Soy Saponin Improves Egg-Laying Performance and Immune Function of Laying Hens

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Research

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Abstract

Background

It is of great significance to clarify the role of biologically active ingredients in soybean meal to solve the shortage of soybean meal resources. The objective of this experiment was to design a low soybean meal basal diet and measure the effects of dietary soya saponin (SS) on egg-laying performance and immune function.

Results

Results showed that egg production rate, feed conversion ratio (FCR), and eggshell quality tended to be improved in the 50 SS groups. Besides, peripheral blood LPS stimulation index and proportion of B lymphocytes ratio at 5th week were increased. Serum follicle stimulating hormone and Interleukin-4 (IL-4) levels were significantly elevated at 10th week in the 50 SS groups. We also found that mRNA levels of follicle stimulating hormone receptor (*FSHR)* in ovarian, nuclear transcription factor kappa B (*NF-κB)*, Interleukin-12 (*IL-12)*, Transforming growth factor *(TGF-β*) and Interferon γ (*IFN-γ*) in spleen were up-regulated at the end of the trial. Beyond that, the relative abundance of *Proteobacteria* in the ileal flora was dropped, and the relative abundances of *Lactobacillus, Romboutsia* and *Lactobacillus delbrueckii* were elevated in the 50 SS group. Similar to the above were that at the end of the 5th week, there are same biological effects on laying hens about 50 and 500 mg/Kg SS. While at the end of the trial, the blood levels of alanine aminotransferase, alkaline phosphatase, IFN-γ, and the ratio of IFN-γ to IL-4 were significantly increased in the 500 SS group. What's more, the gene mRNA levels of *NF-κB, IL-12, IFN-γ*, Interleukin-1β (*IL-1β*), and the ratio of *IFN-γ* to *IL-4* about spleen were up-regulated in the 500 SS group. The mRNA levels of genes such as the gonadotropin releasing hormone 1 (*GnRH1*) in Hypothalamus, the estrogen related receptor (*ERR*) in ovaries were down-regulated with 500 mg/kg SS supplementation.

Conclusions:

The egg production performance was improved by dietary supplementation with 50 mg/kg SS via stimulating ovaries development, increasing ovarian *FSHR* transcription level and serum estrogen level. The intestinal microflora was regulated, and the immune function of laying hens also was improved with 50 mg/kg SS supplementation. Unexpectedly, the immune homeostasis of the laying hens tilted towards the pro-inflammatory direction with 500 mg/kg SS supplementation. It resulted in a negative impact on the laying performance of laying hens and physiological functions of the liver.

Introduction

It is worthwhile mentation that, soybean meal of China relies heavily on imports, and the cost of raising livestock and poultry remains high. We believe that studying of the biologically active substances in

soybean meal is of great significance to explore the reduction alternatives of soybean meal in livestock and poultry formulations. Soya saponin (SS) is a pentacyclic triterpenoid naturally occurring compound in soybeans. Initially, SS was regarded as an anti-nutritional factor in soybean meals for poultry. Nevertheless, it has been widely studied in recent years because of its immunomodulatory effects. SS is composed of soybean sapogen and some glycosides, uronic acid, etc. Due to the difference in glycosides, SS is divided into four different types: A, B, E, DDMP [11].

The major biological roles of SS have been reported to be associated with inhibiting the production of inflammatory factors via regulating toll-like receptor 4 (*TLR4*)-nuclear factor kappa beta (*NF-κB*) and phosphatidylinositol 3-kinase (*PI3K*)-protein kinase B (*PKB*)-*NF-κB* pathway [21; 50]. Some findings also suggested its role in down-regulating the production of reactive oxygen species (ROS) to relieve oxidative stress via regulating NF-E2-related factor 2 (*Nrf-2*)-antioxidant response element (*ARE*) pathway [25]. In addition, it also reduced the production of inflammatory factors via activating peroxisome proliferator-activated receptor-γ (*PPAR-γ*). And *PPAR-γ* directly regulated the nuclear transport of *NF-κB*. Besides, the activation of *PPAR-γ* induced the secretion of IL-10 and inhibited the expression of Claudin-2 to enhance the intestinal barrier function [45]. Likewise, SS could relieve osteoporosis in mice via activating Smads molecules to promote the transcription of runt-related transcription factor 2 (*Runx2*) and osterix (*Osx*) genes, which elevated the expression of alkaline phosphatase (*ALP*) and Osteoclasts (*OCL*) [2; 18]. It was demonstrated in a number of studies that some beneficial effects were shown with SS supplemented to the diets. To illustrate, the antibody titer of Newcastle disease in broiler serum was elevated without affecting the production performance with SS supplementation [32]. Additionally, there were no negative effects on its performance, intestinal morphology and organ index with SS supplementation to the diet of mice [26]. SS alleviated 2,4-dinitro fluoro benzene (DNFB)-induced intestinal microflora imbalance in mice [31]. Most of these studies were concentrated in the medical field. It should be pointed out that there is almost no research about SS in the animal husbandry industry.

Currently, the research on the interaction between SS and gut microbes is extremely limited. Studies showed that the proliferation of Staphylococcus aureus and Escherichia coli were inhibited by SS [9; 16]. SS could also alleviate allergic skin reactions in mice via improving the intestinal microflora [31]. Besides, some scholars tried to ferment soybean meal with Lactobacillus pentosus, and finally detected the production of soyasaponol and genistein. At the same time, these metabolites elevated the expression of brain-derived neurotrophic factors and alleviate memory impairment in mice [47]. SS and ginsenoside are triterpenoid saponins, it was demonstrated that ginsenoside promoted the proliferation of granulosa cells in chicken follicles and stimulated the development of follicles via activating protein kinase C (PKC) and up-regulating the expression of cyclin D1/cyclin dependent kinase 6 (*CDK6*) and cyclin E/cyclin dependent kinase 2 (*CDK2*) genes [24; 48]. Similarly, ginsenosides promoted proliferation of granulosa cells from chicken prehierarchical follicles and alleviated ovaries dysfunction caused by excessive pregnant mare serum gonadotrophin (PMSG) stimulated through PKC activation and up-regulated cyclin gene expression [41]. Ginsenosides not only promoted the development of ovarian cells, but also inhibited their apoptosis. To illustrate, ginsenosides inhibited the apoptosis of ovarian granulosa cells from mice, at the same time inhibited the secretion of inflammatory factors by means of up-regulating Bcl-2 and

down-regulating bax protein levels [14]. We believed that the appropriate dose of SS in the diet might improve the development of the reproductive tract and laying performance of laying hens.

Based on the findings and thinking of those studies, we firmly believed that with an appropriate dose of SS added to the low soybean meal diet, the laying performance, immune function and intestinal microflora of laying hens would be improved. Thus, a low soybean meal basal diet was designed to explored our conjecture by adding SS to the diet.

Materials And Method

All procedures adapted for the experiment were approved by the Animal Ethics Committee of China Agricultural University, Beijing, China.

Experimental design and animal management

A total of 270 Hy-line gray layer hens with 21-week-old weights and similar egg production rates were selected and housed in a conventional stepped cage in a closed house. The cages were arranged in 3 tiers with 5 cages per tier and 3 birds per cage. One week pre-feeding was carried out, the control group was fed during the pre-feeding. The diet formula of laying hens was formulated with reference to NY /T33-2004 (table 1). After an acclimation period, 270 22-week-old Hy-line Grey hens were randomly divided into three treatment groups according to the principle of uniform egg production rate (47% ± 0.02%) and similar weight (1470 ± 10 g). Control group (feeding basic diet with low soybean meal), 50 ppm SS group [Basic diet supplemented with 50 mg/kg SS], 500 ppm SS group [Basic diet supplemented with 500 mg/kg SS]. There were 6 replicates per treatment and 15 birds per replicate. The test SS was purchased from Xi'an Tongze Biotechnology Co., Ltd. (total SS content is 45.1%). The formal test period was 10 weeks, artificial feeding and the nipple drinker supplies water were used during the test. Eggs were collected and weighed once at 4 pm every day. The temperature of laying hen room was controlled at 25 ± 3°C, besides, 16h light: 8h dark lighting program was used. During formal testing, egg quality was measured every three weeks, egg production and feed efficiency were calculated every week. At the end of the 5th week, eight laying hens with uniform body weight and egg production rate were selected to collect whole blood from the underwing vein, and then extracted lymphocytes and separated serum for detection and analysis. At the same time, six layers with uniform body weight and egg production rate from each group were anesthetized with 50 mg/kg BW of sodium pentobarbital, and then slaughtered to obtain ovaries, fallopian tubes and ileal chyme for detection and analysis. At the end of the trial, eight layers with uniform body weight and egg production rate from each group were selected to be collected blood, and then anesthetized and slaughtered to collect ovaries, fallopian tubes, hypothalamus, liver and spleen for testing and analysis.

