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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

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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

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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 NF-E2-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,

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 $(HCO_3)_2$	1.860	Total phosphorus (%)	0.76
NaCl	0.350	Available phosphorus (%)	0.43
Trace minerals[b]	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[a]	0.030		
Antioxidants	0.030		
Phytase	0.016		
Zeolite powder	0.200		
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: 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℃ for 15 min. The ileum, spleen, liver, ovary, 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℃ 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 wattle 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 =(thickness of the right shank after injection thickness of the right shank before injection) / (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**[a]

Gene name[b]		Prime sequence (5′−3′)	Gene Bank	Product size, bp
LYZ	F	CCCAGGCTCCAGGAACCT	NM_205281	102
	R	CACGCTCGCTGTTATGTCTGA		
AVBD7	F	ATGGAATAGGCTCTTGCTGTG	NM_001001194.1	119
	R	GCCAGATAGAATGGAGTTGGAG		
Leap2	F	CTCAGCCAGGTGTACTGTGCTT	NM_001001606.1	66
	R	CGTCATCCGCTTCAGTCTCA		
TNF-α	F	CCCCTACCCTGTCCCACAA	NM_204267.1	67
	R	TGAGTACTGCGGAGGGTTCAT		
IFN-γ	F	CTTCCTGATGGCGTGAAGA	NM_205149.1	127
	R	GAGGATCCACCAGCTTCTGT		
A20	F	GACATCGTGCTAACAGCTTGGA	XM_003640919.2	180
	R	AGAAAAGAGGGTATCAGGCACAAC		
NF-κB	F	TGGAGAAGGCTATGCAGCTT	NM_205134.1	117
	R	CATCCTGGACAGCAGTGAGA		
IL-6	F	GCGAGAACAGCATGGAGATG	AJ621249	82
	R	GTAGGTCTGAAAGGCGAACAG		
IL-8	F	GGCTTGCTAGGGGAAATGA	NM_205498.1	200
	R	AGCTGACTCTGACTAGGAAACTGT		
Nrf-2	F	ATCACCTCTTCTGCACCGAA	NM_205117.1	229
	R	GCTTTCTCCCGCTCTTTCTG		
SOD	F	CCGGCTTGTCTGATGGAGAT	NM_205064.1	125
	R	TGCATCTTTTGGTCCACCGT		
GPX-Px	F	GACCAACCCGCAGTACATCA	NM_001277853.2	204
	R	GAGGTGCGGGCTTTCCTTTA		
β-actin	F	GAGAAATTGTGCGTGACATCA	NM_205518.1	152
	R	CCTGAACCTCTCATTGCCA		

[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℃ 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;

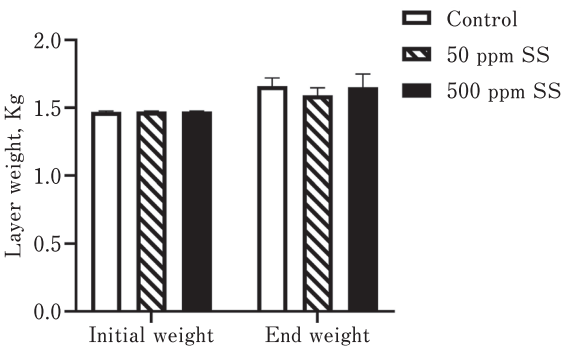

Fig. 1. **The effects of soy saponin (SS) on the body weight of laying hens.** Data represent the mean ± SEM ($n=6$) from our previous study (Li *et al*., 2022).

