Dietary selenium sources alleviate immune challenge induced by *Salmonella Enteritidis* potentially through improving the host immune response and gut microbiota in laying hens

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The aim of this study was to evaluate the effects of different selenium (Se) sources on the immune responses and gut microbiota of laying hens challenged with *Salmonella enteritidis* (*S. Enteritidis*). A total of 240 45-week-old layers were randomly divided into eight groups with six replicates per group according to a 4 × 2 factorial design, including a blank diet without Se supplementation (CON group) and three diets with 0.3 mg/kg Se supplementation from sodium selenite (IS group), yeast Se (YS group), and selenium-enriched yeast culture (SYC group), respectively. After 8 weeks of feeding, half of them were orally challenged with 1.0 ml suspension of 10^9 colony-forming units per milliliter of *S. Enteritidis* daily for 3 days. The serum was collected on days 3, 7, and 14, and the cecum content was collected on day 14 after challenge. There was no significant difference in laying performance among the eight groups before challenge. The *S. Enteritidis* challenge significantly decreased the laying performance, egg quality, GSH-Px, IgG, and IgM and increased the ratio of feed and egg, malondialdehyde (MDA), *Salmonella*-specific antibody (SA) titers, IL-6, IL-2, IL-1β, and INF-γ. However, SYC increased the level of GSH-Px and IgG and decreased IL-6, while YS decreased the level of IL-2 and IL-1β. What is more, Se supplementation decreased the SA titers to varying degrees and reduced the inflammatory cell infiltration in the lamina propria caused by *S. Enteritidis* infection. In addition, the *S. Enteritidis* challenge disrupted the intestinal flora balance by reducing the abundance of the genera *Clostridium innocuum*, *Lachnospiraceae*, and *Bifidobacterium* and increasing the genera *Butyricimonas* and *Brachyspira*, while Se supplementation increased the gut microbial alpha diversity whether
challenged or not. Under the S. Enteritidis challenge condition, the alteration of microbial composition by the administration of different Se sources mainly manifested as IS increased the relative abundance of the genera Lachnospiraceae and Christensenellaceae, YS increased the relative abundance of the genera Megamonas and Sphingomonas, and SYC increased the genera Fusobacterium and Lactococcus. The alteration of gut microbial composition had a close relationship with antioxidant or immune response. To summarize, different Se sources can improve the egg quality of layers challenged by S. Enteritidis that involves elevating the immunity level and regulating the intestinal microbiota.

KEYWORDS
selenium, laying hen, Salmonella Enteritidis, immune responses, gut microbiota, antioxidant

Introduction

Salmonella enterica serovar Enteritidis (S. Enteritidis) is a gram-negative enteric bacterium that is a major animal-infectious pathogen that can not only cause disease in poultry but also infect humans through the food chain, causing food poisoning and even death (1, 2). S. Enteritidis is the most important serotype of Salmonella, causing about 40–60% of Salmonella infections worldwide (3). Eggs and egg products are the main food carriers for S. Enteritidis to spread disease (4, 5). Although the alkaline pH value, high viscosity, and antibacterial protein in albumen create a complex antibacterial environment, S. Enteritidis can also be able to resist these stresses and proliferate in eggs, causing food poisoning (6, 7). In 2010, there was an outbreak of S. Enteritidis-contaminated eggs in the United States, with as many as 2,752 cases of infection, and more than 500 million defective eggs were recalled (8). Between 2015 and 2018, 16 European countries reported 1,209 large outbreaks of salmonellosis caused by the contaminated eggs of S. Enteritidis (9). What is more, previous studies have found that the S. Enteritidis challenge reduced the antioxidant capacity and immune function of laying hens by increasing the serum levels of MDA, IL-1β, and IL-6 (10, 11). Thus, S. Enteritidis was a substantial problem for human and animal health, and some strategies are urgently needed to solve this problem.