Determination of production performance and egg quality

Calculating egg production rate, feed consumption and feed egg ratio in weeks. Egg production rate (%) = total number of eggs laid during the statistical period / (number of housed hens × number of statistical

days) × 100%. Average egg production rate during the test period (%) = total number of eggs laid during the test period / (number of hens housed × total days of the test) × 100%. Feed-to-egg ratio (FCR)= total material consumption during the test / total egg weight during the test.

All eggs from each treatment within 24h were randomly extracted to detect eggshell thickness, eggshell strength, Haugh units, albumen height and egg yolk color. Using the egg quality tester DET-6000 (NABEL Co., Ltd, Japan) to measure the eggshell strength (kg/cm^2) and egg yolk color after weighing the eggs. Specifically, placing the egg vertically on the eggshell strength tester, with the blunt end up, to measure the pressure on the eggshell surface per unit area. albumen height was determined with the albumen height measuring instrument KIYA-818B (SEISAKUSHO, LTD), and then the Haugh unit was calculated according to the formula. This formula=100 Log(H-1.7W0.37+7.57), where H= the albumen height (mm) and W= the egg weight (g). The thickness of the eggshell was measured with a micrometer. Specifically, taking 3 parts (large, medium and small) from each egg after removing the shell membrane from the eggshell to take the average value after the measurement. By the way, the average value was in millimeters, accurate to 0.01 mm.

Organ index and liver morphology

The ovaries, oviducts, liver and spleen were weighted with an electronic balance (accurate 0.01g). Using a ruler (accurate 0.01 mm) to measure the length of ovary, the total length of the oviduct, the length of the magnum and the shell gland. Ovarian, oviduct and liver weight index (%) = weight of ovary, oviduct and liver (g) / live body weight of birds (g) × 100%. Spleen weight index (‰) = weight of spleen (g) / live body weight of birds (g) × 1000%. At the end of the trial, the samples about 1 cm^2 of the liver tip were collected and then suspend it into 4% paraformaldehyde solution. Liver sections were made and then stained with eosin-hematoxylin (HE stain). The OLYMPS BX-41TF microscope was used to observe the infiltration state of inflammatory cells in liver slices. The magnification was 400 times.

Determination of serum hormone levels, immune indexes and biochemical indexes

Blood was collected from the wing vein, and then, serum was separated by centrifugation at 3000 rpm and 4°C for 15 minutes. The contents of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Estradiol (E2) and Progesterone (P4) in serum were detected by radioimmunoassay according to the Protocols of the kit (from Beijing Northern Biotechnology Institute, Beijing). Detection coefficient of variation was less than 10%. According to the steps of the kit instructions of Nanjing Jian cheng Biotechnology Co., Ltd., an automatic biochemical analyzer (Unicel DXC800, Beckman Coulter, USA) were used to determine the total serum protein (TP), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and glucose (GLU) contents of laying hens at the end of the trial. The level of globulin was total protein minus albumin.

At the end of the trial, the kits from Nanjing Jian cheng Biotechnology Co., Ltd. were used to detect the contents of lysozyme (LZM) and complement C3 in the serum. The chicken β-defensin 1 enzyme-linked immunoassay kit from Beijing Konka Hong yuan Biotechnology Co., Ltd. was used to detect the content

of β-defensin in the serum. The kits from Beijing Solarbio Biotechnology Co., Ltd. were used to detect the levels of immunoglobulin G, immunoglobulin A and immunoglobulin M in the serum. ELISA kits (IDEXX laboratories lnc., Weatbrook, Maine, USA) were used to determine the levels of interleukin-2, interleukin-6, IL-4 and IFN-γ in the serum at 5^{th} and 10^{th} week. The ratio of IFN-γ to IL-4 was calculated.

Peripheral blood lymphocyte ratio and stimulation index

According to the method of Fan Hao (2018) [7], chicken peripheral blood lymphocyte separation solution from Tianjin Hao yang Biological Co., Ltd. was used to separate lymphocytes, and then red blood cell lysate was used to lyse red blood cells. The separated lymphocytes were washed twice in hanks buffer without calcium and magnesium, and then resuspended in RPMI-1640 complete medium. The cell counter was used to make sure the cell concentration as 1×10^6 cell/m L. Lymphocytes were mixed with CD3, Monocytes/Macrophages or Bu-1 antibodies purchased from Southern Biotech and bathed in water at 37°C for 30 minutes. Hanks solution was used to wash it twice, and then it was fixed with 3% paraformaldehyde. Four-colour flow cytometric analysis was conducted using a Navios EX flow cytometer with 10 colors (Beckman Coulter Corp., Fullerton, CA, USA) at Xi-Yuan Traditional Chinese Medicine Hospital, Chinese Academy of Medicine Science, China. The percentages of $CD3^+$ T cells, Monocytes/Macrophages, and Bu-1 were subsequently calculated. The result was expressed as a percentage. The MTT method was used to determine the stimulation indexs of Concanavalin A (ConA, 45μg/mL) on T cells and lipopolysaccharide (LPS, 25μg/mL) on B cells. The results were expressed in terms of the stimulus index (SI) value [43]. Both ConA and LPS were purchased from Sigma-Aldrich.

Gene expression measurement and analysis

Hypothalamus, ovarian, liver, and spleen samples were collected and placed in RNase-free Centrifuge tube, and then the samples were quickly placed in liquid nitrogen. Taking 100 mg tissue sample into 1mL trizol (Invitrogen Life Technologies, Carlsbad, USA), Total RNA isolation, quantification, cDNA synthesis, and real-time PCR were carried out as previously described Hou (2013) [53]. Total RNA was quantified by using the NanoDrop® ND-2000 UV-VIS spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at an OD of 260 nm, and the purity was assessed by determining the OD260/OD280 ratio. All the samples had an OD260/OD280 ratio above 1.8, corresponding to 90-100% pure nucleic acids. Meanwhile, the integrity of RNA in each sample was assessed using 1% denatured agarose gel electrophoresis. RNA was used for RT-PCR analysis when it had a 28 S/18 S rRNA ratio ≥ 1.8. Total RNA was reverse-transcribed using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. cDNA was synthesized and stored at -80℃ until use. The RT-PCR analysis of gene expression was performed using primers listed in table 2, and the SYBR® Premix Ex TaqTM (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). The total volume of the PCR reaction system was 20 μL. Amplification products were verified by melting curves, agarose gel electrophoresis, and direct sequencing. Results were analyzed by the cycle threshold (CT) method from Fu (2010) [8].

Microbial sequencing and analysis

The ileum chyme of laying hens was collected at end of 5th week. Sequencing and analysis according to the method described by Zhang (2018) [51]. The method was briefly described as that fecal microbial DNA extraction kit (QIAamp Fast DNA Stool Mini Kit, Qiagen Company, Germany) was used to extract microbial DNA from ileal chyme. NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) was used to determine the concentration of DNA samples, after which 1% agarose gel electrophoresis was used to detect the purity of DNA samples. 16SrDNA gene V3-V4 region universal primers 338 F (5'-ACTCCTACGGGAGGCAGCA-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify bacterial DNA, and then the PCR products were purified, quantified and homogenized to form a sequencing library. HiSeq2500 PE250 was used for on-machine sequencing. Sequencing analysis was completed by Beijing Nuohe Zhiyuan Bio-Information Technology Co., Ltd. Qiime software (Qiime2-2019.7, Nature Biotechnology) was used to generate species abundance tables of different taxonomic levels. The alpha diversity and the beta diversity of the samples were analyzed, and then the UPGMA clustering tree was constructed. Subsequently, LEfSe analysis was performed to find biomarkers (Biomarker) with statistical differences between the groups based on the LDA value. R software (Version 2.15.3) was used to draw Venn diagram, principal coordinate analysis (PCoA) diagram and perform ANOSIM analysis. PICRUSt software was used to perform metagenome function prediction analysis.

