-glucan is not clear. Kim et al. (2010) showed that β-glucan promoted the maturation of dendritic cells by up-regulating the expression of CD40, CD80, and CD86. In addition, Lee and Kim (2014) and Su et al. (2020) reported that β-glucan activated pattern recognition receptors such as Dectin-1 receptor, Toll-like receptor and CR3 (Baert et al., 2015).

Spleen is a critical organ for initiating immune response and plays an essential role in both innate and acquired immunity (Bronte and Pittet, 2013). However,

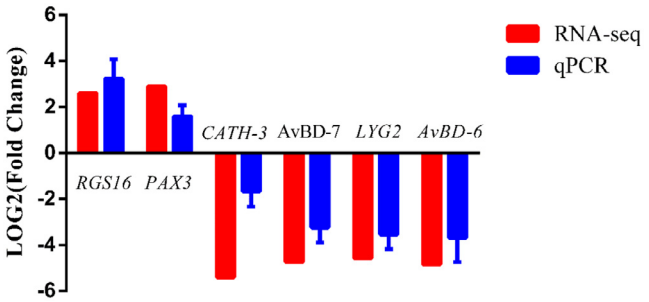

Figure 7. RT-qPCR confirmation of selected DEG candidates. Data are expressed as mean ± SE.

there are only few research based on RNA-seq analysis of mRNA expression in the spleen post oral administration of yeast polysaccharides. In the present study, the Kyoto Encyclopedia of Genes and Genomes pathway analysis suggested that these genes were primarily grouped into 2 pathways, including "Neuroactive ligand-receptor interaction" and "MAPK signaling pathway." The receptors for pattern recognition targeting β-glucan were enriched on the surface of immune cells (Goodridge et al., 2009). Following β-glucan recognition, the tyrosine kinase and nuclear factor kappaB (**NF-kappaB**) were stimulated by these receptors, resulting in the induction of pro-inflammatory cytokine secretion and activation of immune responses (Kankkunen et al., 2010; Masuda et al., 2012; Xu et al., 2016; Bode et al., 2019). The MAPK, as a family of serine-threonine protein kinases, plays crucial roles in inflammation and cytokine production in chickens. Byun et al. (2016) demonstrated that β-glucan elevated the production of interferon-γ and interleukin-2 and triggered significantly greater levels of phosphorylation of MAPK p38 in peritoneal macrophage. Wang et al. (2014) reported that β-glucan attenuates inflammatory responses in THP-1 cells by inhibiting p38 MAPK activation. In this study, 13 DEGs including *DUSP4, CACNA2D1, PDGFD, PTPRR*, ENSGALG00000006351, *FAS, MAP2K3,*

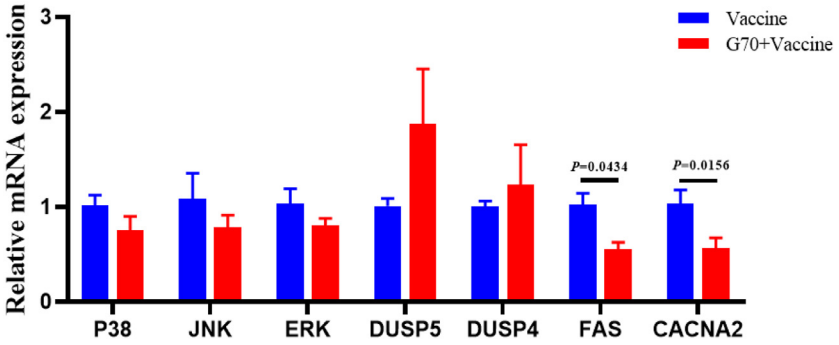

Figure 8. Relative mRNA expression in the spleen. Total RNA was extracted from the spleen. The chicken β-actin was served as the internal control gene. Data are expressed as mean ± SE.

MYC, DUSP5, MAX, SRF, PRKCB, and *EGFR* were identified in MAPK signaling pathway, which suggested that β-glucan might regulated immune response of spleen via MAPK in chickens.

