Dietary Soy Saponin Improves Antioxidant and Immune Function of Layer Hens

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The objective of this study was to determine the effects of dietary soy saponin (SS) on the antioxidant and immune functions of laying hens. Two hundred seventy 22-week-old Hy-line gray layers were randomly allocated into three treatment groups: a control group (Control) fed a basal diet with low soybean meal and groups supplemented with 50 and 500 mg/kg SS (50 SS and 500 SS). After ten weeks, eight chickens from each treatment group were anesthetized and sacrificed to collect tissue samples. In the 50 and 500 SS groups, results showed that the levels of superoxide dismutase (SOD) in serum and spleen were elevated, and the content of malondialdehyde (MDA) in serum decreased. The mRNA levels of genes such as NF-E2-related factor 2 (Nrf-2) in the ileum and Nrf-2 and SOD in the spleen were also upregulated. In addition, the skin irritation index of phytohemagglutinin (PHA), the number of serum white blood cells, and lymphocytes were elevated in the two groups. At the same time, the number of monocytes in the blood increased in the 50 SS group, and it was significantly higher in the 500 SS group. In addition, the mRNA levels of lysozyme (LYZ) and IFN-γ in the spleen were upregulated, similar to the mRNA levels of zinc finger protein A20 (A20) in the ileum. Furthermore, the mRNA levels of NF-κB and IL-6 in the ileum were downregulated. In conclusion, with supplementation of 50 and 500 mg/kg SS in low soybean meal diets, the antioxidant, and immune functions of laying hens were improved. More importantly, the target for SS to exert biological effects on laying hens may be in the intestine and spleen tissues.

Key words: antioxidant, immune, layer, soy saponin

Introduction

In order to reduce the cost and amount of soybean meal used in the animal feed formula, attempts have been made to replace soybean meal with other cheap protein feed ingredients such as palm kernel meal, sunflower meal, and cottonseed meal or to use low-protein diet technology (Mohamed et al., 2019; Chrystal et al., 2020). Some studies have suggested that although amino acid levels are balanced, the poultry production, feed conversion efficiency, antioxidant and immune functions are not satisfactory compared to traditional corn, soybean meal-based diets (Sharifi et al., 2016; Lee et al., 2020). Soybean meal contains many natural active ingredients such as soy isoflavones and genistein that are beneficial for lipid metabolism, anti-oxidation, and immune function in livestock and poultry (Fan et al., 2018). Soy saponin (SS) is another naturally occurring pentacyclic triterpenoid compound in soybeans, with its content ranging from 0.1% to 0.5% in soybean meal (Guang et al., 2014). Initially, SS was regarded as an anti-nutritional factor in soybean meal for poultry (Su et al., 2018). Nevertheless, it has been widely studied in recent years because of its immunomodulatory and antioxidant functions. SS is composed of soybean sapogenin, some glycosides, and uronic acid. Owing to the difference in glycosides, SS is divided into four different types: A, B, E, and DDMP (Guang et al., 2014).

Many studies have focused on the role of SS in the regulation of immune function. Upon SS supplementation, the antibody titer for Newcastle disease in broiler serum was elevated without affecting the production performance (Naveed et al., 2020). A study found that the concanavalin A (Con A) and lipopolysaccharide (LPS) stimulation index of mouse spleen cells increased with SS supplementation (Xie et al., 2018). The major biological roles of SS have been associated with the inhibition of the production of inflammatory factors via regulation of the toll-like receptor 4 (TLR4)-nuclear factor kappa beta (NF-κB) and phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)-NF-κB pathway (Zhang et al., 2016; Xie et al., 2018). Some findings also
suggested that SS could alleviate the inflammatory response in mice caused by LPS and 2,4,6-trinitro-Benzenesulfonic acid (TNBS) (Lee et al., 2010; Zha et al., 2014). The antioxidant function of SS is attributed to its role in downregulating the production of reactive oxygen species (ROS) to relieve oxidative stress by regulating the Nrf-2-related factor 2 (Nrf-2)-antioxidant response element (ARE) pathway (Chen et al., 2014). SS relieves alcohol-induced oxidative stress in cells by stimulating the production of heme hydrogenase 1 (HO-1) (Lijie et al., 2016). Another study found that soy saponin alleviated alcohol-induced oxidative stress by increasing superoxide dismutase (SOD) activity and reducing the content of malondialdehyde (MDA) (Zha et al., 2014). SS can also alleviate LPS-induced lung oxidative damage in mice by improving the activity of SOD and catalase (CAT) in the lungs (Lin et al., 2016).

