Soya saponin fails to improve the antioxidation and immune function of laying hens with antibiotics treated

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ABSTRACT Soya saponin (**SS**) helps to improve antioxidant and immune function of body, and intestinal bacteria might play an important role here. In the present study, the co-occurring network of the ileal flora was analyzed with 50 mg/kg SS supplemented to the diet, and *Romboutsia* was found to have evolved into a dominant flora. In addition, the co-occurring network of the flora was changed with the combined antibiotic treated, and the *unidentified-cyanobacteria* developed into the dominant flora, whereas the relative abundance of *Romboutsia* was dropped. Dietary SS failed to elevate the relative abundance of *Romboutsia* with antibiotics treated, at the same time, it was not helpful for the antioxidant and immune function of laying hens. While dietary SS had a little help on the egg-laying performance. Intestinal bacteria did play a key role in the biological functions of SS on laying hens. In conclusion, SS failed to improve the antioxidation and immune function of laying hens with antibiotics treated.

Key words: soya saponin, laying hen, antioxidation, immune, intestinal flora

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INTRODUCTION

Soya saponin (**SS**) is a biologically active molecule in legumes and which is found in daily foods such as tofu, bean sprouts, soy milk, and other soy products (Guang et al., 2014). Study suggested that SS contributed to the health of human. Specifically, SS was helpful for improving lipid metabolism, antioxidation and regulating immune functions (MacDonald et al., 2005; Lee et al., 2010; Yang et al., 2015). Additionally, SS offered exciting opportunities to antivirus and antitumor (Chen et al., 2014; Wang et al., 2019a). While SS was regarded as an anti-nutritional factor in the animal husbandry field many years ago, which was not conducive to the digestion and absorption of the gastrointestinal tract of livestock and poultry (Su et al., 2018). Interestingly, our previous study found that dietary 50 mg/kg SS improved the egg-laying performance and immune function, and high-dose SS did show a negative impact on the physiological functions of laying hens (Li et al., 2022b). The fact that SS was helpful to the immune function of birds had also been confirmed in the study of other scholars (Naveed et al., 2020). Another study of ours also suggested that SS was able to improve the antioxidation functions of laying hens (Li et al., 2022a). We hold that the intestinal flora was of great importance for the biological importance of SS to the birds.

To gain more insight about the relationship between intestinal flora and nutrients, a model of low intestinal bacteria was established, and combination antibiotics were considered one of the effective strategies. The combination of streptomycin, ampicillin and neomycin could drop the diversity of the cecal flora of Peking ducks (Wang et al., 2019b). The combination of vancomycin, ampicillin, neomycin and metronidazole was widely used to construct low-bacterial models in the intestinal tract of mice (Vijay-Kumar et al., 2010; Wang et al., 2011). In the present study, the co-occurring network of intestinal flora was analyzed to deeply investigate the relationship between intestinal flora and SS. In addition, the combination of vancomycin, ampicillin, neomycin and metronidazole was used to descend the diversity of the intestinal flora, and the biological role of SS on layer hens was studied with antibiotics treated. This study aimed at investigating the effects of SS on the antioxidation and immune function of laying hens with antibiotics treated.

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MATERIALS AND METHODS

All procedures adapted for the experiment were approved by the Animal Ethics Committee of China Agricultural University, Beijing, China. The animal welfare number was AW92601202-1-2.

Experiment Design and Animal Management

To further study the influence of SS on the intestinal flora, the ileal flora data of laying hens with or without SS treated in the previous study (Li et al., 2022b) were used for co-occurrence network analysis. With SS supplementation, the dominant flora in the intestines was identified, and correlation analysis was carried out between this bacteria and related indexes of immunity and egg production performance. The relationship between the dominant flora and the immune function and laying performance were clarified.

And then, combination antibiotics were used to build a low-bacterial model in the intestine, and the effect of SS on the immune function, antioxidant and laying performance of laying hens was investigated. A total of 180 Hy-line gray layer hens with 22-wk-old were randomly assigned to 2 treatment groups, 6 replicates per each and 15 birds per replicates. All birds equipped with uniform egg production rate (47 ± 0.02%) and similar weight (1,470 ± 10 g). The diet formula was formulated with reference to the feeding standards of Chinese chickens (NY/T33-2004) (Table 1), and then 400 mg/kg ampicillin, 400 mg/kg neomycin, 400 mg/kg metronidazole, and 200 mg/kg vancomycin (antibiotics were purchased from Wuhan Dongkang Technology Development Co., Ltd.) were supplemented the diet to prepare the basal diet. The birds in control group (A group) were fed with basal diet, and the basal diet with 50 mg/kg SS (Xi'an Tongze Biotechnology Co., Ltd., China; its purity was 45.1%) supplemented was fed to the birds in treatment group (A+ 50 SS). All birds eat and drink freely, and the temperature of breeding environment was controlled at 25 ± 3°C, and a 16-h light: 8-h dark lighting program was used. The feeding cycle lasted for 5 wk, and the egg production rate was counted once a week. At the end of the trial, all eggs in a replicate of each treatment were collected for egg quality statistics. Additionally, one bird per replicate from each treatment was randomly selected to harvest the blood from the wing vein, and then slaughter under sodium pentobarbital anesthesia (50 mg/kg BW) to collect the spleen, ovaries, ileum, and ileal chyme for analysis.

Table 1. Test diet composition and nutrition level.

Ingredients		Nutritional parameters	Levels[3]
Corn (7.8% protein)	67.550	Metabolizable energy (ME, Mcal/kg)	2.70
Dephenolized cotton-seed 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
$CaHPO_4$	1.860	Total phosphorus (%)	0.76
NaCl	0.350	Available phosphorus (%)	0.43
Trace minerals[2]	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		
Multivitamins[1]	0.030		
Antioxidants	0.030		
Phytase	0.016		
Zeolite powder	0.200		
Total	100		

[1]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.

[2]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.

[3]Calculated value based on the analysis of experimental diets.

Egg-Laying Performance and Egg Quality

According to the method described in previous study (Li et al., 2022b), the egg-laying rate and the feed to egg ratio were calculated. The egg quality tester DET-6000 (NABEL Co., Ltd, Japan) was used to measure the haugh unit, albumen height, eggshell strength (kg/cm^2), and egg yolk color after weighing the eggs. The thickness of the eggshell was measured with a micrometer (accurate to 0.001 mm). Briefly, the shell thickness of the blunt end, the middle and the sharp end of the egg were measured to calculate the average of the three after removing the shell membrane from the eggshell.

Anti-Oxidase and Serum Hormones and Immune Related Indicator

The serum was harvested by centrifugation at 3,000 × *g* and 4°C for 15 min. According to the principle of weight: volume = 1: 9, spleen and ileum were mixed with saline separately to obtain homogenate. The mixture was centrifuged at 3,500 × *g* and 4°C for 10 min, and the supernatant was collected for later use. The kits from Nanjing Jian cheng Biotechnology Co., Ltd., China were used to measure the levels of superoxide dismutase (**SOD**), catalase (**CAT**), and malondialdehyde (**MDA**) in the serum, spleen, and ileum. The contents of luteinizing hormone (**LH**), follicle stimulating hormone (**FSH**), and estradiol (**E2**) in the serum were detected by radioimmunoassay using commercial kit (Beijing Northern Biotechnology Institute, Beijing, China), and the detection coefficient of variation was less than 10%. Following the method described in the kit (Nanjing Jian cheng Biotechnology Co., Ltd.), serum complement (**C3**), lysozyme, and *β*-defensins were measured.

Table 2. List of gene primer sequences[1].