The MAPK is a conserved class of Ser/Thr kinases in cells that are mainly involved in cellular responses such as immune response, apoptosis, and proliferation. The MAPK family includes at least 3 subfamilies: c-Jun N-terminal kinase (**JNK**), p38 MAPK and extracellular signal-regulated kinase (**ERK**) (Hong et al., 2020; Zhang et al., 2021a). *DUSP4* and *DUSP5* are dual-specificity phosphatases that specifically inhibit the activity of MAPK family members such as *p38, JNK*, and *ERK*, playing an important regulatory role in preventing inflammatory overreaction and promoting the regression of inflammation in the body (Imasato et al., 2002; Talreja et al., 2021). In addition, a small non-coding RNA named gga-miR-200a-3p has been reported to suppress the expression of pro-inflammatory factors and thus regulate the host autoimmune response by negatively regulating the MAPK pathway and its downstream signaling molecules (Pham et al., 2017). In the present study, RT-qPCR analysis showed that mRNA expression of *P38, JNK*, and *ERK* decreased and mRNA expression of *DUSP4* and *DUSP5* increased in chickens fed β-glucan (G70). These results suggest that the immune enhancing effect of β-glucan (G70) on Newcastle disease vaccine may be related to the negative feedback regulation of pro-inflammatory factors mediated through MAPK. In addition, *FAS* is an essential substance that mediates apoptosis and is vital for immune homeostasis (Krueger et al., 2003; Guégan and Legembre, 2018). Meanwhile, *CACNA2* is a voltage-dependent calcium channel subunit, which has a promotional effect on cell proliferation, migration, and invasion (Sun et al., 2020). In this study, *FAS* and *CACNA2* were significantly decreased in chickens supplemented β-glucan (G70), indicating that β-glucan (G70) exerts immune enhancing effects mainly by inhibiting immune cell apoptosis and balancing autoimmunity. These findings provide a theoretical reference for the use of β-glucan (G70) as an immune enhancer in clinical settings. However, the mechanism of β-glucan enhancing immune response through the negative feedback effect of MAPK pathway needs further proof.

It is interesting to note that genes encoding cathelicidin comprising *CATH3, CATH1, CATH2* and genes encoding β-defensin including *AvBD6, AvBD7, AvBD1*, and *AvBD4* were markedly down-regulated in the comparison H (Group G70 + Vaccine) vs. Control (Group Vaccine). Host Defense Peptides (**HDPs**) are effector molecules that a crucial role in the innate immune system (Cuperus et al., 2013). These peptides have been discovered in a variety of animal species of from mammals to birds. There are 4 cathelicidins in chicks, consisting of CATH1, CATH2, CATH3, and CATHB1, which effectively kill various bacteria (Hamad et al., 2017). Avian beta-defensins (**AvBDs**), alternatively named gallinacins, which are small cationic peptides with 3 cysteine disulfide bonds between their cysteine residues and perform an important role in the innate immune system (Yoshimura, 2015). The reduction in expression of cathelicidins and beta-defensin was possibly explained by feedback regulation, where bacterial and parabacterial populations were reduced by yeast cell wall polysaccharides (Bi et al., 2020). Similar results were revealed in other studies. Shao et al. (2016) showed that supplementation of yeast β-glucan in broiler chicken suppressed *Salmonella* infection and decreased the expression of HDPs via quantitative real-time PCR analysis.

In this study, a product G70 mainly composed of β-glucan enhanced the level of NDV-specific antibody in serum, increased the NDV stimulation index of peripheral blood lymphocytes and intestinal lymphocytes, and promoted the differentiation of peripheral blood T lymphocytes into CD4 + T cells. In addition, RNA-seq analysis showed that G70 upregulated the mRNA expressions related to G-protein coupled serotonin receptor and MHC class I polypeptide and downregulated the mRNA expressions related to cathelicidin and beta-defensin. Considering the enhanced effect of G70 on ND vaccine in chicken, the effect of G70 on other poultry vaccines such as avian influenza vaccine can be further studied.

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Author Contributions: SB and LC conceived and designed the experiments. LC, JL, JZ, and LZ performed the experiments. JL, HH, WX, LZ, and FG analyzed the data. SB and LC wrote the manuscript. All authors reviewed and approved the final paper.

DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102414.

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