Statistical Analysis

Data were presented as means ± SD and analyzed using one-way ANOVA. Differences among treatment means were determined by Duncan's post hoc test. All statistical analyses were performed by the SPSS 23.0 software (Chicago, IL, USA). Possibility values < 0.05 were taken to indicate statistical significance. Graphpad prism 8.0 software was used to graph the data.

Results

Compared with the control group, the laying rate and the feed-to-egg ratio tended to be improved in the 50 SS group (0.05< P< 0.1) (Figure 2B). It should be mentioned that egg production rate of laying hens was significantly elevated from 2th to 4th week (P< 0.05), and there was a tendency to be elevated from 5th week to 10th week (0.05< P< 0.1) (Figure 2A). We also found that with the age of the laying hens increased, the average egg weight gradually increased. While the average egg weight and the body weight of the laying hens were not affected by the addition of SS (Figure 1A, 2 C). With the supplementation of 50 mg/Kg SS, the ovarian mass index of laying hens at the end of the trial was raised (Table 3). The thickness of the eggshell from 4th to 6th week was increased, and the thickness and strength of the eggshell from 7th to 9th week were raised in the 50 SS group (P< 0.05) (Table 5). With supplementation of 500 mg/Kg SS, although the eggshell thickness and eggshell strength were improved during the 1-3 weeks of the trial (P< 0.05), the protein height tended to be reduced during the 4-6 weeks of the test (p= 0.07).

The levels of ALP and FSH in the serum were heightened at the end of the trial in the 50 SS group (P< 0.05) (Table 6, 7). The contents of E2, P and LH in the serum were significantly elevated at the end of 5th week in the 500 SS group (P< 0.05). However, at the end of the trial, the levels of E2 and LH in the serum were reduced numerically. At the same time, the levels of ALT and ALP in the serum were increased significantly with supplementation of 500 mg/Kg SS (P< 0.05). At the end of 5th week, the peripheral blood LPS stimulation index (Figure 4A) and the proportion of B lymphocytes were significantly increased in the 50 and 500 SS group (Figure 4B). At the same time, the contents of serum IgA and lysozyme tended to be heightened (0.05< P< 0.1) (Table 8), Besides, the level of serum IL-4 was elevated at the end of the trial in the 50 SS group (P< 0.05). Similarly, serum IgA, lysozyme, IL-4 and IFN-γ levels were significantly increased at the end of 5th week in the 500 SS group (P< 0.05) (Table 8, 9). Unexpectedly, at the end of the trial, the level of serum IFN-γ and the ratio of IFN-γ to IL-4 were raised significantly with 500 mg/Kg SS supplementation (P< 0.05) (Figure 3B). Therefore, we observed from the liver tissue morphology that there was more immune cell infiltration of liver in the 500 SS group (Figure 3A).

The flora composition of the ileal chyme in the 50 and 500 SS groups was similar, and there was a certain difference from the control group (Figure 5D). Compared with the control group, the relative abundance of *Lactobacillus*, *Romboutsia* (Figure 5G, 5H) and *Lactobacillus delbrueckii* (Figure 5J, 5K, 5M) in the ileum chyme at the end of 5th week were elevated, and the relative abundance of *Proteobacteria* (Figure 5E, 5F) was descended in the 50 SS group (P< 0.05). Similar to this was that the relative abundance of *Lactobacillus salivarius* was increased, and the relative abundance of *Proteobacteria* was reduced (P< 0.05). the relative abundance of *Lactobacillus* tended to be elevated with 500 mg/Kg SS supplementation (P= 0.081). Based on the function prediction, we found that the metabolic process of the microbiota heterotrophic trophism was enriched, and the biological metabolic process of auto-trophism, parasites and symbionts were down-regulated with SS supplementation (Figure 5N).

The mRNA levels of follicle stimulating hormone receptor (*FSHR)* in ovarian, *IL-4* and *IFN-γ* in Liver, Nuclear transcription factor kappa B (*NF-κB),* Interleukin-12 (*IL-12),* Transforming growth factor *(TGF-β*) and *IFN-γ* in spleen were up-regulated at the end of the trial in the 50 SS group (P< 0.05) (Figure 6A-6G). With 500 mg/Kg SS supplementation, the gene mRNA levels of B cell lymphoma/leukemia-2 associated X Protein (*Bax*) and the ratio of *Bax* to B cell lymphoma/leukemia-2 (*Bcl2*) about ovaries, cysteinyl aspartate specific proteinase 1 (*Caspase-1*) about liver, *NF-κB, IL-12, IFN-γ*, Interleukin-1β (*IL-1β*) and the ratio of *IFN-γ* to *IL-4* about spleen were up-regulated. Beyond that, the mRNA levels of genes such as the gonadotropin releasing hormone 1 (*GnRH1*) in Hypothalamus, the estrogen related receptor (*ERR*) mRNA expression in ovaries, and *IL-4* in the spleen were down-regulated (P< 0.05) (Figure 6A-6G).

Discussion

1. The effect of SS on egg production performance and egg quality

In the present study, the egg production rate and FCR were improved with 50 mg/kg SS supplementation, while there was a negative impact on egg production rate with 500 mg/kg SS added to the diet. As mentioned in the introduction, ginsenosides promoted the proliferation of ovarian cells from chicken [24; 41; 48]. A possible explanation is that SS might improve the development of the reproductive tract to regulate hormone secretion, thus egg production rate was changed. To gain more insight, we carried out experiments on the physiological and molecular levels to study it.

The physiological factors, especially endocrine factors, regulate egg production via gonadotropin releasing hormone (GnRH), prolactin (PRL), follicle stimulating hormone (FSH) and luteinizing hormone (LH). Importantly, the secretion of these hormones is controlled via the "hypothalamus-pituitary-ovarian" axis [4]. In the present study, the contents of LH and FSH in the serum from laying hens in the tenth week of the experiment were significantly elevated with50 mg/Kg SS addition. As the research reported, LH mainly regulates the production of regulatory steroids and promotes ovulation, while FSH promotes the production of sperm in poultry or other vertebrates and stimulates the maturation of ovarian follicles [37; 42]. Beyond that, study showed that the ovarian FSH receptor (FSHR) stimulated the growth of follicles and regulates ovarian development via mediating estrogen synthesis. Additionally, FSHR response to FSH [40]. In the present study, the ovarian and oviduct weight indexes and the mRNA level of FSHR in the ovarian tissue were elevated at the end of trial with 50 mg/Kg SS addition. To explain the observed activity, we might consider that SS might improve egg production rate via improving the development of ovary and ovulation and stimulating the secretion of LH and FSH.

Surprisingly, this study showed that supplementation with 500 mg/Kg SS in the diet, E2, P, LH levels in the serum at 5th week were significantly increased. By contrast, the content of E2 in the serum tended to decrease in the 10th week. It has been reported that progesterone stimulates the pituitary gland to secrete FSH and LH to promote follicular maturation and ovulation. The increase in progesterone levels followed by higher levels of FSH and LH could be considered as an important indicator of ovarian function decline [39]. Likewise, E2 regulates ovarian activity through the feedback function of hypothalamus and pituitary. Decreased serum E2 level may cause ovarian dysfunction, premature ovarian failure and Schieren's syndrome [17]. In addition to the difference in hormone levels, we also found the mRNA levels of hypothalamus *GnRH1* and ovary *ERR* were significantly down-regulated at the end of trial. An explanation might be that the high-dose SS in the early stage of the experiment excessively stimulated the laying hens to secrete P, E2 and LH, which caused the hen ovarian function to decline. At the same time, the high-dose estrogen negative feedback regulated the "hypothalamus-pituitary-ovarian" axis. Thus, ovarian estrogen secretion was reduced.