SS has shown good immune regulation and antioxidant functions, but the studies are mainly conducted on mice and cell lines. We believe that an appropriate dose of soy saponin may also have a beneficial biological effect on poultry. Thus, the following study was designed using a low-soybean-meal-based diet to test the effects of dietary SS supplementation on the antioxidant and immune functions of laying hens.

Materials and Methods

All procedures adapted for the experiment were approved by the Animal Ethics Committee of the China Agricultural University, Beijing, China. The animal welfare number was AW92601202-1-1.

**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 three tiers with five cages per tier and three birds per cage. One week of pre-feeding was carried out, and all birds were fed with the diet of control group during pre-feeding. The diet formula of laying hens was formulated according to the feeding standards of Chinese chickens (NY/T33-2004) (Table 1). After an acclimation period, all the 22-week-old Hy-line gray hens were divided into three treatment groups according to the principle of uniform egg production rate (47±0.02%) and similar weight (1470±10 g). The following groups were formed: a control group that was fed a basic diet with low soybean meal, 50 ppm soy saponin (SS) group which was given a basic diet supplemented with 50 mg/kg SS, and 500 ppm SS group with a basic diet and 500 mg/kg SS. There were six replicates per treatment and 15 birds per replicate. The test SS was purchased from Xi’an Tongze Biotechnology Co., Ltd. (total SS content was 45.1%). The formal test period was 10 weeks, and artificial feeders were used with a nipple drinker to supply water. The temperature of the laying hen room was controlled at 25±3℃, and a 16-h light: 8-h dark lighting program was used. Eight laying hens with uniform weight from each group were randomly selected to collect blood from the wing vein. Additionally, all birds were weighed at the end of the 5th and 10th week. At the end of the trial, eight birds with uniform weight from each group were sacrificed under sodium pentobarbital anesthesia (50 mg/kg BW) to obtain the ileum, spleen, liver, and kidney for analysis.

**Table 1. Test diet composition and nutrition level**

| Ingredients | Nutritional parameters | Levels |
|-------------|------------------------|--------|
| Corn (7.8% protein) | 67.550 | Metabolizable energy (ME, Mcal/kg) 2.70 |
| Dephenolized cottonseed protein (50% protein) | 14.000 | Crude protein (%) 16.53 |
| Limestone powder | 8.154 | Lysine (%) 0.79 |
| Corn gluten meal (51.3% protein) | 5.000 | Methionine (%) 0.41 |
| Soybean meal (48% protein) | 2.000 | Calcium (%) 3.63 |
| Ca (HCO₃)₂ | 1.860 | Total phosphorus (%) 0.76 |
| NaCl | 0.350 | Available phosphorus (%) 0.43 |
| Trace minerals | 0.300 | Methionine (%) 0.68 |
| L-Lysine HCl (78%) | 0.250 | Threonine (%) 0.58 |
| DL-Methionine | 0.120 | Tryptophan (%) 0.16 |
| Choline chloride (50%) | 0.120 | |
| Tryptophan | 0.020 | |
| Multi-vitamins | 0.030 | |
| Antioxidants | 0.030 | |
| Phytase | 0.016 | |
| Zeolite powder | 0.200 | |
| Total | 100 | |

*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. |

*bTrace element premix (provided per kilogram of feed) the following substances: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; selenium, 0.15 mg; iodine, 0.35 mg.
liver, ovary, and shell gland.