Gene name		Prime sequence (5′-3′)	Product size, bp
GnRH1	F	GGCTCAACACTGGTCTTATGG	202
	R	TCTTCTGGCTTCTCCTTCG	
ERR	F	GTACGGCTCTACTACACTCAGTTATGC	160
	R	CTGCTGGCTGTGGTGATGGATG	
LHR	F	CGTCCTCATAACCAGCCACTACAAG	119
	R	TCTGAGCATCCACCGAAGCAATG	
FSHR	F	GTCTCACCTGCTTGCTGATTCTCC	99
	R	CCTTGATCTCCTGGCAGATGAATATCC	
β-actin	F	GAGAAATTGTGCGTGACATCA	152
	R	CCTGAACCTCTCATTGCCA	

Abbreviations: *ERR*, estrogen related receptor, *FSHR*, follicle stimulating hormone receptor; *GnRH1*, gonadotropin releasing hormone, *LHR*, luteinizing hormone receptor.
[1]Primers designed using Primer Express software (Sangon Biotech, Shanghai, China).

ELISA kits (IDEXX laboratories lnc., Weatbrook, ME) were used to determine the levels of serum interleukin-4 (**IL-4**) and interferon-γ (**IFN-γ**). Elisa kits (Beijing Solarbio Biotechnology Co., Ltd., China) were used to evaluate the contents of immunoglobulin G (**IgG**), IgA, and IgM in the serum.

Peripheral Blood Lymphocyte Stimulation Index and Ratio

Based on the method of previous study (Fan et al., 2018), the chicken peripheral blood lymphocyte separation solution from Tianjin Hao yang Biological Co., Ltd., China was used to separate lymphocytes, and then those lymphocytes were washed for 3 times by the RPMI 1640 (Invitrogen Corp., Grand Island, NY) in complete culture medium. Finally, these cells were resuspended into 2 mL of RPMI 1640 complete culture medium, and the concentration of cells was adjusted to 1×10^7 cell/m L. A 1 mL clean tube was stained with IgG1κ mouse anti-chicken-Bu-1-PB-labeled antibody (8395−26), IgG1κ mouse anti-chicken- CD45-PE- labeled antibody (8270-09), and IgG1κ mouse anti-chicken-CD3-APC-labeled antibody (8200−11) (Southern Biotechnology Associates Inc., Birmingham, AL), and a volume of 100 μL of lymphocytes extracted above was added into this tube for incubation about 45 min at room temperature. The cells were washed twice and adjusted to a final volume of 500 μL, and then the 3-color flow cytometric analysis was conducted using a Navios EX flow cytometer with 10 colors (Beckman Coulter Corp., Fullerton, CA) at Xi-Yuan Traditional Chinese Medicine Hospital, Chinese Academy of Medicine Science, China. As for data analysis, the CD45 ring gate was used to eliminate the interference of red blood cells, and then the ratio of $CD3^+$ and $Bu\text{-}1^+$ were obtained in the gate of $CD45^+$. Additionally, concanavalin A (ConA, 45μg/mL, Sigma-Aldrich, St. Louis, Missouri) and LPS (25 μg/mL, Sigma-Aldrich, St. Louis, Missouri) were used to stimulate lymphocytes extracted above according to the MTT method (Wagner et al., 1999), and the results were expressed in terms of the stimulus index (**SI**) value.

Gene Transcription Level

One mL Trizol (Invitrogen Life Technologies, Carlsbad, CA) was added into a 2 mL sterilized centrifuge tube, and then 0.1 g ovary was added into it for well mixed on ice. According to the method of previous study (Zhao et al., 2017), the total RNA was extracted and tested for purity, and the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) was used to reverse-transcribe the total RNA. The RT-PCR analysis of gene expression was performed using primers listed in Table 2, and the cDNA, primers, and SYBR Premix Ex TaqTM (Takara) were mixed to carry out the real-time PCR on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA). β-actin was used as an internal reference to analyze all genes, and the results were analyzed by the cycle threshold (**CT**) method from Fu et al. (2010).

Intestinal Flora

At the end of the trial, the chyme in the distal region of the ileum was collected. Based on the method described by (Zhang et al., 2018), sequencing and analysis were carried out. Briefly, the microbial DNA was extracted following the protocol of the fecal microbial DNA extraction kit (QIAamp Fast DNA Stool Mini Kit, Qiagen Company, Germany), and then the concentration of DNA was measured and quality controlled. The universal primers of 16SrDNA gene V3−V4 region were used to amplify bacterial DNA, and the primer sequence as follows, 338 F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). After the PCR products were purified and quantified, the sequencing library was established for on-machine sequencing on HiSeq2500 PE250. Sequencing analysis was completed by Beijing Nuohe Zhiyuan Bio-Information Technology Co., Ltd., China. The alpha diversity of the samples was analyzed, and R software

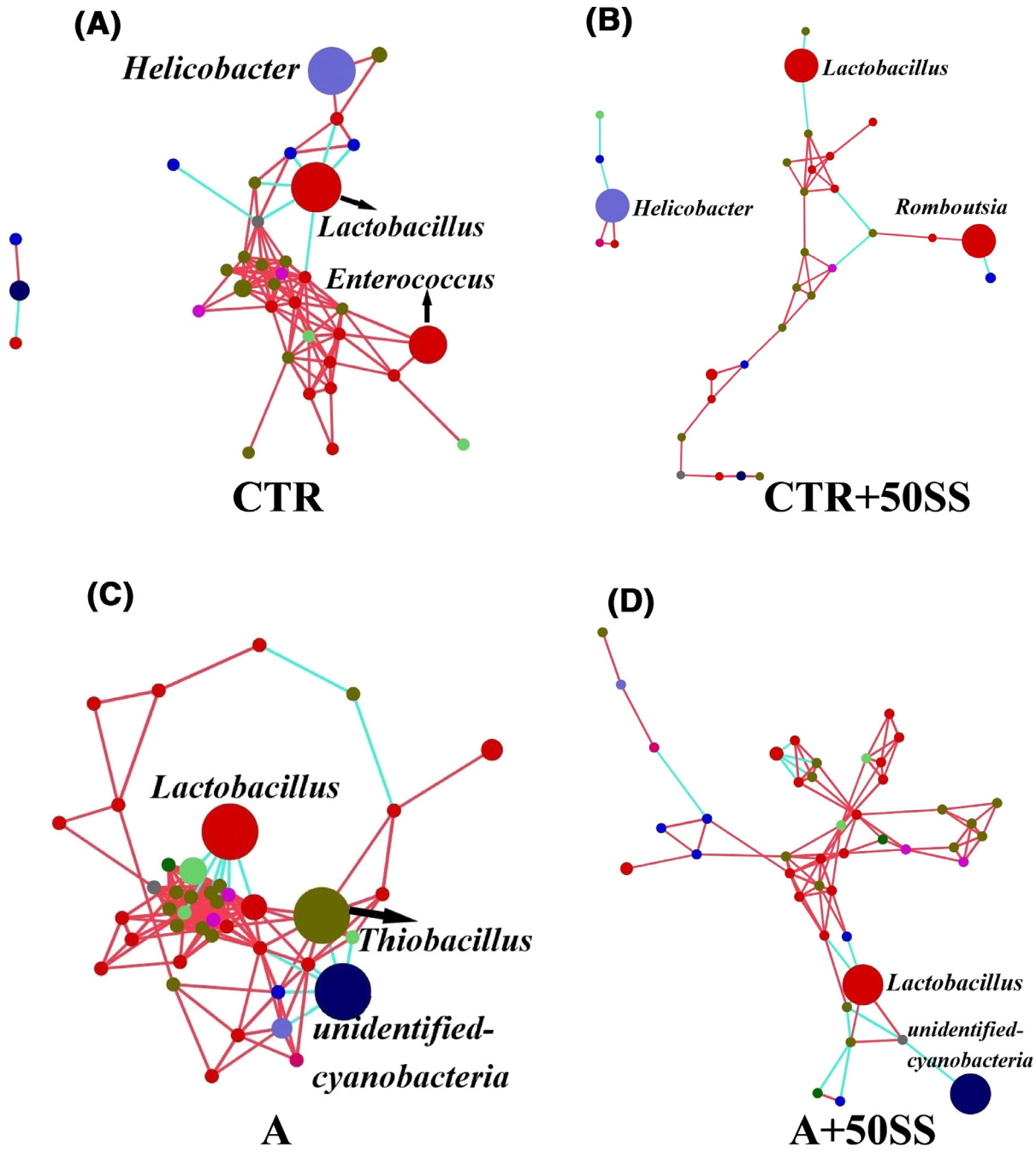

Figure 1. The results of ileal flora co-occurrence network analysis. The results of the ileal flora without antibiotic treatment were showed in (A) and (B), among them, the one without SS supplemented was showed in (A), and with SS treated in (B), and the analysis of these two sets of data were based on the results of previous studies (Li et al., 2022b). The results of the ileal flora with antibiotic treatment were showed in (C) and (D), among them, the one without SS supplemented was showed in (C), and with SS treated in (D). The nodes with the same color in the figure represented the same phylum level, and the larger the node, the higher the average relative abundance of the genus in the ileum, and only the dominant flora was marked in the figure. It should be noted that the node sizes cannot be compared between groups, only within groups. In addition, the red connecting lines between nodes indicated positive correlations and blue indicated negative correlations. (n = 6).