Abnormal expression of estrogen levels often negatively affected the organ development of the reproductive tract [35]. Apoptosis of the reproductive tract weakened the reproductive performance of livestock and poultry. *Bcl2* family proteins are mainly involved in the apoptosis pathway. Specifically, there are two major types of proteins in the *Bcl-2* family: pro-apoptotic proteins (*Bax* family) and anti-apoptotic proteins (*Bcl-2*), the value of *Bax/Bcl2* is generally adopted to reflect the state of apoptosis [5]. Study told us that SS relieved MPP (1-methyl-4-phenylpyridine) -induced increase in *Bax/Bcl2* of cells

[12]. In the present study, with 50 mg / kg SS supplementation, the *Bax* transcription level of the ovary was increased, while there was no effect on the *Bax/Bcl2* ratio. Unexpectedly, ovary *Bax* mRNA level and *Bax/Bcl2* ratio were increased at the end of trial with 500 mg/kg SS supplementation. To explain the observed activity, we might consider that high-dose SS promoted the apoptosis of ovarian, which affected the secretion of ovarian hormones and negatively affected egg production performance.

This study found that the eggshell thickness and eggshell strength in 1th -3th week of trial were increased with 500 mg/Kg SS supplementation, while the albumen height in the 4th -6th week of trial was reduced. Particularly, the eggshell thickness in 4th -6th week of trial, the eggshell thickness and strength in the 7th -9th week of trial were improved with 50 mg/kg SS supplementation. A study showed that SS Bb promoted the absorption of zinc via increasing the expression of zinc transporter protein 4 (*Zip4*) protein in cells [13]. SS might further promote the absorption and utilization of calcium via promoting the absorption and utilization of zinc [29]. As a result, the quality of eggshell was improved in our study.

Based on the existing research results, we could draw a conclusion that with 50 mg/kg SS supplementation, the egg-laying performance was improved via stimulating the development of laying ovaries, increasing the transcription level of ovarian *FSHR* and serum estrogen levels. Additionally, the quality of eggshells was improved. While the addition of 500 mg/kg SS promoted ovarian cell apoptosis, down-regulated the mRNA levels of hypothalamus *GnRH1* and ovarian *ERR*, at the same time caused abnormal serum estrogen levels, thus egg-laying performance was reduced numerically.

2. The effect of SS on the intestinal microflora and immune function of laying hens

Serum globulin and lysozyme levels were used as indicators to assess the innate immunity of animals [19]. In the present study, the serum globulin and lysozyme contents were significantly elevated in the 50 SS group. The serum lysozyme level was also increased in the 500 SS group. In addition, the proliferation and differentiation of peripheral blood lymphocytes directly determine immune function of the body. It is generally accepted that concanavalin A (Con A) promotes the proliferation of T cells, while LPS could directly stimulate the proliferation and differentiation of mature B cells [43]. We found that the stimulation index of LPS to peripheral blood lymphocytes and proportion of B lymphocytes were significantly raised with 50 and 500 mg/Kg SS supplementation. It is commonly known that B cells are mainly involved in mediating fluid immune function, some scholars regarded the serum levels of total IgG, IgM and IgA as the basis for assessing the humoral immunity of poultry [6]. In the present study, the level of serum IgA in laying hens was significantly increased with 50 and 500 mg/Kg SS supplementation. It suggested that SS might improve the body's immune function via regulating the humoral immune response.

Cytokines play an important role in regulating the immune function of the body. These cytokines are usually divided into pro-inflammatory cytokines and anti-inflammatory cytokines. IL-1β, IFN-γ and TNF-α

are typical representatives of pro-inflammatory factors. There are many anti-inflammatory cytokines in the body, such as IL4, IL10 and TGF-β, etc. Pro-inflammatory cytokines activate the immune system to help eliminate pathogenic microorganisms in the host at an appropriate level. While the health of the body will be affected by excessive pro-inflammatory cytokines [38]. Anti-inflammatory cytokines are involved in reducing inflammation and promoting the body's antibody synthesis, but there is also the risk of increasing disease susceptibility [34]. The typical representatives of pro-inflammatory and anti-inflammatory cytokines are IFN-γ and IL-4 respectively. The ratio of IFN-γ to IL-4 was used to reflect the immune homeostasis of body [20]. In the present study, the mRNA levels of genes such as *IFN-γ* and *IL-4* in the liver, and *IL-12, TGF-β, IFN-γ* in spleen were significantly up-regulated in the 50 SS group. At the same time, the level of IL-4 in the serum was also elevated. A possible explanation was that the complex polysaccharide molecular structure of SS stimulated immune cells to secrete cytokines, and the immune function of body was improved.

NF-κB is the central regulator of cellular stress in all cell types of the body, it participates in the regulation of innate and adaptive immunity, as well as cell turnover [46]. Under non-pathological conditions, the body's immune system is activated by *NF-κB* to resist the stimulation of pathogens. However, once *NF-κB* is excessively activated, the overexpression of pro-inflammatory factors lead body to an inflammatory response [30]. We found that with the level of *IFN-γ/IL-4* balance, the mRNA level of *NF-κB* in spleen was up-regulated. At the same time, the levels of cytokines and lysozyme were heightened in the 50 SS group, which indicated that the immune function of the birds was improved. One unanticipated finding was that the mRNA levels of *NF-κB, IL-12, IFN-γ, IL-1β* and the ratio of *IFN-γ/IL-4* in spleen were significantly up-regulated in the 500 SS group. In addition, the transcription level of spleen *IL-4* was down-regulated. To explain the observed activity, we might consider that high-dose SS causes the immune stress response of spleen, and then *NF-κB* was activated to induce the release of pro-inflammatory factors. It tilted the body's immune homeostasis towards pro-inflammatory. Caspase 1 is involved in the proteolytic activation of IL-1β family cytokines. When the body is in a state of inflammatory stress, Caspase 1 will be activated [27]. The levels of ALT and AST in the blood are often used as indicators to evaluate liver function. Once the liver is damaged, the levels of ALT, AST and ALP in the blood will be significantly elevated [3]. In the present study, the mRNA level of liver *Caspase1* and the contents of ALT and ALP in serum were significantly raised in the 500 SS group. Besides, we also found more inflammatory cell infiltration in the liver of this group. Our findings lead us to conclude that high doses of SS have a negative effect on the liver of laying hens.

It is well established that the structure of the intestinal flora is closely related to the host's immune function. In the intestinal flora, bacteria of the genus *Lactobacillus* tend to metabolize to produce SCFAs and functional oligosaccharides, etc. These beneficial metabolites improve host immune function. However, some harmful bacteria, for instance pathogenic *Escherichia coli*, *Campylobacter jejuni* and *Helicobacter pylori* in the phylum Proteus metabolize and produce some endotoxins which affect the health of the host [15; 23]. We found that the relative abundance of *Lactobacillus* in the ileal chyme was elevated, and the relative abundance of *Proteobacteria* was decreased in the 50 and 500 SS group. On the

basis of these results we concluded that an appropriate level of SS could improve the intestinal microflora of laying hens.

Romboutsia is a group of bacteria that ferment multiple carbohydrates and metabolize to produce SCFAs, oligosaccharides and other prebiotics. Studies found that *Romboutsia* was closely related to obesity, Crohn's disease and diabetes [10; 36; 49]. Another scholar found that the relative abundance of *Fischeri* and *Romboutsia* in the stool of patients with colorectal cancer was significantly dropped. *Romboutsia* was supposed to be the key role in regulating immune function [28]. In the present study, the relative abundance of *Romboutsia* and *Lactobacillus delbrueckii* in ileal chyme were significantly heightened with 50 mg/Kg SS supplementation. *Lactobacillus delbrueckii* is a probiotic that is widely used in yogurt to improve human health. Studies suggested that with 1×10^7 CFU/g of *Lactobacillus delbrueckii* supplementation, the serum lysozyme and IgM levels of Yellow River carp were elevated, and the mRNA levels of *IL-10* and *TGF-β* in the spleen are also up-regulated [52]. Other scholars found that oral administration of *Lactobacillus delbrueckii* improved the intestinal barrier function of mice, and the mRNA levels of *IL-4, IL-10*, and *TGF-β* were up-regulated [1; 22]. We believed that 50 mg/Kg SS might improve the immune function of laying hens via increasing the relative abundance of *Romboutsia* and *Lactobacillus delbrueckii.* At the same time, *Romboutsia* and *Lactobacillus delbrueckii* might participate in the metabolic process of SS in the intestine, and then produce some biologically active substances to regulate immune function. Our team is currently conducting further research on the content of this section.