Selenium (Se) is an essential trace element for the synthesis of some antioxidant enzymes and selenoproteins. It can clean up active oxidative substances in the body and has biological functions such as anti-oxidation, anti-stress, and improving immunity (12–15). Historically, sodium selenite (SS) was the most widely used inorganic Se in animal feed. However, organic Se has higher deposition efficiency and bioavailability, stronger biosafety, and lower toxicity than inorganic Se (16, 17). The sources of organic Se include microorganisms, plants, and animals that absorb inorganic Se and convert it to organic selenium (14, 18, 19). Liao et al. compared the effects of dietary supplementation of SS, yeast Se (YS), and selenoprotein on broiler chicks and found that YS was more effective in increasing Se retention in the liver and muscle than IS and selenoprotein (20). Sun et al. found that adding 1.0 mg/kg of selenium-enriched earthworms power to laying hens increased the levels of glutathione peroxidase, IgG, and IL-2, further promoting antioxidant activity and immune response (14).

However, there is little information about whether supplementation of different forms of Se could alleviate the adverse effect of laying hens caused by S. Enteritidis. The purpose of this experiment was to investigate the effects of dietary supplementation of different Se sources on the performance, immune response, and gut microbiota of laying hens challenged with S. Enteritidis to evaluate the effect of different Se sources in resisting the inflammatory response caused by Salmonella infection and provide a theoretical basis for Se to defend against Salmonella infection in the production practice of laying hens.

Materials and methods

Animal experimental ethics

The experiment was allowed by the China Agricultural University Animal Care and Use Committee (A0041011202-1-1, Beijing, China).

Chemicals and treatments

The common yeast culture and selenium-enriched yeast culture (SYC) used in this experiment were both fermented
from the same yeast strain (preservation number: ACCC20060), but with different levels of sodium selenite (Se content was 0 and 30 mg/kg, respectively) in their medium. Both cultures were air-dried at 60°C to inactivate the yeast. Common yeast culture was added to the diet to balance the effect of yeast culture in different treatment diets. The sodium selenite premix (IS), containing 1% of inorganic Se, was purchased from Hebei Yuanda Zhongzheng Biotechnology Co., Ltd. (Hebei, China). The yeast Se (YS), named Alkosel, contains 1,000 mg/kg of organic Se, which was extracted from inactivated whole cell yeast (Lallemand Inc., Montreal, Quebec, Canada). The *Salmonella Enteritidis* (**S. Enteritidis**) strain (preservation number CVCC3377) was purchased from China Institute of Veterinary Drug Control (Beijing, China).

### Animals and experimental design

Before the feeding trial, a total of 240 45-week-old laying hens (Peking Pink, Huadu Yukou Poultry Industry Co., Ltd., Beijing) were confirmed as double-negative for *S. Enteritidis* by using PCR method and plate-agglutination assay to test the cloacal swab and serum samples, respectively (21, 22). The birds were randomly divided into eight groups, with six replicates in each group of five birds each, according to a 4 × 2 factorial design. The chickens were housed in wire cages (length, 45 cm × width, 45 cm × height, 45 cm), with one hen per cage, which were equipped with nipple water and a V-shaped feeding trough. The diets of different treatments consisted of a blank diet without Se supplementation (CON group) and three diets with 0.3-mg/kg Se supplementation, which was supplied from sodium selenite (IS group), yeast Se (YS group), and selenium-enriched yeast culture (SYC group), respectively. The whole experimental period consisted of 8 weeks of normal feeding, followed by a 3-day continuous challenge with 10⁹ colony-forming units (CFU)/ml (11, 23), or they received the same volume of physiological saline solution (PS), and then the samples were collected at 3, 7, and 14 days after challenge (Figure 1). In *S. Enteritidis*, in order to control the horizontal transmission of pathogenic microorganisms, the layer challenged with PS or *S. Enteritidis* was reared respectively in two houses with exactly the same conditions, with four groups of birds in each house. The feed and water were provided *ad libitum*, and the diet composition and nutrient levels are shown in Supplementary Table S1.