(Version 2.15.3) was used to analyze and draw the diagrams of petal diagram, PCoA, and Anosim. Qiime software (Qiime2-2019.7, Nature Biotechnology) was used to draw UPGMA clustering tree, and to generate species abundance tables of different taxonomic levels, and LEfSe analysis was performed to find the dominant flora based on the LDA value. Additionally, top 50 microbial genera were selected for calculation of Pearson's correlation coefficient, and then connections with a correlation coefficient greater than 0.6 and a significance level $P < 0.05$ were selected for network drawing (graphviz-2.38.0). It should be pointed out that the birds without antibiotics treated from our previous study (Li et al., 2022b) were bred together with the birds of the 2 treatment groups in this study, and their physical condition, diet and living environment are consistent.

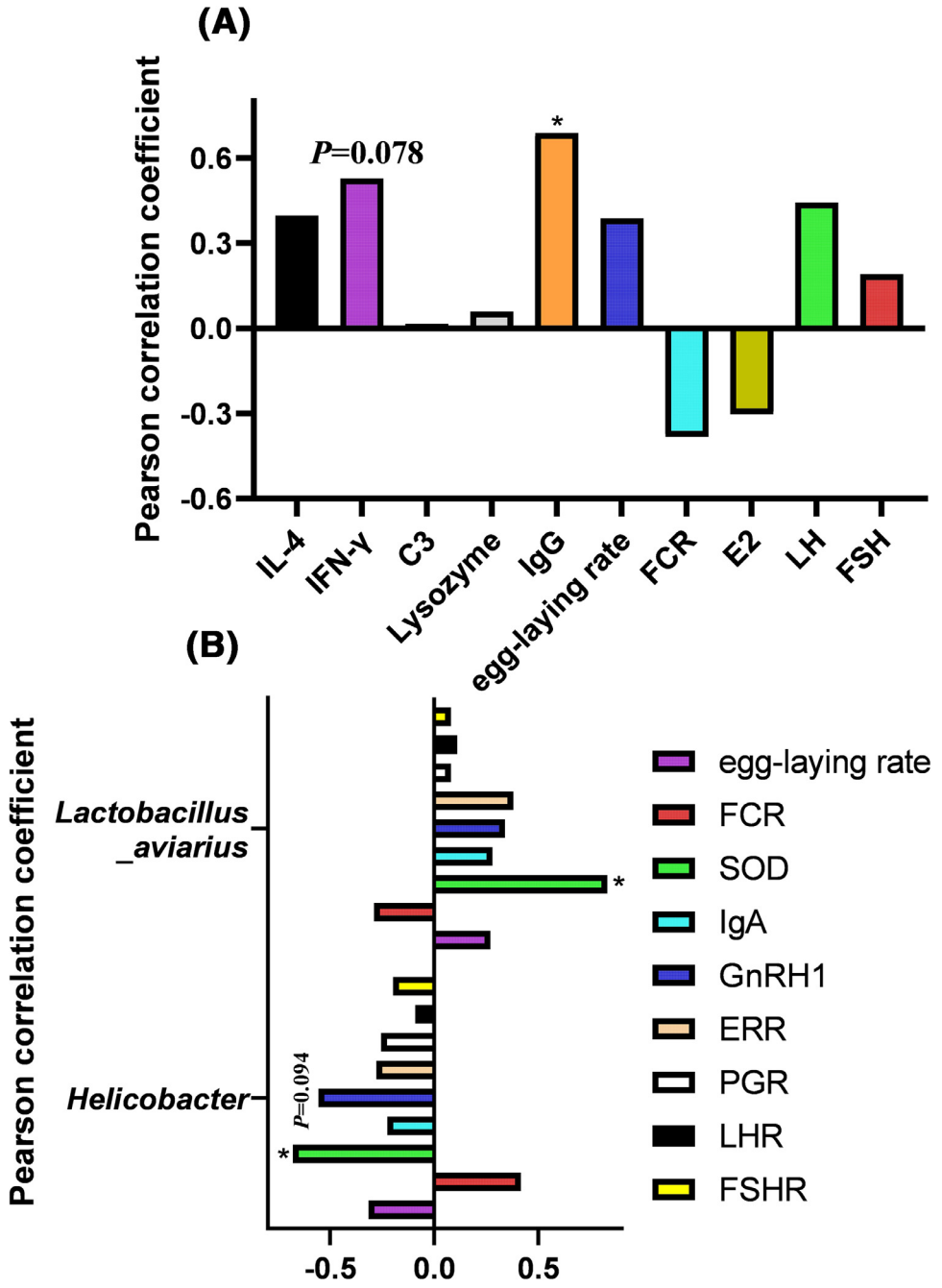

Figure 2. The results of correlation analysis between ileal flora and detection indicators. The results in (A) showed the correlation between *Romboutsia* and indicators of immune and egg production performance, the analysis of these data was based on our previous work (Li et al., 2022b). With antibiotics and SS treated, the results of correlation analysis between ileal flora and detection indicators were showed in (B). (n = 6).

Statistical Analysis

One-way ANOVA and Duncan's multiple comparisons of SPSS 23.0 software (SPSS Inc., Chicago, IL) were used to analyze the data of Shannon index and relative abundance of *Romboutsia*, and the independent sample t test was used to analyze the other data. The results were displayed in the form of mean ± standard deviation, and $P < 0.05$ was considered to be significant. Pearson's correlation analysis was used to analyze the correlation between bacteria and indicators, and Graphpad prism 8.0 software was used to graph the data.

RESULTS

Intestinal Bacterial Structure

The ileal flora data in our previous study (Li et al., 2022b) were subjected to a co-occurrence network analysis, and the 2 groups were the control group (**CTR**, basal diet without antibiotic) and the SS addition group (CTR+50 SS, the diet of CTR with 50 mg/kg SS supplemented). Results showed that dietary SS contributed to the development of *Romboutsia* as the dominant ileal flora (Figure 1B). Based on the date of our previous study (Li et al., 2022b),