Lactobacillus salivarius stimulates immune cells to secrete anti-allergic-related cytokines and regulate the body's immune function. Studies found that with *Lactobacillus salivarius* supplementation, the levels of serum IFN-γ and lymphocyte proliferation and differentiation of chickens were raised. In addition, the antibody titer of the serum IBDV vaccine was also increased [44]. Beyond that, the number of *Campylobacter jejuni* and *Salmonella enteritidis* in the gastrointestinal tract of broilers were dropped with *Lactobacillus salivarius* supplementation. By the way, the level of serum lysozyme was elevated [33]. We found that the relative abundance of *Lactobacillus salivarius* in the ileal chyme was heightened in the 500 SS group. It told us that high-dose SS might improve the immune function of laying hens in the early stage of the trial via promoting the proliferation of *Lactobacillus salivarius*. It must also be mentioned that *Romboutsia, Lactobacillus delbrueckii* and *Lactobacillus salivarius* are all chemo-heterotrophic microorganisms. We found in this study that the addition of SS promoted the fermentation process of heterotrophic microorganisms. It illustrated us that the flora closely related to SS metabolism in the intestine might be chemoheterotrophic bacteria. In a word, with 50 mg/kg SS supplementation, the immune function of laying hens was improved via improving the intestinal microflora. However, with 500 mg/kg SS supplementation, the immune homeostasis of laying hens got unbalanced, which had a negative impact on the liver.

There are many active ingredients in soybean meals, such as soybean isoflavone, genistein, daidzein, various functional sugars and amino acids, etc. As an active ingredient in soybean meals, SS has been widely studied in the medical field. Unfortunately, it was regarded as an anti-nutritional factor in the

livestock field. Its regulation of immune function, scavenging of oxygen free radicals, and improvement of lipid metabolism were ignored by researchers of livestock field. Although a detailed investigation of mechanisms is beyond the scope of this work, we acknowledge that a suitable dose of SS surprisingly improved the egg-laying performance and immune function. Further detailed studies and the development of the functions about SS are ongoing in our laboratory.

Conclusion

The egg production performance was improved by dietary supplementation with 50 mg/kg SS via stimulating ovaries development, increasing ovarian *FSHR* transcription level and serum estrogen level. The intestinal microflora was regulated, and the immune function of laying hens also was improved with 50 mg/kg SS supplementation. Unexpectedly, the immune homeostasis of the laying hens tilted towards the pro-inflammatory direction with 500 mg/kg SS supplementation. It resulted in a negative impact on the laying performance of laying hens and physiological functions of the liver.

Abbreviations

LPS: Lipopolysaccharide, Con A: Concanavalin A, DNFB: 2,4-dinitro fluoro benzene, LYZ: Lysozyme, FSH: Follicle Stimulating Hormone, LH: Luteinizing Hormone, E2: Estradiol, P4: Progesterone, ALB: Albumin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GLU: Glucose, GnRH1: Gonadotropin Releasing Hormone 1, ERR: Estrogen Related Receptor, PGR: Progesterone Receptor, LHR: Luteinizing Hormone Receptor, FSHR: Follicle Stimulating Hormone Receptor, Bcl-2: B cell Lymphoma/leukemia-2, Bax: B cell Lymphoma/leukemia-2 associated X Protein, PPAR-γ: Peroxisome proliferator activated receptor γ, TNF-α: Tumor necrosis factor α, IL-1β: Interleukin 1β, IFN-γ: Interferon γ, IL-4: Interleukin 4, NF-κB: Nuclear Transcription Factor Kappa B, Caspase1: Cysteinyl Aspartate Specific Proteinase 1, IL-12: Interleukin 12, TGF-β: Transforming Growth Factor β.

Declarations

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Authors' contributions

Guo Yuming and Li Peng designed the study, Li Peng wrote the manuscript. Li Peng, Zhao Yizhu, Yan Shaojia, Song Bocheng, Liu Yongfa, Gao mingkun, Tang Dazhi collected and analyzed experimental results. All authors contributed to the data interpretation and approved the final version of the manuscript.

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Availability of data and materials

The datasets produced and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All study procedures were approved by the Animal Care and Use Committee of China Agriculture University and were in accordance with the Beijing Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Test diet composition and nutrition level (air-dry basis)

ingredients	contents（%）	Nutritional parameters[c]	Levels
Corn (7.8% pro)	67.55	ME MC/kg	2.7
Dephenolized cottonseed protein(50% pro)	14	Crude protein %	16.53
Limestone powder	8.154	Lysine%	0.79
corn gluten meal (51.3% pro)	5	Methionine%	0.41
Soybean meal (48% pro)	2	Calcium %	3.63
$Ca(HCO_3)_2$	1.86	Total phosphorus %	0.76
NaCl	0.35	Available phosphorus %	0.43
Trace minerals[b]	0.3	Methionine %	0.68
L-Lysine HCl (78%)	0.25	Threonine %	0.58
DL-Methionine	0.12	Tryptophan %	0.16
Choline chloride （50%）	0.12		
Tryptophan	0.02		
multi-vitamins[a]	0.03		
Antioxidants	0.03		
Phytase	0.016		
Zeolite powder	0.2		
Total	100		

[a]Vitamin premix (provided per kilogram of feed) the following substances: vitamin A, 12,500 IU, vitamin D3, 2,500 IU, vitamin K3, 2.65 mg, vitamin B1, 2 mg, vitamin B2, 6 mg, vitamin B12, 0.025 mg, vitamin E, 30 IU, biotin, 0.0325 mg, folic acid, 1.25 mg, pantothenic acid, 12 mg, niacin, 50 mg. [b]Trace element premix (provided per kilogram of feed) the following substances: copper, 8 mg, zinc, 75 mg, iron, 80 mg, manganese, 100 mg, selenium, 0.15 mg, iodine, 0.35 mg. [c]Calculated value based on the analysis of experimental diets.

Table 2. List of gene primer sequences [a]

Gene Name	Prime sequence（5'-3'）		Product size, bp
GnRH1	F	GGCTCAACACTGGTCTTATGG	202
	R	TCTTCTGGCTTCTCCTTCG	
ERR	F	GTACGGCTCTACTACACTCAGTTATGC	160
	R	CTGCTGGCTGTGGTGATGGATG	
PGR	F	GTGTCGCTTGAGGAAGTGCTGTC	116
	R	CGGCTGGCTGCTGAAGTGC	
LHR	F	CGTCCTCATAACCAGCCACTACAAG	119
	R	TCTGAGCATCCACCGAAGCAATG	
FSHR	F	GTCTCACCTGCTTGCTGATTCTCC	99
	R	CCTTGATCTCCTGGCAGATGAATATCC	
Bcl-2	F	CAACGGAGGATGGGATGC	97
	R	CAGGCTCAGGATGGTCTT	
Bax	F	TCCTCATCGCCATGCTCAT	69
	R	CCTTGGTCTGGAAGCAGAAGA	
PPAR-γ	F	TCCTTCCCGCTGACCAAA	212
	R	TCCTGCACTGCCTCCACA	
TNF-α	F	GAGCGTTGACTTGGCTGTC	64
	R	AAGCAACAACCAGCTATGCAC	
IL-1β	F	ACTGGGCATCAAGGGCTA	131
	R	GGTAGAAGATGAAGCGGGTC	
IFN-γ	F	AGCTGACGGTGGACCTATTATT	259
	R	GGCTTTGCGCTGGATTC	
IL-4	F	AGACAAATAACAAAACTGAGC	212
	R	TTGGTGGAAGAAGGTACG	
NF-κB	F	GTGTGAAGAAACGGGAACTG	203
	R	GGCACGGTTGTCATAGATGG	
Caspase1	F	CGGCCAGCGCCATCTTCATT	347
	R	AGGGAGCTGTCACAGTGCGT	

IL-12	F	AAGGTGCAGAAGCAGAGGAC	88
	R	TTGTGTTGCTCTGACTGTTGG	
TGF-β	F	TCATCACCAGGACAGCGTTA	109
	R	TGTGATGGAGCCATTCATGT	
β-actin	F	GAGAAATTGTGCGTGACATCA	152
	R	CCTGAACCTCTCATTGCCA	

[a]Primers designed using Primer Express software (Sangon Biotech, Shanghai, China).