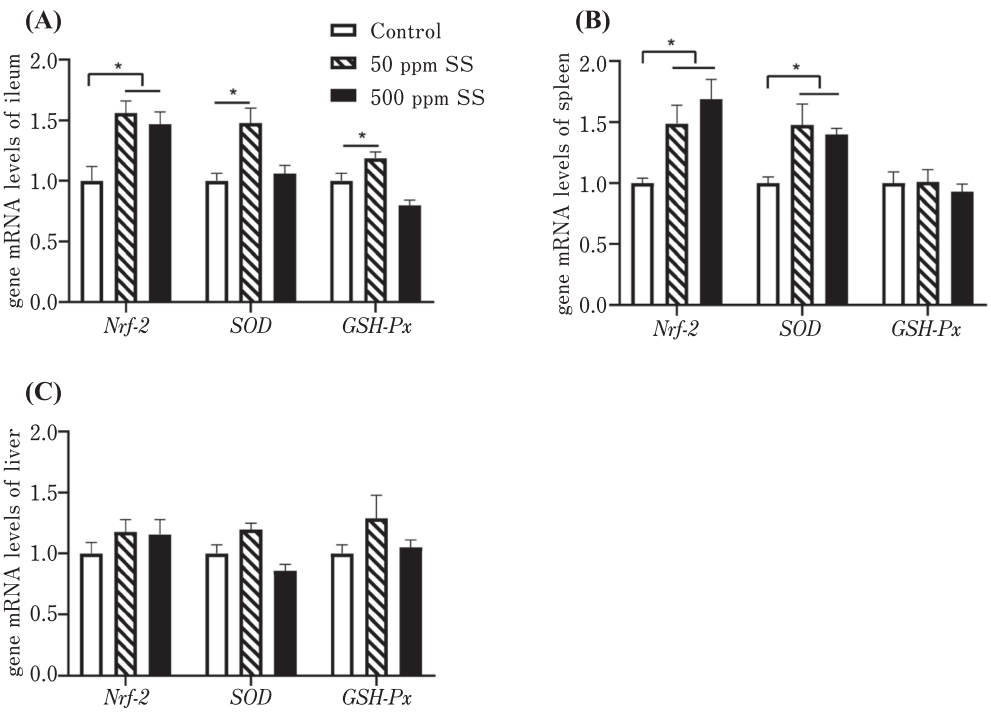

Fig. 2. **The effect of soy saponin (SS) on the gene mRNA levels of *Nrf-2*, *SOD*, and *GSH-px* in the ileum (A), spleen (B), and liver (C). Data represent the mean ± SEM ($n=8$).** * represents a significant difference ($P<0.05$).

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±0.33[a]	4.03±0.20[ab]	4.56±0.22[b]	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

[a~b] 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±0.29[a]	5.26±0.29[a]	6.87±0.51[b]	<0.001
	MDA (nmol/mL)	6.58±0.36[b]	4.81±0.21[a]	4.95±0.28[a]	<0.001
	SOD (U/mL)	201.09±1.67[a]	210.15±2.79[b]	203.18±2.69[ab]	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±1.46[a]	24.12±1.40[b]	19.35±1.27[a]	0.012
	MDA (nmol/mg protein)	1.86±0.09[b]	1.29±0.09[a]	1.78±0.16[b]	0.005
	SOD (U/mg protein)	217.07±11.32[a]	260.93±16.19[b]	255.06±9.71[b]	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±0.13[b]	0.97±0.05[a]	1.03±0.11[ab]	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

[a~b] 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

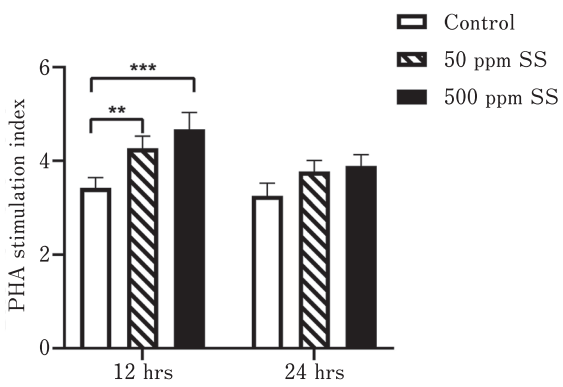

Fig. 3. **The effect of soy saponin (SS) on the stimulus index of PHA. Data represent the mean ± SEM (n=8).** ** and *** represent a significant difference ($P<0.01$ and $P<0.001$), respectively.