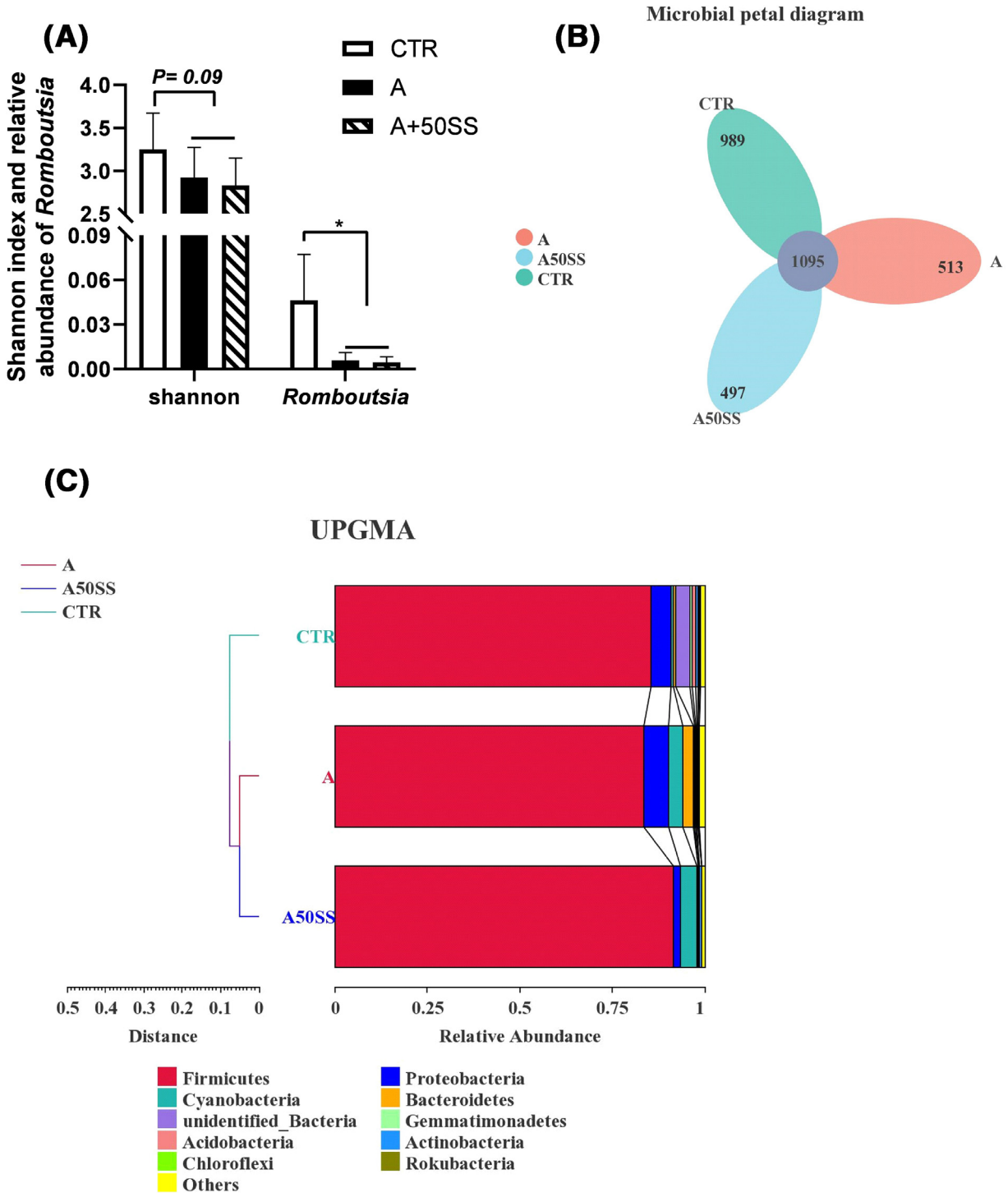

Figure 3. The results of diversity analysis about ileal flora. Among them, the Shannon index and the relative abundance of *Romboutsia* were arranged in (A). The petal diagram and UPGMA-weighted_unifrac clustering tree at phylum level based on the OTU analysis were shown in (B) and (C), respectively. CTR = the birds without antibiotics and SS treated, and the date of CTR were based on our previous work (Li et al., 2022b). A50SS = A+50 SS, and * was judged as a significant difference ($P < 0.05$). (n = 6).

Romboutsia was analyzed in relation to immunity and egg production performance, and the results showed that *Romboutsia* was positively correlated with serum IgG and IFN-γ, but not significantly correlated with egg production performance (Figure 2A). It illuminated us that *Romboutsia* might play an essential role in SS to improve immune immunity rather than egg production performance on laying hens.

To gain more insight, antibiotics were used to reduce the abundance of intestinal flora, results showed that antibiotic treatment altered the co-occurrence network relationships of the flora, and *unidentified-cyanobacteria* became the dominant flora in the intestine (Figure 1C). In addition, the α-diversity and the relative abundance of *Romboutsia* about the ileal flora were dropped with antibiotics treated, while dietary SS failed to raise the relative abundance of *Romboutsia* with antibiotics

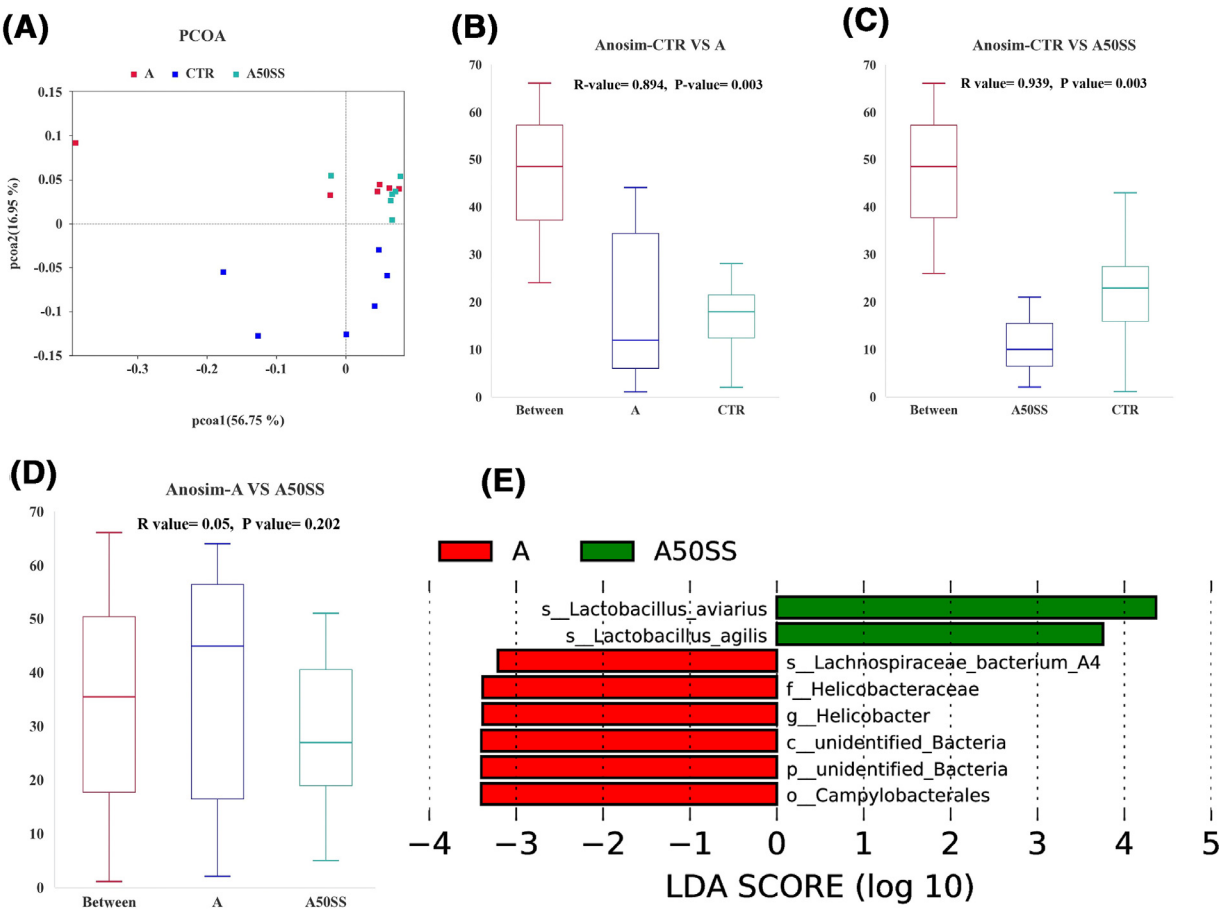

Figure 4. The results of PCoA, Anosim and LEFSe analysis about ileal flora. Among them, the result of PCoA-weighted_unifrac was arranged in (A), and the results of Anosim were shown from (B) to (D). The result of LEFSe analysis between A and A50SS group was arranged in (E). CTR= the birds without antibiotics and SS treated, and the date of CTR were based on our previous work (Li et al., 2022b). A50SS = A+50 SS, and * was judged as a significant difference ($P < 0.05$). (n = 6).

supplementation (Figure 3A). The number of OTUs in the antibiotic-treated groups was much lower than that of control (Figure 3B). Additionally, the bacterial structure of the antibiotic-treated group was different from that of control (Figure 3C), and the results of PCoA and Anosim analysis also came to a consistent conclusion (Figures 4A−4C). These evidence suggested that antibiotics perturbed the intestinal flora. Dietary SS had a little effect on the intestinal flora with antibiotics treated (Figure 4D), whereas LEFse analysis showed *Lactobacillus-aviarius* was the dominant flora in the A+ 50 SS group (Figure 4E). In addition, the relative abundance of *Lactobacillus-aviarius* was elevated (Figure 5F), and the relative abundance of *Helicobacter* was descended with SS supplementation (Figure 5D). In order to deeply investigate the influence of these changes in the flora on laying hens, the egg-laying performance, antioxidant, and immune function were analyzed.