Table 3. ***Organs weight index***

Time	Items	Control	50 ppm SS	500 ppm SS	*P*-value
5th week（n=6）	Liver %	2.75±0.44[b]	2.25±0.44[b]	2.12±0.20[a]	0.029
	Spleen ‰	1.27±0.17	1.17±0.15	1.17±0.16	0.482
	oviduct %	2.69±0.43	2.90±0.51	2.78±0.63	0.791
	oviducts %	2.41±1.28	3.43±0.31	2.90±0.97	0.208
10th week（n=8）	Liver %	2.40±0.42	2.25±0.22	2.22±0.52	0.625
	Spleen ‰	0.91±0.22	1.05±0.03	0.95±0.11	0.176
	oviduct %	2.39±0.69[a]	3.20±0.64[b]	2.20±0.67[a]	0.017
	oviducts %	2.47±0.44	3.19±0.48	2.99±1.13	0.169

The datas in Table 3 different shoulder letters indicate significant difference ($P < 0.05$), same shoulder letters indicate no difference ($P > 0.1$), the same below.

Table 4. Results of the length of the oviducts

		the length of the oviducts（cm）		
Time	Group	Total length	magnum	shell gland
5th week（n=6）	Control	47.98±18.78	22.45±13.25	4.67±1.43
	50 ppm SS	58.33±6.18	33.64±4.40	5.87±1.06
	500 ppm SS	51.73±11.75	27.80±7.36	5.45±1.34
	P-value	0.414	0.138	0.291
10th week（n=8）	Control	54.07±4.16	26.34±6.52	7.32 ± 0.23^{ab}
	50 ppm SS	58.40±5.21	32.01±3.58	8.00 ± 0.49^{b}
	500 ppm SS	53.88±14.52	29.25±8.14	6.69 ± 1.30^{a}
	P-value	0.55	0.228	0.015

Table 5. Egg quality results (n=11)

Time (Week)	Group	Eggshell strength (Kg/cm^2)	albumen hight (mm)	Haugh unit	Egg yolk color	Eggshell thickness (mm)
1th-3th	Control	4.27±0.28^{a}	8.93±1.00	94.61±5.82	7.85±0.42	0.351±0.015^{a}
	50 ppm SS	4.50±0.28^{ab}	9.05±1.71	93.51±8.61	7.73±0.36	0.349±0.016^{a}
	500 ppm SS	4.73±0.33^{b}	9.16±1.45	97.37±6.42	7.82±0.34	0.364±0.007^{b}
	P-value	0.004	0.931	0.428	0.744	0.026
4th-6th	Control	4.53±0.18	8.21±1.48^{ab}	90.85±8.29	7.48±0.28	0.366±0.010^{a}
	50 ppm SS	4.73±0.53	8.72±0.49^{b}	92.59±3.98	7.67±0.37	0.375±0.009^{b}
	500 ppm SS	4.52±0.10	7.38±0.89^{a}	88.49±6.50	7.73±0.25	0.365±0.008^{a}
	P-value	0.263	0.017	0.345	0.161	0.03
7th-9th	Control	4.47±0.47^{a}	8.85±0.71	94.31±5.57	7.21±0.40	0.363±0.015^{a}
	50 ppm SS	4.94±0.48^{b}	8.90±0.73	94.37±4.02	7.31±0.27	0.378±0.013^{b}
	500 ppm SS	4.24±0.42^{a}	9.35±1.14	97.20±4.31	7.09±0.37	0.359±0.009^{a}
	P-value	0.004	0.362	0.271	0.346	0.003

Table 6. Serum E2, P, LH, FSH levels

Time	Group	E2 (pg/mL)	P(ng/ml)	LH (mIU/ml)	FSH (mIU/ml)
5th week（n=6）	Control	682.26±160.43[a]	0.25±0.04[a]	3.54±0.60[a]	2.48±0.55
	50 ppm SS	782.42±166.81[ab]	0.25±0.07[a]	4.63±0.95[a]	2.85±0.53
	500 ppm SS	928.54±133.90[b]	0.41±0.10[b]	8.76±1.68[b]	3.17±0.73
	P-value	0.026	0.001	< 0.001	0.132
10th week（n=8）	Control	646.17±128.77[ab]	0.23±0.08[a]	5.11±1.74	2.35±0.62[a]
	50 ppm SS	701.59±98.47[b]	0.37±0.08[b]	5.76±1.16	3.11±0.81[b]
	500 ppm SS	537.52±115.01[a]	0.30±0.08[ab]	4.38±1.43	2.13±0.49[a]
	P-value	0.029	0.007	0.197	0.017

Table 7. the results of serum biochemical indexs in 10th week (n=8)

Item	Control	50 ppm SS	500 ppm SS	*P*-value
TP (g/L)	32.63±1.27	32.46±0.78	32.47±0.76	0.927
ALB (g/L)	12.57±0.98	11.88±0.88	12.25±1.01	0.377
globulin (g/L)	19.70±0.70[a]	20.58±0.74[b]	20.22±0.91[ab]	0.106
ALT (U/L)	14.02±1.55[a]	13.44±1.54[a]	15.56±1.07[b]	0.019
AST (U/L)	111.80±8.07	111.70±8.12	117.69±14.38	0.447
ALP (U/L)	313.08±21.85[a]	369.94±13.68[b]	379.88±28.54[b]	< 0.001
GLU (m mol/L)	12.15±0.15	12.23±0.33	12.19±0.50	0.904

Table 8. Serum immunoglobulins and immune molecules in 5th week (n=6)

Item	Control	50 ppm SS	500 ppm SS	*P*-value
IgG (ng/mL)	1156.99±52.49	1149.89±35.00	1142.72±46.55	0.863
IgA (ng/mL)	296.73±14.41^{a}	306.59±4.67^{ab}	309.71±5.80^{b}	0.072
IgM (ng/mL)	86.33±14.59	100.28±22.81	98.09±15.22	0.374
C3 (g/L)	0.82±0.13	0.86±0.19	0.79±0.20	0.769
Lysozyme (g/L)	125.33±13.62^{a}	155.39±29.44^{ab}	172.88±49.25^{b}	0.081
β-DF1 (ng/L	8.05±0.60	8.21±1.10	7.88±0.69	0.789

Table 9. Serum cytokines levels in 5th week (n=6)

Item	Control	50 ppm SS	500 ppm SS	*P*-value
IL-2 (pg/mL)	71.98±3.88	67.23±4.52	70.02±4.49	0.146
IL-6 (pg/mL)	81.60±9.91	88.06±13.29	91.67±16.63	0.390
IL-4 (pg/mL)	20.08±2.59^{a}	23.27±4.11^{a}	30.04±7.84^{b}	0.008
IFN-γ (pg/mL)	62.86±19.47^{a}	67.01±9.71^{ab}	82.82±19.28^{b}	0.090
IFN-γ/IL-4	3.11±0.80	2.94±0.55	2.78±0.24	0.586