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[a]	74.40±1.06[a]	77.75±1.02[b]	0.003
NETU%	27.63±0.67	26.75±0.47	27.25±0.50	0.543
NETU#	19.95±0.34[a]	19.89±0.38[a]	21.18±0.30[b]	0.024
LYMPH%	56.61±0.70	58.69±0.54	57.44±0.86	0.140
LYMPH#	40.95±0.63[a]	43.68±0.86[b]	44.65±0.69[b]	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

[a~b] 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; LYMPH%, ratio of lymphocytes to WBC; LYMPH#, 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[a]	76.74±1.56[b]	76.98±1.49[b]	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
LYMPH%	57.13±0.62	58.21±0.62	57.66±1.32	0.706
LYMPH#	40.55±0.70[a]	44.69±1.12[b]	44.33±1.03[b]	0.011
MONO%	7.29±0.47	8.31±0.42	8.76±0.77	0.202
MONO#	5.16±0.32[a]	6.39±0.37[ab]	6.80±0.68[b]	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

[a~b] 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; LYMPH%, the ratio of lymphocytes to WBC; LYMPH#, 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.

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-1are 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

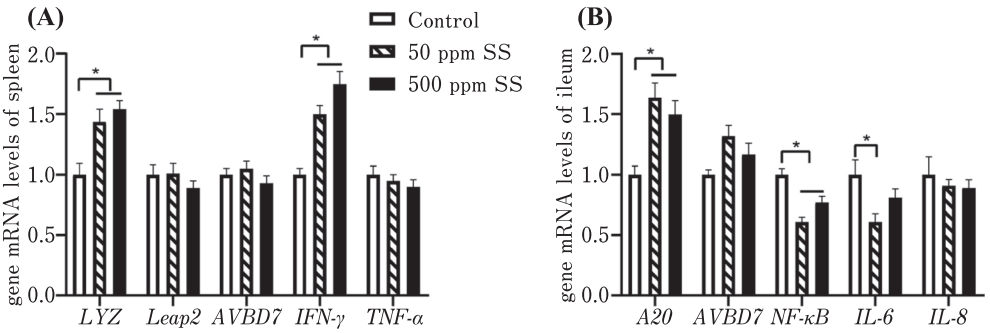

Fig. 4. **The effects of soy saponin (SS) on the mRNA levels of immune-related genes in the spleen (A) and ileum (B).** The data of *IFN-γ* and *TNF-α* in spleen were from our previous study (Li *et al*., 2022). Data represent the mean ± SEM (n=8). * represents a significant difference (P<0.05).

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 saponols, 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 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, lysozymes 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-1*β and *IL-6* (Beyaert *et al*., 2000). In the present study, the mRNA level of *A20* was upregulated; conversely, the mRNA levels of *NF-*κ*B* 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-*κ*B* 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.

References

Baird L and Yamamoto M. The molecular mechanisms regulating the KEAP1-NRF2 pathway. Molecular and Cellular Biology, 40. e00099−20. 2020.

Beyaert R, Heyninck K and Van-Huffel S. A20 and A20-binding proteins as cellular inhibitors of nuclear factor-kappa B-dependent gene expression and apoptosis. Biochemical Pharmacology, 60: 1143−1151. 2000.

Carrick JB and Begg AP. Peripheral blood leukocytes. Veterinary Clinics of North America: Equine Practice, 24: 239−259. 2008.

Chen J, Sun S, Zha D, Wu J, Mao L, Deng H, Chu X, Luo H and Zha L. Soyasaponins prevent H_2O_2-induced inhibition of gap junctional intercellular communication by scavenging reactive oxygen species in rat liver cells. Nutrition and Cancer, 66: 1342−1351. 2014.