### Laying performance and egg quality

The egg weight and the number of egg mass were recorded daily based on each replicate. Feed consumption was recorded weekly based on each replicate. The rate of egg production, the mean egg weight, the average feed intake, and the feed/egg ratio were then calculated. At the end of 3, 7, and 14 days after the *S. Enteritidis* challenge, three eggs of each replicate were randomly selected and collected to analyze the egg quality. Egg Haugh units (HU) and egg yolk color were measured by using an egg analyzer (EA-01, Orka Teachnology Ltd., Ramat Hasharon, Israel). The eggshell strength was determined by an egg force reader (EFR-01, Orka Teachnology Ltd., Ramat Hasharon, Israel). The eggshell thickness was determined by a digital egg tester (ESTG-1; Orka Technology Ltd., Ramat Hasharon, Israel).

### Blood collection and serum analysis

At the end of 3, 7, and 14 days after the *S. Enteritidis* challenge, blood samples of five chicken hens in each replicate were collected into heparin treated tubes for 3 h and then centrifuged at 3,000 revolutions per minute for 20 min to get
the sera, which were stored at -20°C for further analysis. All samples of five chickens in each replicate were mixed in equal proportions into one sample before analysis (24). The S. Enteritidis -specific antibody titer of the serum was determined by using avian Salmonella ELISA antibody test kit (catalog number SALS-SP, Biovetest Biotechnology Co., Ltd., Tianjin, China), following the instructions provided by the manufacturer. Serum MDA (catalog number A003-1-2), superoxide dismutase (SOD, catalog number A001-3-2), glutathione peroxidase (GSH-Px, catalog number A005-1-2), immunoglobulin A (IgA, catalog number H108-1-2), immunoglobulin G (IgG, catalog number H106), immunoglobulin M (IgM, catalog number H109), interleukin-1β (IL-1β, catalog number H002), interleukin-2 (IL-2, catalog number H003), interleukin-6 (IL-6, catalog number H007-1-2), and interferon-γ (IFN-γ, catalog number H025) were measured by using corresponding kits (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China) according to instructions.

Histological examination

At 14 days after S. Enteritidis challenge, the duodenum, jejunum, and ileum of chicken were collected and fixed in 4% paraformaldehyde for 24 h. The method of histological examination referred to that in Li et al. (25). Images were collected by using the CaseViewer 2.4 software (3DHISTECH Ltd., Budapest, Hungary). The villus height and crypt depth were determined by ImageJ software.

Immunohistochemistry

The small intestine tissues were paraffin-embedded and cut into slices with a thickness of 4 µm. The slides were dewaxed, dehydrated, and then underwent antigen retrieval. The endogenous peroxidases were blocked with 3% H₂O₂ for 15 min at room temperature. The samples were incubated overnight at 4°C with a CD4 mouse-anti-chicken primary antibody (catalog number 8210-26, Southern Biotech; 1:2,000). After three washes, the samples were incubated with a goat-anti-mouse secondary antibody (Beyotime, Beijing, China) for 15 min at room temperature. The villus height and crypt depth were determined by ImageJ software.

16S rRNA gene sequencing

At 14 days after the S. Enteritidis challenge, theecal contents of chicken were collected in tubes and stored at -80°C for further analysis. Theecal contents from five chicken in each replicate were also mixed in equal proportions into one sample (27) to make the microbiome correspond to the phenotypic indicators and serum indices. The total DNA was extracted by using the Omega Bio-tek stool DNA kit (Omega, Norcross, GA, USA) following the manufacturer’s instructions. The V3–V4 region of the 16S rRNA gene was amplified with 338F (5′-ACTCTACGGGAGGCAGCAG-3′) and 806R (5′-ACTACHVGGGTWTCTAAT-3′). The PCR products were recovered by 2% agarose gel, purified using AxyPrep DNA Gel Extraction Kit (AxygenBiosciences, Union City, CA, USA), and quantified with QuantiFluor™-ST (Promega, USA). The purified PCR products were sequenced on the Illumina MiSeq PE300 platform (Shanghai MajorBio Biopharma Technology Co., Ltd., Shanghai, China).