Figures

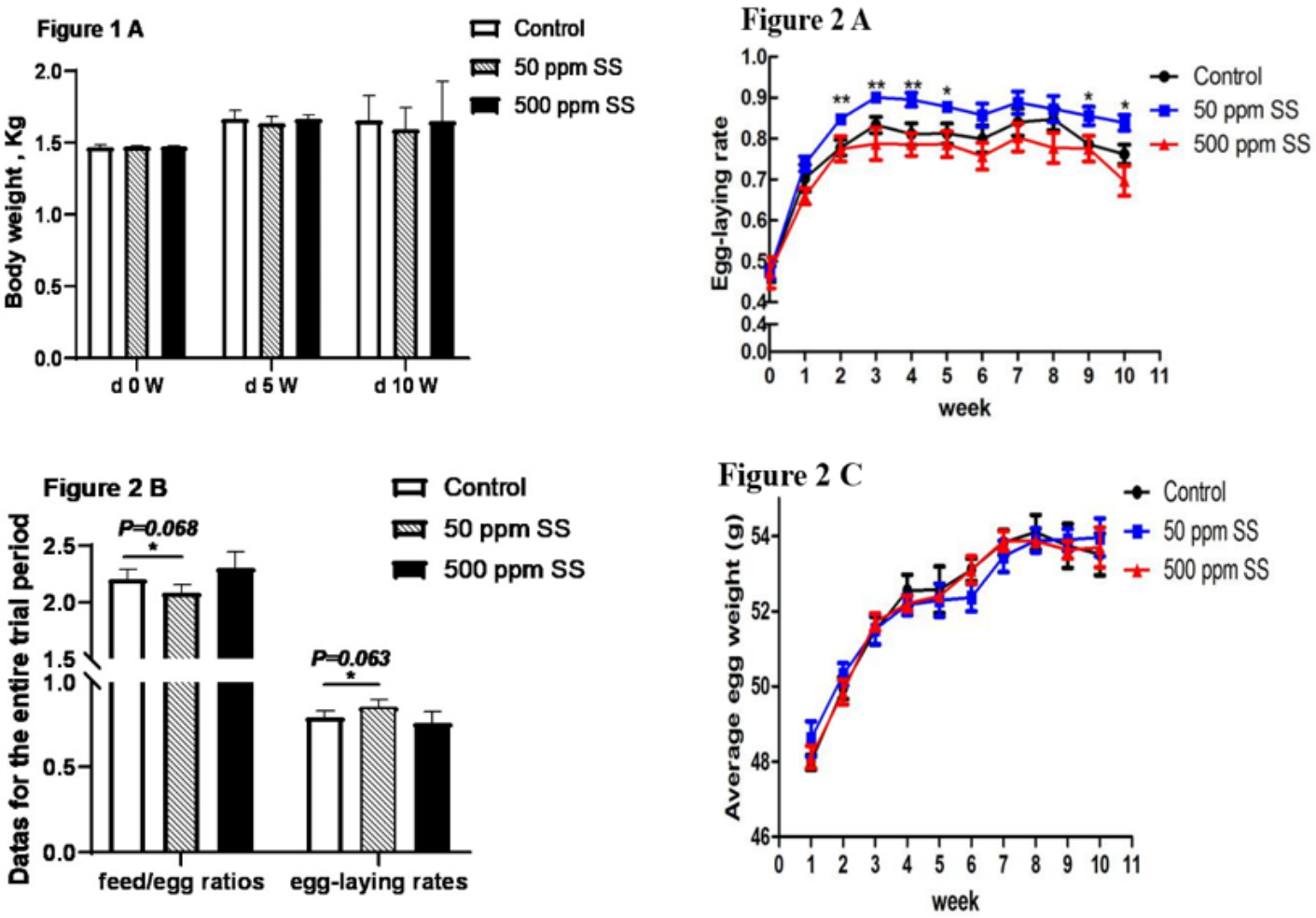
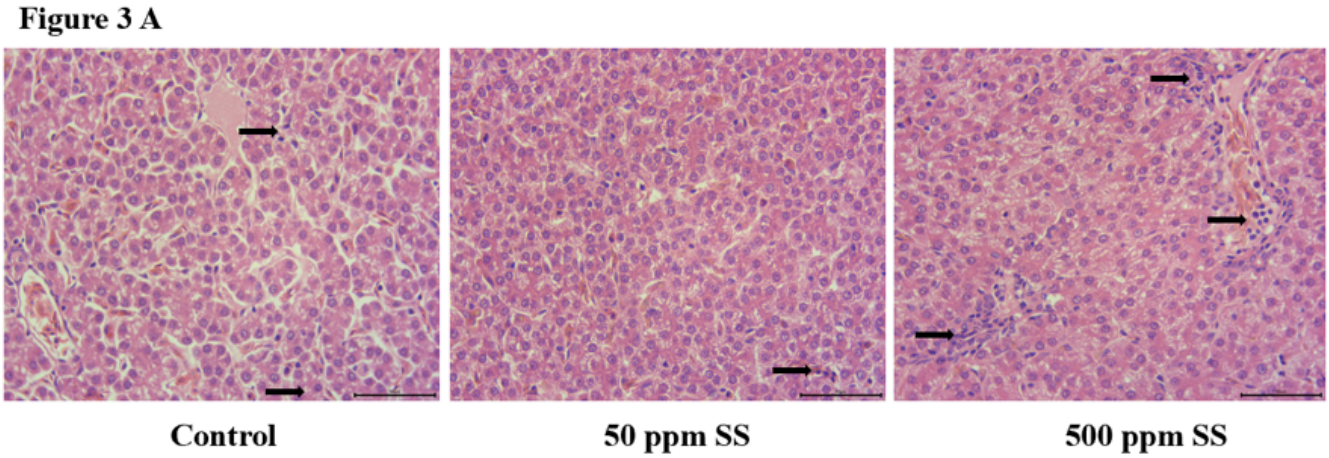
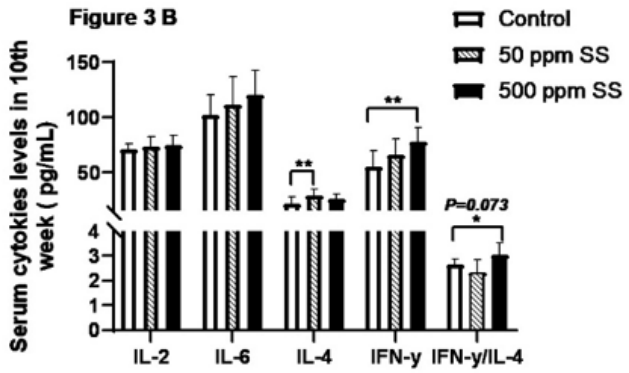
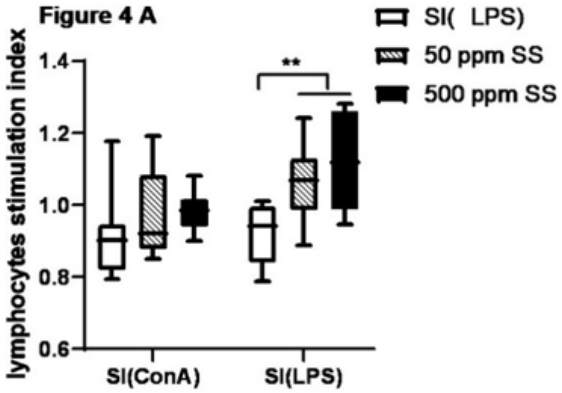


Figure 1

The effects of soy saponin on laying rate and body weight of laying hens Fig 1A, 2A, 2B, and 2C represent the results of body weight, egg production rate, average egg production rate, FCR, and average egg weight during the experiment. Among them, ** represents a significant difference (P <0.05), * was judged as a trend with difference (0.05 <P <0.1), the same below, (n=6).







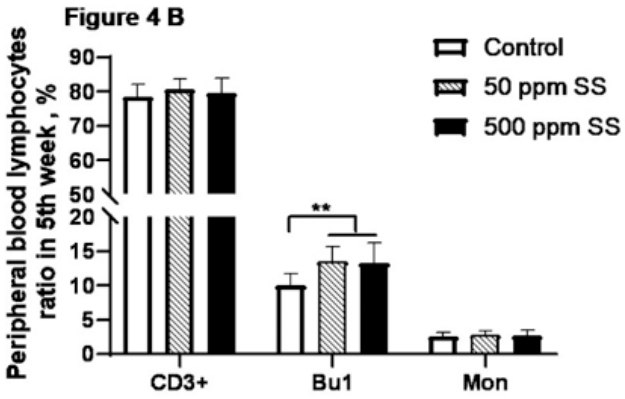


Figure 2

The effects of soy saponin on the liver morphology and blood immunity related indexes The morphological observation results about the liver of laying hens at the end of the trial in Figure 3A above. The arrow points to the infiltration of immune cells. The above Fig 3B represent the results of serum cytokine levels at the of the trial (n=8). Peripheral blood lymphocyte stimulation index and lymphocyte ratio at the end of 5th week were shown in Fig 4A and 4B (n=8).