Egg-Laying Performance

With antibiotics treated, the egg production during the trial was increased by 2.5% with SS supplementation (Figure 6B), this might be useful for producers. We also measured the levels of gene transcription related to egg production performance, and found the mRNA levels of *GnRH1* and *ERR* in the ovary tended to be upregulated, and the transcription of *FSHR* was heightened with SS added (Figure 6D), whereas the changes in hormone levels had not been observed (Figure 6C). Additionally, the eggshell thickness also tended to be improved with SS supplemented (Table 3). There was no correlation between the different intestinal flora and the indicators related to egg production performance (Figure 2B).

Antioxidant and Immune Function

Compared with the antibiotic treatment group, the level of serum SOD was elevated in the A+50 SS group (Table 4). However, there is no difference in the contents of antioxidant enzymes and MDA in the spleen and ileum did not change, hence we could not hold that dietary SS helped with the antioxidant function of laying hens with antibiotics treated. The increase in serum SOD level might be related to the increase in the relative abundance of *Lactobacillus-aviarius* and the decrease in the relative abundance of *Helicobacter* (Figure 2B). With antibiotics treated, dietary SS failed to improve the ratio of lymphocytes and the stimulation index of immunogen to lymphocytes (Figure 7), and there were

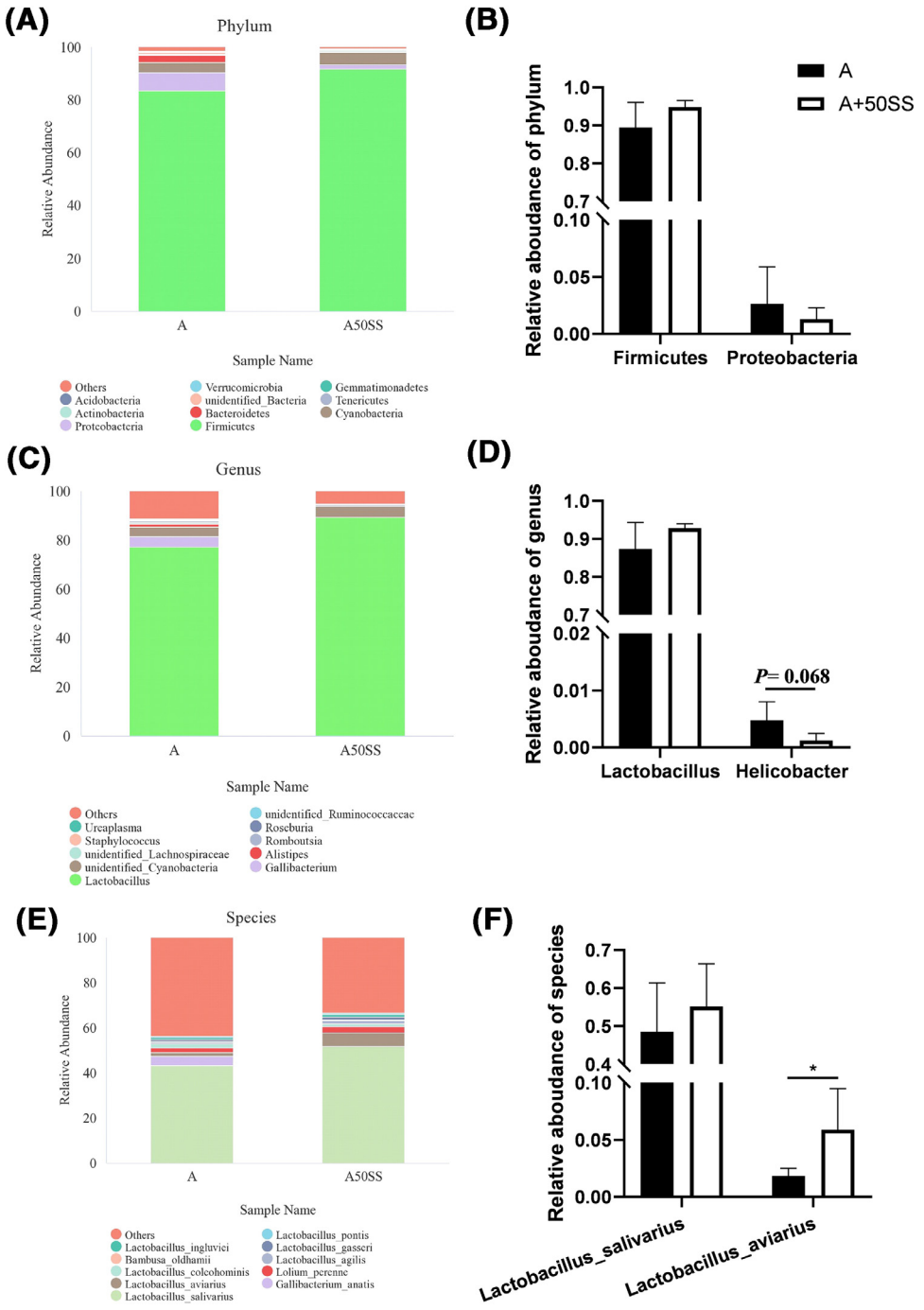

Figure 5. The effect of dietary SS on the relative abundance of ileal flora with antibiotics treated. Among them, A50SS = A+50 SS, and * was judged as a significant difference ($P < 0.05$). (n= 6).

no changes in the levels of serum immune molecules and cytokines (Table 5).

DISCUSSION

Intestinal bacteria regulate the health of the host by directly participating in the regulation of intestinal physiology, an example of this is the absorption and transport of nutrients, and the secretion of hormones (Abd El-Tawab et al., 2016). The intestinal flora also contributes to the host indirectly by metabolizing the nutrients in the diet. For instance, hericium erinaceus polysaccharide relieved colitis with the help of intestinal flora (Shao et al., 2019), and the improvement of lipid metabolism by tea polyphenols was attributed to the intestinal flora (Chen et al., 2019). Our previous study found that SS improved the immune function of laying hens might be related to the improvement of the structure of intestinal flora (Li et al., 2022b). In the present study, we further analyzed the influence of SS on the co-occurring network of intestinal flora, and found *Romboutsia* was not only the dominant flora in the SS addition group, but also positively correlated with immune-related indicators without antibiotics treated. Study suggested that *Romboutsia* had the ability to metabolize