**Immune Cells**

At the end of the 5th and 10th week, blood samples were collected from the wing vein and an automatic blood cell analyzer (Siemens ADVIA® 2120i, Germany) was used to measure the number of white blood cells, neutrophils, lymphocytes, eosinophils, basophils, and monocytes. The ratios of neutrophils, lymphocytes, eosinophils, basophils, and monocytes were calculated and the ratio of the number of immune cells to the number of white blood cells was determined.

**Antioxidant Enzymes and Related Product Levels**

Blood was collected from the wing vein, and the serum was separated by centrifugation at 3000×g and 4°C for 15 min. The ileum, spleen, liver, and shell gland were collected and placed into liquid nitrogen at the end of the trial. It should be noted that after follicles larger than 2 mm in diameter were removed, the entire ovaries were shredded and mixed for later use. Samples (0.8 g samples in 10 mL tubes) and 7.2 mL saline were homogenized on ice. The supernatant was collected by centrifugation at 3000 rpm at 4°C for 15 min. The levels of MDA, T-SOD, GSH-Px, and CAT were determined according to the manufacturer’s protocol. The kits were purchased from Nanjing Jiancheng Biotechnology Co., Ltd.

**Skin Irritation Index of PHA**

The method described by Sullivan et al. (2017) was used to detect the skin irritation index. Briefly, phytohemagglutinin (PHA, Sigma-Aldrich L1668) was dissolved in sterile saline (0.9%) to obtain a final concentration of 2 µg/mL. In the 10th week of the trial, eight laying hens were randomly selected from each group and 50 µL of PHA solution was injected into their right shank, and an equal volume of sterile saline was injected into the left shank. The thickness of the wattles was measured using a Vernier caliper (Peacock, Ozaki MFG. Co. Ltd, Tokyo, Japan) with an accuracy of 0.01 mm before the injection and at 12, 24 h after the injection. The irritation index (SI) was calculated using the following formula: $SI = (\text{thickness of the right shank after injection thickness of the right shank before injection}) / (\text{thickness of the left shank after injection thickness of the left shank before injection})$.

**Gene Expression Levels and Analysis**

The ileum, liver, and spleen were collected and placed in RNase-free centrifuge tubes, and the samples were immediately placed in liquid nitrogen. Taking 100 mg tissue sample in 1 mL TRIzol (Invitrogen Life Technologies, Carlsbad, USA), the total RNA isolation, quantification, cDNA synthesis, and real-time PCR were carried out as previously described in Zhao et al. (2017). Briefly, after total RNA was quantified, the purity was assessed by determining the OD

### Table 2. List of primer sequences for genes

| Gene name | Prime sequence (5′→3′) | Gene Bank | Product size bp |
|-----------|-------------------------|------------|----------------|
| LYZ       | CCCAGGCTCCAGGGAACCT     | NM_205281  | 102            |
|           | R CACGGCTGCTTATAGTCTGA |            |                |
| AVBD7     | ATGGAAATAGGCTTCCTGCCTGGT | NM_001001194.1 | 119          |
|           | R GCCGACAGATACCGGTGGG    |            |                |
| Leap2     | CTAGCCACGGTTGACTGGCTTT  | NM_001001606.1 | 66            |
|           | R GCTCTACGCAAGTCTCA     |            |                |
| TNF-α     | CCTCACTACCTGGCACA       | NM_204267.1 | 67            |
|           | R TGACTACGGAGGTTCTCAT    |            |                |
| IFN-γ     | CTTCCTCTAGTGCGAGGAAGA   | NM_205149.1 | 127           |
|           | R GAGGATCCACAGTGCTCTTG  |            |                |
| A20       | GACATCGTGCTAAGGCAAGTCTGG | XM_003640919.2 | 180          |
|           | R AGAAGGGGATACGACGGCACAC |            |                |
| NF-κβ     | TGGAGAAGGGCTATGCAGCTTT  | NM_205134.1 | 117           |
|           | R CATCCTGGACAGCAGTGAGA  |            |                |
| IL-6      | GCGAGAAAGACGATGGAGAT    | AJ621249   | 82            |
|           | R GTAGGGTCTGAAGGGGAACAC |            |                |
| IL-8      | GGGTTGCTAGGGAAGAATGA    | NM_205498.1 | 200           |
|           | R AGTCAGTCTCATAGGAAACTG |            |                |
| Nrf-2     | ATCAACTTTTCGACGGGAAA    | NM_205117.1 | 229           |
|           | R GTTCTCCGGCTCTTCTTG    |            |                |
| SOD       | CCGGCTTGTGTAGGAGAT      | NM_205064.1 | 125           |
|           | R TGCACTTTGGTCCACCGT    |            |                |
| GPX-Px    | GACAAACCGCGATCATA       | NM_00277853.2 | 204          |
|           | R GAGGAGGGGCTCTTCTTTTA  |            |                |
| β-actin   | CCTGAGAACCTTCTATTGCA    | NM_205518.1 | 152           |