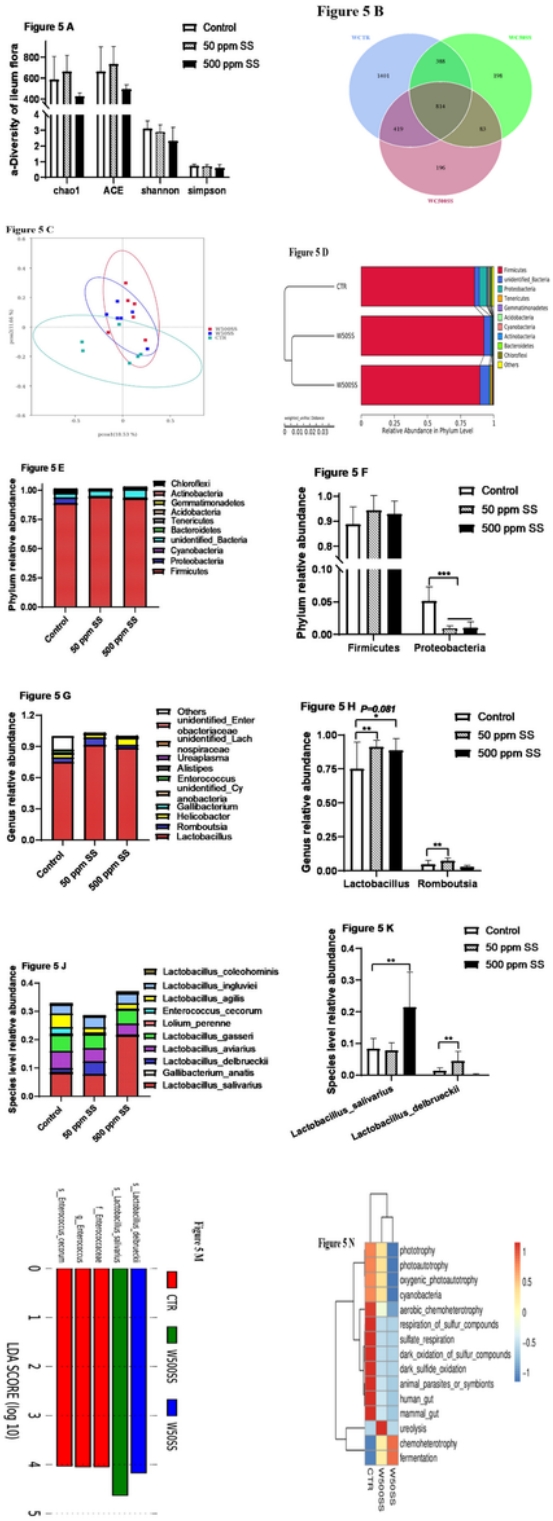


Figure 3

The effect of soybean saponins on the microbial structure of the ileum intestine of laying hens at the end of the 5th week (n=6) Fig 5A, 5B, 5C, and 5D represent the α-diversity of the flora, the venn diagram of different species, the β-diversity (PcoA) and the cluster structure of the flora. The below Fig 5E-5K represent the differences in flora at the level of phylum, genus, and species. Fig 5M represents the results

of the differential flora analyzed by LEFSe. Fig 5N represents the prediction result of flora function. WCTR= Control, WC50SS= 50 ppm SS, WC500SS= 500 ppm SS.

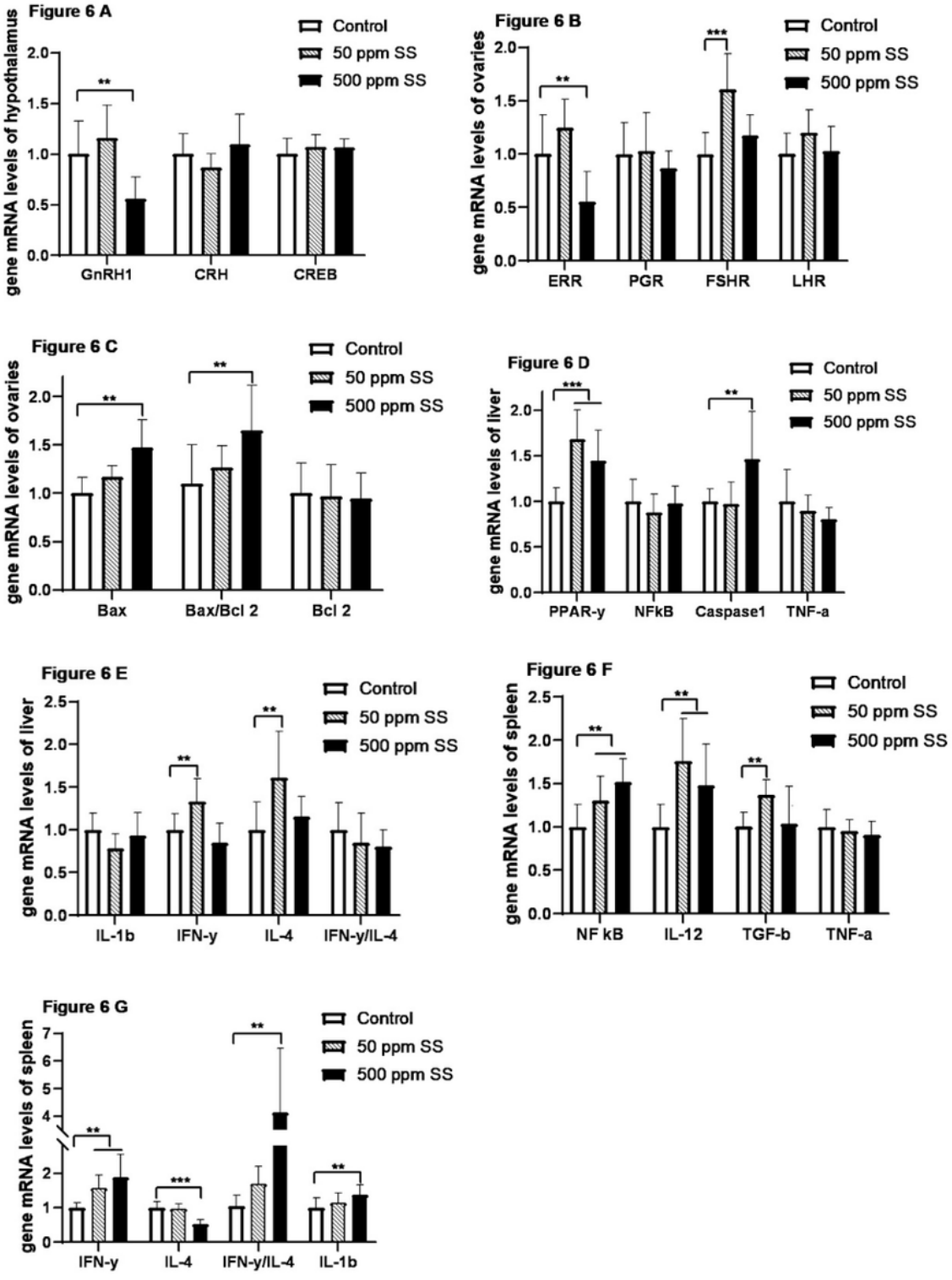


Figure 4

Effect of soy saponin on the gene mRNA levels of hypothalamus, ovary, liver and spleen in Layers Fig 6A, 6B, and 6C represent the results of gene mRNA levels about hypothalamus and ovary at the end of the trial (n=8). The above Fig 6D and 6E represent the results of gene mRNA levels about liver at the end of

the trial (n=8). The results of gene mRNA levels about spleen at the end of the trial were shown in the above Fig 6F and 6G (n=8).