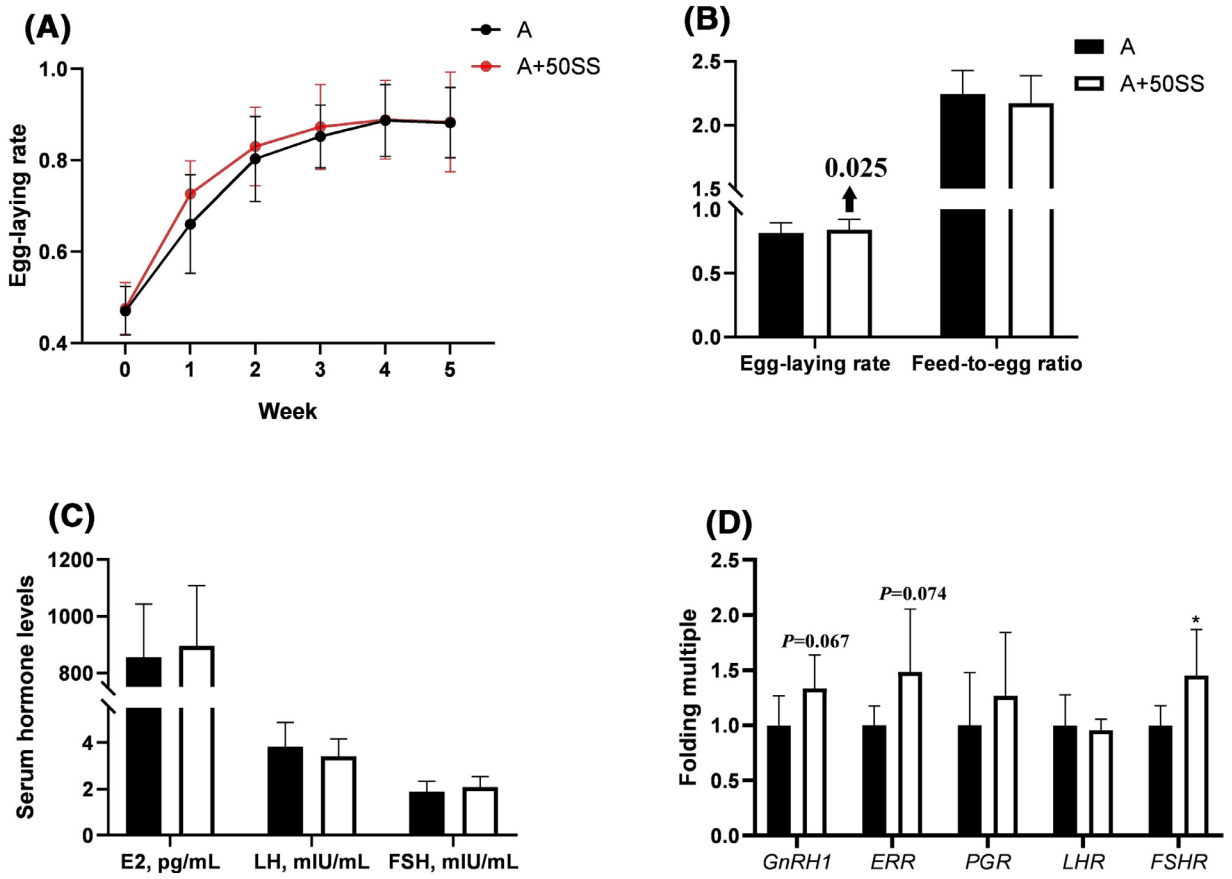

Figure 6. The effect of dietary SS on egg production performance with antibiotics treated. Among them, * was judged as a significant difference ($P < 0.05$). (n = 6).

a variety of carbohydrates into short-chain fatty acids, oligosaccharides and other prebiotics, and these metabolites were beneficial to the host (Zeng et al., 2019). Study also suggested *Romboutsia* was related to many diseases, by way of example, the relative abundance of *Romboutsia* in the intestine of patients with type I diabetes and ulcerative colitis was lower than that of healthy individuals (Gao et al., 2019; Russell et al., 2019). In this study, the relative abundance of *Romboutsia* was decreased with antibiotics treated, and dietary SS failed to raise the relative abundance of *Romboutsia*. Strikingly, dietary SS also failed to improve the immune function of laying hens with antibiotics treated. On the basis of our findings, it could be concluded that dietary SS might be helpful for the immune function of laying hens by increasing the relative abundance of *Romboutsia*. The direct interaction mechanism between SS and *Romboutsia* needed to be further investigated.

Study suggested that *Lactobacillus aviaries* helped to enhance the vitality of intestinal stem cells, and improve the intestinal flora structure and laying performance of laying hens (Hong et al., 2021). The relative abundance of *Lactobacillus aviaries* in healthy intestinal flora was far higher than that in sub-healthy (Zhu et al., 2020). Additionally, some scholars hold that *Lactobacillus aviaries* improve the absorption and utilization of dietary nutrients via reinforcing the activity of starch hydrolyzing enzymes (Meng et al., 2018). *Helicobacter* is one of the bacteria that normally exist in the gastrointestinal tract of humans and animals. As a conditional disease, the health of the host would also be adversely affected by changes in its relative abundance. Studies found that the excessive proliferation of *Helicobacter* could cause a variety of diseases, such as pancreatitis (Sjödin et al., 2011), cholangiohepatitis (Greiter-Wilke et al., 2006),

Table 3. The results of egg quality (n = 6).

Item	A	A+50SS	*P*-value
Eggshell thickness, mm	0.354 ± 0.009	0.360 ± 0.007	0.071
Eggshell strength, kg/cm^2	4.518 ± 0.348	4.540 ± 0.360	0.882
Albumen height, mm	9.679 ± 1.666	9.246 ± 2.231	0.595
Haugh unit	96.662 ± 8.879	93.606 ± 12.491	0.497
Egg yolk color	8.000 ± 0.402	8.028 ± 0.361	0.860

Table 4. The levels of antioxidant enzymes and products (n = 6).

Item		A	A+50SS	*P*-value
Serum	CAT, U/mL	5.71 ± 0.87	5.80 ± 1.28	0.895
	MDA, nmol/mL	5.84 ± 1.02	4.99 ± 0.55	0.102
	SOD, U/mL	200.77 ± 4.66	208.04 ± 3.68	0.013
Spleen	CAT, U/mg pro	5.58 ± 0.68	5.29 ± 0.60	0.458
	MDA, nmol/ mg pro	6.10 ± 1.04	6.59 ± 1.11	0.445
	SOD, U/ mg pro	358.01 ± 35.86	353.95 ± 33.19	0.843
Ileum	CAT, U/ mg pro	13.07 ± 3.19	15.48 ± 1.78	0.136
	MDA, nmol/ mg pro	1.25 ± 0.35	1.14 ± 0.32	0.613
	SOD, U/ mg pro	254.12 ± 41.40	260.51 ± 43.01	0.798