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

*b* Abbreviations: LYZ, lysozyme; AVBD7, avian beta-defensin 7; Leap2, liver-expressed antimicrobial peptide 2; TNF-α, tumor necrosis factor α; IFN-γ, interferon γ; A20, zinc finger protein A20; NF-κB, nuclear transcription factor-kappa B; IL-6, interleukin 6; IL-8, interleukin 8; Nrf-2, NF-E2-related factor 2; SOD, superoxide dismutase; GPX-Px, glutathione peroxidase.
260/280 ratio (>1.8 corresponds to 90–100% pure nucleic acids) (Zhao et al., 2017). The integrity of the RNA in each sample was assessed using 1% denatured agarose gel electrophoresis. Total RNA was reverse-transcribed using the Prime Script® RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer’s instructions. cDNA was synthesized and stored at −80°C until use. The quantitative PCR analysis of gene expression was performed using the primers shown in Table 2 and SYBR® Premix Ex Taq™ (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). The total volume of the PCR reaction was 20 µL. Amplification products were verified by melting curves, agarose gel electrophoresis, and direct sequencing. The results were analyzed according to the method described by Fu et al. (2010).

Statistical Analysis

A computer program SPSS 23.0 (Chicago, IL, USA) was used for the statistical analysis. Differences between the group were analyzed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer’s procedure for multiple comparisons. All data in tables are presented as mean±SEM. Results were considered statistically significant when the P-value was <0.05.

Results

There was no effect on the bodyweight of laying hens treated with 50 and 500 mg/kg SS (Fig. 1). The mRNA levels of Nrf-2, SOD, and GPX-Px in the ileum and Nrf-2 and SOD in the spleen were upregulated in the 50 SS group (P<0.05) (Figs. 2A and 2B). Additionally, the content of SOD in serum and the levels of CAT and SOD in the spleen increased. The MDA levels in the serum, spleen, and ileum reduced with 50 mg/kg SS supplementation (P<0.05) (Tables 3 and 4) while the levels of genes such as Nrf-2 in the ileum and Nrf-2 and SOD in the spleen were upregulated. At the same time, serum CAT and spleen SOD levels were also elevated in the 500 SS group (P<0.05). The MDA content in the serum was reduced;
Table 3. The effects of soy saponin (SS) on the levels of antioxidant-related enzymes and products in liver, ovaries, and shell gland (n=8)