Chrystal PV, Moss AF, Khoddami A, Naranjo VD, Selle PH and Liu SY. Effects of reduced crude protein levels, dietary electrolyte balance, and energy density on the performance of broiler chickens offered maize-based diets with evaluations of starch, protein, and amino acid metabolism. Poultry Science, 99: 1421−1431. 2020.

Delhaye J, Salamin N, Roulin A, Criscuolo F, Bize P and Christe P. Interspecific correlation between red blood cell mitochondrial ROS production, cardiolipin content and longevity in birds. Age (Dordr), 38: 433−443. 2016.

Fan H, Lv Z, Gan L and Guo Y. Transcriptomics-related mechanisms of supplementing laying broiler breeder hens with dietary daidzein to improve the immune function and growth Performance of offspring. Journal of Agricultural and Food Chemistry, 66: 2049−2060. 2018.

Fu WJ, Stromberg AJ, Viele K, Carroll RJ and Wu G. Statistics and bioinformatics in nutritional sciences: analysis of complex data in the era of systems biology. Journal of Nutritional Biochemistry, 21: 561−572. 2010.

Goto N, Kodama H, Okada K and Fujimoto Y. Suppression of phytohemagglutinin skin response in thymectomized chickens. Poultry Science, 57: 246−250. 1978.

Guang C, Chen J, Sang S and Cheng S. Biological functionality of soyasaponins and soyasapogenols. Journal of Agricultural and Food Chemistry, 62: 8247−8255. 2014.

Haque MM, Murale DP, Kim YK, and Lee JS. Crosstalk between Oxidative Stress and Tauopathy. International Journal of Molecular Sciences, 20: 1959. 2019.

Huang Y, Li WJ, Su ZY, and Kong AN. The complexity of the Nrf2 pathway: beyond the antioxidant response. Journal of Nutritional Biochemistry, 26: 1401−1413. 2015.

Kim YR, Kim EY, Choi SY, Hossain MT, Oh R, Heo WS, Lee IM, Cho YC, and Kong IS. Effect of a probiotic strain, Enterococcus faecium, on the immune responses of olive flounder (Paralichthys olivaceus). Journal of Microbiology and Biotechnology, 22: 526−529. 2012.

Kovac S, Angelova PR, Holmstrom KM, Zhang Y, Dinkova-Kostova AT and Abramov AY. Nrf2 regulates ROS production by mitochondria and NADPH oxidase. Biochimica et Biophysica Acta-General Subjects, 1850: 94−801. 2015.

Lee CY, Song AA, Loh TC and Abdul Rahim R. Effects of lysine and methionine in a low crude protein diet on the growth performance and gene expression of immunity genes in broilers. Poultry Science, 99: 2916−2925. 2020.

Lee IA, Park YJ, Yeo HK, Han MJ and Kim DH. Soyasaponin I attenuates TNBS-induced colitis in mice by inhibiting NF-kappaB pathway. Journal of Agricultural and Food Chemistry, 58: 10929−10934. 2010.

Lee MO, Jang HJ, Rengaraj D, Yang SY, Han JY, Lamont SJ, and Womack JE. Tissue expression and antibacterial activity of host defense peptides in chicken. BMC Veterinary Research, 12: 231. 2016.

Li P, Zhao Y, Yan S, Song B, Liu Y, Gao M, Tang D and Guo Y. Soy saponin improves egg-laying performance and immune function of laying hens. Journal of Animal Science and Biotechnology, 12: 126. 2022.

Lijie Z, Ranran F, Xiuying L, Yutang H, Bo W and Tao M. Soyasaponin Bb protects rat hepatocytes from alcohol-induced oxidative stress by inducing heme oxygenase-1. Pharmacognosy Magazine, 12: 302−306. 2016.

Lin J, Cheng Y, Wang T, Tang L, Sun Y, Lu X and Yu H. Soyasaponin

Ab inhibits lipopolysaccharide-induced acute lung injury in mice. International Immunopharmacology, 30: 121−128. 2016.