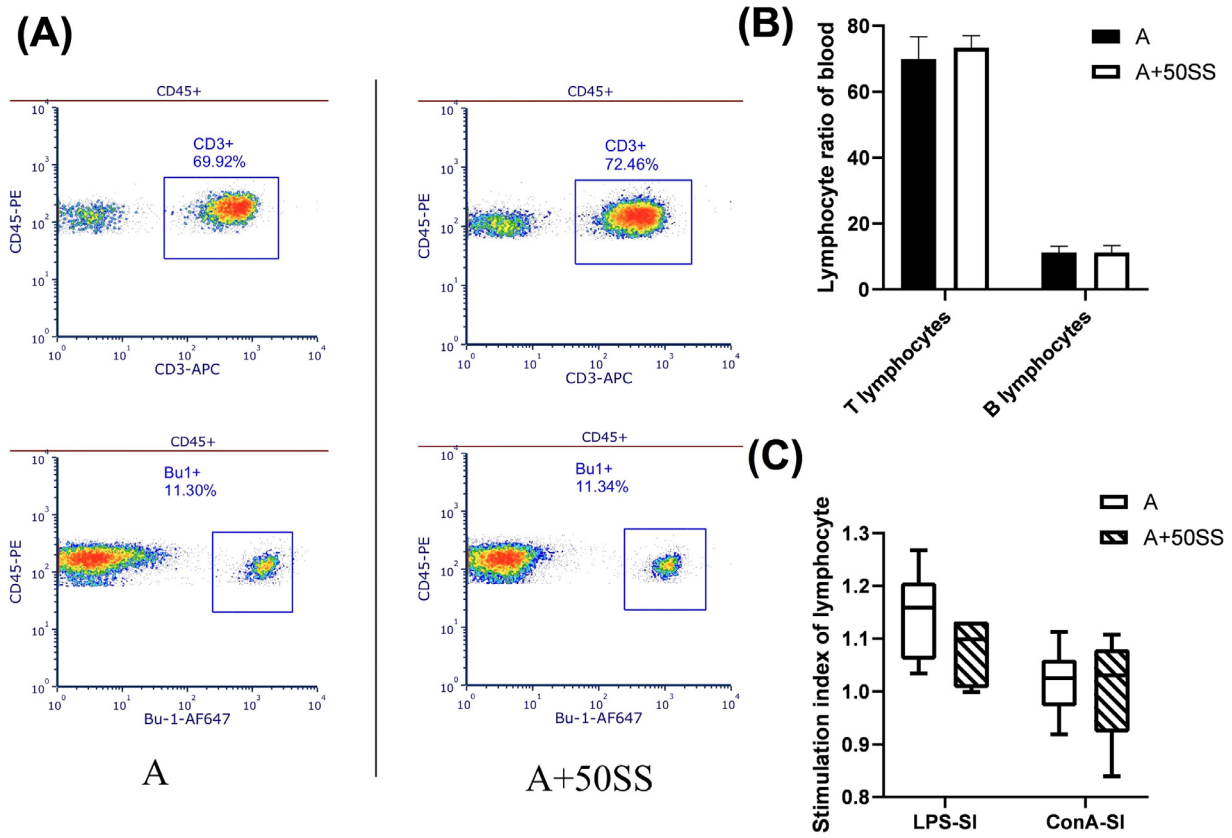

Figure 7. The effect of dietary SS on the stimulation index and ratio about peripheral blood lymphocyte with antibiotics treated. T lymphocytes were labeled with $CD3^+$, and $Bu1^+$ was used to B lymphocytes. (n = 6).

and some gastrointestinal diseases (Elyasi et al., 2020). In the present study, with antibiotics treated, dietary SS increased the relative abundance of *Lactobacillus aviaries* and decreased the relative abundance of *Helicobacter*. This seemed to help the birds, and we also observed that the changes of *Lactobacillus aviaries* and *Helicobacter* were encouragingly correlated with the level of serum SOD. Whereas combined with our overall results, we hold that dietary SS failed to improve the antioxidant function of laying hens with antibiotics treated. This was demonstrated in a number of studies that dietary SS contributed to improving the antioxidant function (Lijie et al., 2016; Elyasi et al., 2020). It illuminated us that intestinal flora played a leading role in improving the antioxidant function of laying hens by SS. This would be another research topic worthy of our attention.

Egg-laying performance was comprehensively regulated by environmental, endocrine, and genetic factors. The gonadotropin releasing hormone (**GnRH**), estradiol (**E2**), follicle stimulating hormone (**FSH**), and luteinizing hormone (**LH**) were regarded as the main endocrine factors. Among them, LH helps the ovaries to ovulate, and FSH contributes to stimulating the maturation of ovarian follicles (Tsutsui et al., 2010). During the laying period, the ovaries are cyclically ovulating and accompanied by periodic changes in the secretion of ovarian sex hormones (Sharp and Blache, 2003). Study suggested that the ovarian FSH receptor (**FSHR**) stimulated the growth of follicles and regulated the development of ovaries by mediating the synthesis of estrogen and responding to FSH stimulation (Stilley and Segaloff, 2018). Our previous study found that dietary SS might improve the egg-laying performance via upregulating the transcription level of *FSHR* in the ovarian (Li et al., 2022b). In the present study, dietary SS was still helpful for egg production performance with antibiotics treated, and we tried to attribute this effect to the increase in the relative abundance of *Lactobacillus aviaries* (Hong et al., 2021). Unexpectedly, its relative abundance in this study did not establish an encouraging correlation with egg production performance. We speculated that the improvement in egg production performance might be attributed to the functional groups in the SS structure. It is well known that the molecular structure of SS is similar to ginsenosides. Study suggested that ginsenosides helped the proliferation of bird

Table 5. The levels of serum immune related indicators (n = 6).

Item	A	A+50SS	*P*-value
Complement C3, g/L	0.69 ± 0.21	0.66 ± 0.13	0.752
Lysozyme, U/mL	146.77 ± 26.24	154.03 ± 28.89	0.659
β-defensins, ng/L	8.97 ± 0.53	8.25 ± 1.84	0.377
IgA, ng/mL	302.91 ± 2.88	306.47 ± 3.52	0.085
IgG, ng/mL	1203.22 ± 25.05	1195.46 ± 22.58	0.586
IgM, ng/mL	110.95 ± 24.22	121.60 ± 37.88	0.574
IL-4, pg/mL	20.79 ± 7.68	22.30 ± 3.70	0.676
IFN-γ, pg/mL	65.65 ± 14.51	68.16 ± 13.89	0.765
IL-4/IFN-γ	0.31 ± 0.06	0.33 ± 0.06	0.524

follicular granulosa cells to stimulate the development of follicles (Liu et al., 2005), and ginsenosides could also alleviate ovarian dysfunction caused by the overstimulation of pregnant mare serum gonadotropin (Tan et al., 2010). Therefore, it seemed reasonable that dietary SS could improve the laying performance via elevating the expression of *FSHR* in the ovary without involvement of intestinal flora.

Although a detailed investigation of the interaction mechanism between intestinal flora and SS was beyond the scope of this work, we acknowledged that dietary SS did not improve the antioxidant and immune functions of laying hens with antibiotics treated. Intestinal flora, especially *Romboutsia*, might played an active role in the biological effects of SS on laying hens. It might be very interesting to explore the improvement effect of SS on the immune and antioxidant functions based on the intestinal flora.

CONCLUSIONS

With antibiotics treated, dietary SS failed to improve the immune and antioxidant functions of laying hens, whereas SS had a little help on the egg-laying performance. Intestinal flora, especially *Romboutsia*, might be supposed to play a key role in the biological effects of SS on laying hens.

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DISCLOSURES

The authors have no conflicts of interest to report.

REFERENCES

Abd El-Tawab, M. M., I. M. Youssef, H. A. Bakr, G. C. Fthenakis, and N. D. Giadinis. 2016. Role of probiotics in nutrition and health of small ruminants. Pol. J. Vet. Sci 19:893–906.

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

Chen, T., A. B. Liu, S. Sun, N. J. Ajami, M. C. Ross, H. Wang, L. Zhang, K. Reuhl, K. Kobayashi, J. C. Onishi, L. Zhao, and C. S. Yang. 2019. Green tea polyphenols modify the gut microbiome in db/db mice as co-abundance groups correlating with the blood glucose lowering effect. Mol. Nutr. Food Res 63:e1801064.

Elyasi, B., A. Rezaie, N. Moori Bakhtiari, and B. Mosallanejad. 2020. Helicobacter genus in the intestine and liver of stray cats: the molecular, histopathological, and immunohistochemical study. Braz. J. Microbiol 51:2123–2132.

Fan, H., Z. Lv, L. Gan, and Y. Guo. 2018. Transcriptomics-related mechanisms of supplementing laying broiler breeder hens with dietary daidzein to improve the immune function and growth performance of offspring. J. Agric. Food Chem 66:2049–2060.

Fu, W. J., A. J. Stromberg, K. Viele, R. J. Carroll, and G. Wu. 2010. Statistics and bioinformatics in nutritional sciences: analysis of complex data in the era of systems biology. J. Nutr. Biochem. 21:561–572.

Gao, H., Q. Jiang, H. Ji, J. Ning, C. Li, and H. Zheng. 2019. Type 1 diabetes induces cognitive dysfunction in rats associated with alterations of the gut microbiome and metabolomes in serum and hippocampus. Biochim. Biophys. Acta Mol. Basis Dis 1865:165541.

Greiter-Wilke, A., E. Scanziani, S. Soldati, S. P. McDonough, P. L. McDonough, S. A. Center, M. Rishniw, and K. W. Simpson. 2006. Association of Helicobacter with cholangiohepatitis in cats. J. Vet. Intern. Med. 20:822–827.