| Item               | Control       | 50 ppm SS     | 500 ppm SS    | P-value |
|--------------------|---------------|---------------|---------------|---------|
| Liver              |               |               |               |         |
| CAT (U/mg protein) | 4.90±0.44     | 4.97±0.30     | 5.34±0.25     | 0.622   |
| MDA (nmol/mg protein) | 3.43±0.33a   | 4.03±0.20ab   | 4.56±0.22ab   | 0.019   |
| SOD (U/mg protein) | 25.63±2.18    | 28.60±3.03    | 29.22±1.90    | 0.544   |
| GSH (U/mg protein) | 20.27±1.13    | 18.63±0.66    | 18.85±2.23    | 0.704   |
| Ovaries            |               |               |               |         |
| CAT (U/mg protein) | 5.43±0.60     | 5.65±0.31     | 4.87±0.44     | 0.486   |
| MDA (nmol/mg protein) | 4.01±0.54    | 4.63±0.14     | 3.99±0.50     | 0.504   |
| SOD (U/mg protein) | 42.76±5.54    | 39.28±1.99    | 37.73±2.06    | 0.608   |
| GSH (U/mg protein) | 20.99±5.03    | 23.68±2.80    | 22.68±4.64    | 0.904   |
| Shell gland        |               |               |               |         |
| CAT (U/mg protein) | 5.58±0.31     | 5.09±0.24     | 4.90±0.34     | 0.270   |
| MDA (nmol/mg protein) | 3.19±0.25    | 3.15±0.22     | 3.19±0.41     | 0.995   |
| SOD (U/mg protein) | 58.01±2.95    | 62.76±2.59    | 63.36±0.80    | 0.224   |
| GSH (U/mg protein) | 17.44±1.21    | 18.51±1.38    | 16.43±1.18    | 0.517   |

Values with the same superscripts are not significantly different from each other at P<0.05.

Abbreviations: CAT, Catalase; MDA, Malondialdehyde; SOD, Superoxide dismutase; GSH, Glutathione peroxidase.

Table 4. The effects of soy saponin (SS) on the levels of antioxidant-related enzymes and products in serum, spleen, and ileum (n=8)

| Item       | Control       | 50 ppm SS     | 500 ppm SS    | P-value |
|------------|---------------|---------------|---------------|---------|
| Serum      |               |               |               |         |
| CAT (U/mL) | 4.25±0.29a    | 5.26±0.29a    | 6.87±0.51ab   | <0.001  |
| MDA (nmol/mL) | 6.58±0.36a   | 4.81±0.21a    | 4.95±0.28a    | <0.001  |
| SOD (U/mL) | 201.09±1.67a  | 210.15±2.79a  | 203.18±2.69ab | 0.040   |
| GSH (U/mL) | 28.54±1.91    | 31.06±1.63    | 29.67±2.51    | 0.689   |
| Spleen     |               |               |               |         |
| CAT (U/mg protein) | 17.96±1.46a  | 24.12±1.40a   | 19.35±1.27a   | 0.012   |
| MDA (nmol/mg protein) | 1.86±0.09a    | 1.29±0.09a    | 1.78±0.16b    | 0.005   |
| SOD (U/mg protein) | 217.07±11.32a | 260.93±16.19b | 255.06±9.71b  | 0.048   |
| GSH (U/mg protein) | 19.40±1.74    | 24.05±2.01    | 21.23±1.92    | 0.238   |
| Ileum      |               |               |               |         |
| CAT (U/mg protein) | 14.04±0.96    | 15.08±0.68    | 15.68±0.93    | 0.414   |
| MDA (nmol/mg protein) | 1.33±0.13b    | 0.97±0.05a    | 1.03±0.11ab   | 0.049   |
| SOD (U/mg protein) | 219.91±7.12   | 264.60±20.23 | 261.23±28.26 | 0.251   |
| GSH (U/mg protein) | 63.05±4.96    | 67.27±3.64    | 59.42±3.29    | 0.402   |

Values with the same superscripts are not significantly different from each other at P<0.05.

Abbreviations: CAT, Catalase; MDA, Malondialdehyde; SOD, Superoxide dismutase; GSH, Glutathione peroxidase.

However, the levels in the liver increased (P<0.05). The levels of antioxidant-related enzymes and oxidation-related products in the liver, ovaries, and fallopian tubes were not changed by treatment with 50 and 500 mg/kg SS.