Liu D, Guo Y, Wang Z, and Yuan J. Exogenous lysozyme influences Clostridium perfringens colonization and intestinal barrier function in broiler chickens. Avian Pathology, 39: 17−24. 2010.

Liu X, Chen K, Zhu L, Liu H, Ma T, Xu Q and Xie T. Soyasaponin Ab protects against oxidative stress in HepG2 cells via Nrf2/HO-1/NQO1 signaling pathways. Journal of Functional Foods, 45: 110−117. 2018.

Mohamed LA, El Hindawy MM, Alagawany M, Salah AS and El Sayed SAA. Effect of low- or high-CP diet with cold-pressed oil supplementation on growth, immunity and antioxidant indices of growing quail. Journal of Animal Physiology and Animal Nutrition, 103: 1380−1387. 2019.

Naveed G, Ehtisham-Ul-Haque S, Khan I, Rahman SU, Anam S, Usman M, Shakir MZ, Naveed A, Abbas G and Anjum FR. Enhancement in humoral response against inactivated Newcastle disease vaccine in broiler chickens administered orally with plant-derived soyasaponin. Poultry Science, 99: 1921−1927. 2020:

Newsholme P, Cruzat VF, Keane KN, Carlessi R, de Bittencourt PJ. Molecular mechanisms of ROS production and oxidative stress in diabetes. Biochemical Journal, 473: 4527−4550. 2016.

Reiter RJ, Tan DX, Rosales-Corral S, Galano A, Zhou XJ and Xu B. Mitochondria: Central organelles for melatonin's antioxidant and anti-aging Actions. Molecules, 23. 2018.

Schoenborn JR, and Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Advances in Immunology, 96: 41−101. 2007.

Sharifi MR, Khajali F and Hassanpour H. Antioxidant supplementation of low-protein diets reduced susceptibility to pulmonary hypertension in broiler chickens raised at high altitude. Journal of Animal Physiology and Animal Nutrition, 100: 69−76. 2016.

Sullivan KS and Erf GF. CD4+ T cells dominate the leukocyte infiltration response initiated by intra-dermal injection of phytohemagglutinin into growing feathers in chickens. Poultry Science, 96: 3574−3580. 2017.

Su LW, Cheng YH, Hsiao FS, Han JC and Yu YH. Optimization of mixed solid-state fermentation of soybean meal by lactobacillus species and clostridium butyricum. Polish Journal of Microbiology, 67: 297−305. 2018.

Tella JL, Lemus JA, Carrete M and Blanco G. The PHA test reflects acquired T-cell mediated immunocompetence in birds. PLoS One, 3: e3295. 2008.

Xie Q, Gu X, Chen J, Liu M, Xiong F, Wu X, Zhang Y, Chen F, Chen H, Li M, Sun S, Chu X and Zha L. Soyasaponins reduce inflammation and improve serum lipid profiles and glucose homeostasis in high fat diet-induced obese mice. Molecular Nutrition & Food Research, 62: e1800205. 2018.

Zha L, Chen J, Sun S, Mao L, Chu X, Deng H, Cai J, Li X, Liu Z and Cao W. Soyasaponins can blunt inflammation by inhibiting the reactive oxygen species-mediated activation of PI3K/Akt/NF-kB pathway. PLoS One, 9: e107655. 2014.

Zhang Y, Chen F, Chen J, Huang S, Chen J, Huang J, Li N, Sun S, Chu X and Zha L. Soyasaponin Bb inhibits the recruitment of toll-like receptor 4 (TLR4) into lipid rafts and its signaling pathway by suppressing the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent generation of reactive oxygen species. Molecular Nutrition & Food Research, 60: 1532−1543. 2016.

Zhao D, Wu T, Yi D, Wang L, Li P, Zhang J, Hou Y and Wu G. Dietary supplementation with Lactobacillus *casei* alleviates lipopolysaccharide-induced liver injury in a porcine model. International Journal of Molecular Sciences, 18. 2017.