Guang, C., J. Chen, S. Sang, and S. Cheng. 2014. Biological functionality of soyasaponins and soyasapogenols. J. Agric. Food Chem. 62:8247–8255.

Hong, Y., Z. Zhou, L. Yu, K. Jiang, J. Xia, Y. Mi, C. Zhang, and J. Li. 2021. Lactobacillus salivarius and Lactobacillus agilis feeding regulates intestinal stem cells activity by modulating crypt niche in hens. Appl. Microbiol. Biotechnol 105:8823–8835.

Lee, I. A., Y. J. Park, H. K. Yeo, M. J. Han, and D. H. Kim. 2010. Soyasaponin I attenuates TNBS-Induced colitis in mice by inhibiting NF-κB pathway. J. Agric. Food Chem. 58:10929–10934.

Li, P., Y. Liu, M. Gao, J. Fu, and Y. Guo. 2022a. Dietary soy saponin improves antioxidant and immune function of layer hens [e-pub ahead of print]. J. Poult. Sci, doi:10.2141/jpsa.0210073. Accessed June 2, 2022.

Li, P., Y. Zhao, S. Yan, B. Song, Y. Liu, M. Gao, D. Tang, and Y. Guo. 2022b. Soya saponin improves egg-laying performance and immune function of laying hens. J. Anim. Sci. Biotechnol 12:126.

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

Liu, H., C. Zhang, X. Tang, W. Zeng, and Y. Mi. 2005. Stimulating effects of androgen on proliferation of cultured ovarian germ cells through androgenic and estrogenic actions in embryonic chickens. Domest. Anim. Endocrinol. 28:451–462.

MacDonald, R. S., J. Guo, J. Copeland, J. D. Browning Jr., D. Sleper, G. E. Rottinghaus, and M. A. Berhow. 2005. Environmental influences on isoflavones and saponins in soybeans and their role in colon cancer. J. Nutr. 135:1239–1242.

Meng, X., J. Gangoiti, N. de Kok, S. S. van Leeuwen, T. Pijning, and L. Dijkhuizen. 2018. Biochemical characterization of two GH70 family 4,6-α-glucanotransferases with distinct product specificity from Lactobacillus aviarius subsp. aviarius DSM 20655. Food Chem. 253:236–246.

Naveed, G., S. Ehtisham-Ul-Haque, I. Khan, S. U. Rahman, S. Anam, M. Usman, M. Z. Shakir, A. Naveed, G. Abbas, and F. R. Anjum. 2020. Enhancement in humoral response against inactivated Newcastle disease vaccine in broiler chickens administered orally with plant-derived soyasaponin. Poult. Sci. 99:1921–1927.

Russell, J. T., L. F. W. Roesch, M. Ördberg, J. Ilonen, M. A. Atkinson, D. A. Schatz, E. W. Triplett, and J. Ludvigsson. 2019. Genetic risk for autoimmunity is associated with distinct changes in the human gut microbiome. Nat. Commun. 10:3621.

Shao, S., D. Wang, W. Zheng, X. Li, H. Zhang, D. Zhao, and M. Wang. 2019. A unique polysaccharide from Hericium erinaceus mycelium ameliorates acetic acid-induced ulcerative colitis rats by modulating the composition of the gut microbiota, short chain fatty acids levels and GPR41/43 respectors. Int. Immunopharmacol. 71:411–422.

Sharp, P. J., and D. Blache. 2003. A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long- and short-day photoperiods. Can. J. Physiol. Pharmacol. 81:350–358.

Sjödin, S., G. Trowald-Wigh, and M. Fredriksson. 2011. Identification of Helicobacter DNA in feline pancreas, liver, stomach, and duodenum: comparison between findings in fresh and formalin-fixed paraffin-embedded tissue samples. Res. Vet. Sci. 91:e28–e30.

Stilley, J. A. W., and D. L. Segaloff. 2018. FSH actions and pregnancy: looking beyond ovarian FSH receptors. Endocrinology 159:4033–4042.

Su, L. W., Y. H. Cheng, F. S. Hsiao, J. C. Han, and Y. H. Yu. 2018. Optimization of mixed solid-state fermentation of soybean meal by lactobacillus species and clostridium butyricum. Pol. J. Microbiol. 67:297–305.

Tan, T. Q., C. Ge, Y. Mi, Y. Jin, and C. Zhang. 2010. Ginsenosides promote proliferation of granulosa cells from chicken prehierarchical follicles through PKC activation and up-regulated cyclin gene expression. Cell Biol. Int. 34:769–775.

Tsutsui, K., G. E. Bentley, G. Bedecarrats, T. Osugi, T. Ubuka, and L. J. Kriegsfeld. 2010. Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. Front. Neuroendocrinol. 31:284–295.

Vijay-Kumar, M., J. D. Aitken, F. A. Carvalho, T. C. Cullender, S. Mwangi, S. Srinivasan, S. V. Sitaraman, R. Knight, R. E. Ley, and A. T. Gewirtz. 2010. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 328:228–231.

Wagner, U., E. Burkhardt, and K. Failing. 1999. Evaluation of canine lymphocyte proliferation: comparison of three different colorimetric methods with the 3H-thymidine incorporation assay. Vet. Immunol. Immunopathol. 70:151–159.

Wang, L., J. Wang, H. Zhao, G. Jiang, X. Feng, W. Sui, and H. Liu. 2019a. Soyasapogenol B exhibits anti-growth and anti-metastatic activities in clear cell renal cell carcinoma. Naunyn Schmiedebergs Arch. Pharmacol. 392:551–563.

Wang, W., S. Zhai, Y. Xia, H. Wang, D. Ruan, T. Zhou, Y. Zhu, H. Zhang, M. Zhang, H. Ye, W. Ren, and L. Yang. 2019b. Ochratoxin A induces liver inflammation: involvement of intestinal microbiota. Microbiome. 7:151.

Wang, Z., E. Klipfell, B. J. Bennett, R. Koeth, B. S. Levison, B. Dugar, A. E. Feldstein, E. B. Britt, X. Fu, Y. M. Chung, Y. Wu, P. Schauer, J. D. Smith, H. Allayee, W. H. Tang, J. A. DiDonato, A. J. Lusis, and S. L. Hazen. 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472:57–63.

Yang, S. H., E. K. Ahn, J. A. Lee, T. S. Shin, C. Tsukamoto, J. W. Suh, I. Mei, and G. Chung. 2015. Soyasaponins Aa and Ab exert an anti-obesity effect in 3T3-L1 adipocytes through downregulation of PPARγ. Phytother. Res. 29:281–287.

Zeng, Q., D. Li, Y. He, Y. Li, Z. Yang, X. Zhao, Y. Liu, Y. Wang, J. Sun, X. Feng, F. Wang, J. Chen, Y. Zheng, Y. Yang, X. Sun, X. Xu, D. Wang, T. Kenney, Y. Jiang, H. Gu, Y. Li, K. Zhou, S. Li, and W. Dai. 2019. Discrepant gut microbiota markers for the classification of obesity-related metabolic abnormalities. Sci. Rep. 9:13424.

Zhang, B., Z. Lv, Z. Li, W. Wang, G. Li, and Y. Guo. 2018. Dietary l-arginine supplementation alleviates the intestinal injury and modulates the gut microbiota in broiler chickens challenged by clostridium perfringens. Front. Microbiol 9:1716.

Zhao, D., T. Wu, D. Yi, L. Wang, P. Li, J. Zhang, Y. Hou, and G. Wu. 2017. Dietary supplementation with lactobacillus casei alleviates lipopolysaccharide-induced liver injury in a porcine model. Int. J. Mol. Sci. 18:2535.

Zhu, C., L. Gong, K. Huang, F. Li, D. Tong, and H. Zhang. 2020. Effect of heat-Inactivated compound probiotics on growth performance, plasma biochemical indices, and cecal microbiome in yellow-feathered broilers. Front. Microbiol 11:585623.