Some immune-related indicators were measured to evaluate the effect of SS on the immune function of laying hens. The results showed that after 12 h of injection, the skin irritation index of PHA significantly increased in the 50 and 500 SS groups (P<0.05) (Fig. 3). Moreover, with 50 mg/kg SS supplementation, the number of serum lymphocytes at the 5th week and the number of serum white blood cells and lymphocytes at the 10th week were elevated (P<0.05). The number of serum monocytes increased at the end of the trial (P=0.065) (Tables 5 and 6). Similarly, the number of serum white blood cells, neutrophils, and lymphocytes at the 5th week was increased, and the number of white blood cells, lymphocytes, and monocytes at the end of the trial also significantly increased in the 500 SS group (P<0.05). Other
immune-related results showed that the mRNA levels of *LYZ* and *IFN-γ* in the spleen were upregulated, as well as the levels of *A20* in the ileum with 50 and 500 mg/kg SS supplementation (*P*<0.05) (Fig. 4A, 4B). In addition, the mRNA levels of *NF-κB* and *IL-6* in the ileum were downregulated in the 50 and 500 SS groups (*P*<0.05).

**Discussion**

Oxidative stress is a state of imbalance between oxidation and antioxidant effects in cells and tissues. A large number of oxidative intermediate products are produced, and inflammatory infiltration of immune cells occurs once the body is under oxidative stress. At this time, antioxidant enzymes such as SOD, CAT, and HO-1 are secreted to alleviate the stress (Haque et al., 2019). It is generally accepted that oxidative stress is an important factor leading to aging and disease (Newsholme et al., 2016). In the present study, the levels of SOD and CAT in the serum, ileum, and spleen were significantly increased with 50 and 500 mg/kg SS supplementation. This is consistent with the results of previous studies in mice (Zha et al., 2014; Lijie et al., 2016; Lin et al., 2016). Peroxide products such as MDA and myeloperoxidase (MPO) are generally produced in large quantities in the body under stress (Delhaye et al., 2016), which causes cytotoxicity

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**Table 5.** The effects of soy saponin (SS) on the levels of blood cells at the end of the 5th week (n=8)

| Item     | Control          | 50 ppm SS        | 500 ppm SS       | *P*-value |
|----------|------------------|------------------|------------------|-----------|
| WBC      | 72.34±0.86<sup>a</sup> | 74.40±0.16<sup>b</sup> | 77.75±1.02<sup>b</sup> | 0.003     |
| NETU%    | 27.63±0.67       | 26.75±0.47       | 27.25±0.50       | 0.543     |
| NETU#    | 19.95±0.34<sup>a</sup> | 19.89±0.38<sup>b</sup> | 21.18±0.30<sup>b</sup> | 0.024     |
| LYMHP%   | 56.61±0.70       | 58.69±0.54       | 57.44±0.86       | 0.140     |
| LYMHP#   | 40.95±0.63<sup>a</sup> | 43.68±0.86<sup>b</sup> | 44.65±0.69<sup>b</sup> | 0.005     |
| MONO%    | 7.71±0.51        | 7.03±0.36        | 7.69±0.66        | 0.586     |
| MONO#    | 5.61±0.41        | 5.21±0.27        | 6.00±0.55        | 0.442     |
| EO%      | 6.53±0.53        | 6.19±0.32        | 6.11±0.52        | 0.803     |
| EO#      | 4.73±0.41        | 4.61±0.26        | 4.78±0.44        | 0.953     |
| BASO%    | 1.53±0.12        | 1.36±0.08        | 1.48±0.14        | 0.603     |
| BASO#    | 1.10±0.09        | 1.01±0.05        | 1.15±0.11        | 0.556     |

<sup>a-b</sup> Values with the same superscripts are not significantly different from each other at *P*<0.05.

Abbreviations: WBC, white blood cell; NETU%, ratio of neutrophils to WBC; NETU#, number of neutrophils; LYMHP%, ratio of lymphocytes to WBC; LYMHP#, number of lymphocytes; MONO%, ratio of monocytes to WBC; MONO#, number of monocytes; EO%, ratio of eosinophils to WBC; EO#, number of eosinophils; BASO%, ratio of basophils to WBC; BASO#, number of basophils.

**Table 6.** The effects of soy saponin (SS) on the levels of blood cells at the end of the 10th week (n=8)

| Item     | Control          | 50 ppm SS        | 500 ppm SS       | *P*-value |
|----------|------------------|------------------|------------------|-----------|
| WBC      | 71.00±1.02<sup>a</sup> | 76.74±1.56<sup>b</sup> | 76.98±1.49<sup>b</sup> | 0.009     |
| NETU%    | 28.59±0.74       | 26.83±0.74       | 26.09±0.93       | 0.105     |
| NETU#    | 20.31±0.68       | 20.54±0.53       | 20.01±0.45       | 0.803     |
| LYMHP%   | 57.13±0.62       | 58.21±0.62       | 57.66±1.32       | 0.706     |
| LYMHP#   | 40.55±0.70<sup>a</sup> | 44.69±1.12<sup>b</sup> | 44.33±1.03<sup>b</sup> | 0.011     |
| MONO%    | 7.29±0.47        | 8.31±0.42        | 8.76±0.77        | 0.202     |
| MONO#    | 5.16±0.32<sup>a</sup> | 6.39±0.37<sup>b</sup> | 6.80±0.68<sup>b</sup> | 0.065     |
| EO%      | 5.71±0.57        | 5.35±0.75        | 6.01±0.88        | 0.821     |
| EO#      | 4.05±0.41        | 4.11±0.60        | 4.70±0.75        | 0.705     |
| BASO%    | 1.31±0.16        | 1.33±0.18        | 1.45±0.23        | 0.854     |
| BASO#    | 0.93±0.11        | 1.01±0.14        | 1.14±0.19        | 0.619     |

<sup>a-b</sup> Values with the same superscripts are not significantly different from each other at *P*<0.05.

Abbreviations: WBC, white blood cell; NETU%, the ratio of neutrophils to WBC; NETU#, the number of neutrophils; LYMHP%, the ratio of lymphocytes to WBC; LYMHP#, the number of lymphocytes; MONO%, the ratio of monocytes to WBC; MONO#, the number of monocytes; EO%, the ratio of eosinophils to WBC; EO#, the number of eosinophils; BASO%, the ratio of basophils to WBC; BASO#, the number of basophils.
and leads to oxidative damage (Reiter et al., 2018). Our study found that the MDA content in the serum, ileum and spleen of laying hens reduced with 50 and 500 mg/kg SS added to the diet. This suggests that an appropriate dose of SS could improve the antioxidant performance of laying hens.

To explain the observed activity, we consider that the antioxidant effect of SS on laying hens might be related to the Nrf-2 pathway. The Kelch-like ECH-associated protein-1 (Keap-1)-Nrf-2 signaling pathway is regarded as one of the important mechanisms of cell defense against oxidative stress damage. Under normal circumstances, the activity of Nrf-2 is inhibited (Huang et al., 2015). Once the cell undergoes oxidative stress, Nrf-2 is activated to bind to antioxidant response element (ARE) in the nucleus, and the transcription of antioxidant genes is initiated (Baird and Yamamoto, 2020). A study also found that the Keap1-Nrf 2 pathway regulates the production of ROS in the mitochondria and cytoplasm by regulating the activity of NADPH oxidase (Kovac et al., 2015). In the present study, the mRNA levels of Nrf-2 and SOD in the ileum and spleen were upregulated in the 50 and 500 SS groups. This was consistent with a previous finding that SS could alleviate cellular oxidative stress by activating the Nrf-2-ARE pathway (Liu et al., 2018). A study also suggested that SS alleviated the oxidative stress induced by LPS by inhibiting the activity of NADPH oxidase (Zhang et al., 2016). We may consider that the mechanism by which SS improves the antioxidant function of laying hens might be related to the activation of Nrf-2 and the regulation of NADPH oxidase activity. Further studies are needed to determine the specific mechanism.

Based on previous studies (Guang et al., 2014; Lee et al., 2010; Zha et al., 2014), we believe that SS may be degraded by intestinal bacteria into functional sugars, soy saponins, and other substances in the chicken intestine. Some of these substances are used by intestinal microbes in the regulation of intestinal microflora and improve the antioxidant function of the ileum. The other part is absorbed into the blood by intestinal epithelial cells and reaches specific target organs to perform its biological functions. It is worth noting that there was no significant effect on the antioxidant function of the liver, ovary, and shell glands of laying hens with SS supplementation. In contrast, SS improved the antioxidant function of the ileum and spleen. This indicated that the target tissues for SS to play a biological role in the birds might be mainly concentrated in the spleen and intestines.

White blood cells (WBCs) in the blood participate in the functional immune response. It is well established that lymphocytes are mainly involved in the regulation of specific immune processes while monocytes improve the body’s innate immune function via phagocytosis of invading pathogens (Carrick and Begg, 2008). In the present study, with 50 and 500 mg/kg SS supplementation, the number of white blood cells, lymphocytes, and monocytes in the serum of laying hens were raised in the physiological range. A study also suggested that the skin irritation index of PHA was positively correlated with T-cell activity, and the skin irritation index of PHA was used to evaluate the cellular immune function of poultry (Tella et al., 2008; Goto et al., 1978). We found 12 hours after PHA injection, the skin irritation index of PHA was significantly increased in the 50 and 500 SS groups. Based on the above observations, we concluded that SS has great potential to improve the immune function of laying hens.

In a host, lysozmes and defensive peptides constitute the first line of defense against the invasion of pathogenic microorganisms (Liu et al., 2010; Lee et al., 2016). Some studies have also regarded the levels of lysozyme and defensive peptides as important indicators for evaluating the innate immune function of poultry (Kim et al., 2012). In the present study, the mRNA levels of LYZ in the spleen were upregulated in the 50 and 500 SS groups. In addition, a study suggested that the expression of an appropriate level of IFN-γ activates the innate immune system (Schoenborn and Wilson, 2007). In our study, the mRNA level of IFN-γ in the spleen was upregulated with 50 and 500 mg/kg SS supplementation, while TNF-α was not (Li et al., 2022). To explain the observed activity, we considered the characteristics of the polysaccharide structure, which played the role of immune antigens to stimulate the immune system of laying hens. A previous
study also suggested that the activation of NF-κB could be inhibited by A20, which inhibits the expression of pro-inflammatory factors such as IL-1β and IL-6 (Beyaert et al., 2000). In the present study, the mRNA level of A20 was upregulated; conversely, the mRNA levels of NF-κB and IL-6 were downregulated in the 50 and 500 SS groups. This suggests that an appropriate dose of SS can regulate intestinal inflammation. A possible explanation is that SS might be metabolized by bacteria into other substances in the intestine to regulate the A20-NF-κB pathway, which regulates the immune function of the intestinal mucosa.

Overall, we believe that the immune and antioxidant functions of laying hens can be improved with an appropriate dose of SS supplementation. In contrast, high doses of SS might have a negative effect on laying hens. The elevation of MDA content in the liver that we found in our study might be due to this effect. Our previous work illustrated that eggshell quality improved with 50 and 500 mg/kg SS supplementation, and dietary 50 mg/kg SS for 10 weeks could improve egg-laying performance. Unexpectedly, dietary 500 mg/kg SS for 10 weeks was harmful to the hens (Li et al., 2022). Our team is currently conducting further research in this area. Although a detailed investigation of the mechanisms is beyond the scope of this work, we acknowledge that a suitable dose of SS surprisingly improved the antioxidant and immune functions of birds. Our research provides a theoretical reference for the alternative use of soybean meal in animal diet formulations.

Conclusion

The antioxidant and immune functions of laying hens were improved by adding 50 and 500 mg/kg SS to low soybean meal diets. Additionally, the spleen and intestine were found to be potential target tissues for SS to play a biological role in the body.

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Competing of Interests

The authors declare that they have no competing interests.

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Li et al.: Effects of Soy Saponin on Layers

205

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