Statistical analysis

The data were analyzed by using GraphPad Prism, version 7.01 (GraphPad Software, Inc., CA, USA). The results were analyzed by two-way ANOVA, followed by Duncan’s multiple comparison when the data were in Gaussian distribution. Otherwise, the Kruskal–Wallis test, followed by Duncan’s multiple comparison, was used for non-normally distributed data. The data were presented as mean ± SEM. P < 0.05 was considered as significantly different.

The alpha-diversity of the microbiome was calculated by sampling-based OUT analysis by using the MOTHUR program (version v.1.30.1). The beta diversity of the microbiome was displayed by a principal coordinate analysis (PCoA), which was conducted based on the Bray–Curtis distance using QIIME (version 1.17). The difference of bacterial genera that were predominant in bacterial communities among different treatment groups was identified by linear discriminant analysis effect size (LEfSe).

Results

Effects of different SE sources on the laying performance of laying hens challenged with S. Enteritidis

During the 8-week normal feeding period, no differences of laying performance were observed among the different treatment groups (P > 0.05) (Table 1). As shown in Table 2, the egg production rate and egg mass at 0–3 and 4–7 days were reduced markedly, and the feed-to-egg ratio at 4–7 days was increased after the S. Enteritidis challenge (P < 0.05). In addition, compared to IS, SYC supplementation significantly increased the egg production rate from 8 to 14 days after the S. Enteritidis challenge (P < 0.05). There were no differences in the mean weight of eggs and average feed intake of laying hens during 0–3, 4–7, and 8–14 days among different treatment groups (P > 0.05).
Effects of different SE sources on the egg quality of laying hens challenged with *S. Enteritidis*

As demonstrated in Table 3, the *S. Enteritidis* challenge had no significant effect on eggshell strength, egg yolk color, egg yolk percent, and eggshell thickness at 3, 7, and 14 days (*P* > 0.05). The egg Haugh unit on day 3 was significantly reduced after the *S. Enteritidis* challenge (*P* < 0.05). However, YS supplementation significantly increased the egg yolk color compared to IS (*P* < 0.05), SYC supplementation significantly increased the egg yolk percent at 7 and 14 days, and IS increased the eggshell thickness at 7 days after the *S. Enteritidis* challenge compared to CON (*P* < 0.05).

Effects of different SE sources on the serum antioxidant status of laying hens challenged with *S. Enteritidis*

As shown in Figure 2, the serum GSH-Px at 7 days in the IS group was significantly higher than that in CON, YS, and IS+SE groups (*P* < 0.05 and *P* < 0.001), and the GSH-Px in the SYC+SE group was significantly higher than that in the IS+SE group (*P* < 0.05). There was no obvious difference in the serum SOD of laying hens among the eight groups at 3, 7, and 14 days after the challenge with PS or *S. Enteritidis* (*P* > 0.05); however, the serum MDA at 7 days in the CON+SE group was significantly increased compared to that in the CON group (*P* < 0.05).

Effects of different SE sources on serum *Salmonella*-specific antibody titers of laying hens challenged with *S. Enteritidis*

As shown in Figure 3, the level of *Salmonella*-specific antibody (SA) titers at 3 days in the YS+SE group was significantly higher than that in the YS group (*P* < 0.05); however, no obvious difference was observed among the other groups (*P* > 0.05). The SA titers at 7 days in the CON+SE, IS+SE, and YS+SE groups were significantly higher than those in the CON, IS, and YS groups (*P* < 0.001), and the titer in SYC+SE was significantly lower than those in the CON+SE, IS+SE, and YS+SE groups (*P* < 0.001). The SA titers at 14 days...
in the four groups challenged with *S. Enteritidis* were significantly higher than those in the four groups challenged with PS (P < 0.05, P < 0.01, and P < 0.001), and the titers in the IS+SE group were significantly decreased compared to that in the CON+SE group (P < 0.05).

**Effects of different SE sources on the immune response of laying hens challenged with *S. Enteritidis***

As shown in Figure 4, there was no obvious difference in the serum IgA of laying hens among the eight groups at 3, 7, and 14 days after the challenge with PS or *S. Enteritidis* (P > 0.05) (Figures 4A–C). The serum IgG in the SYC group was significantly higher than those in the CON, IS, and YS groups (P < 0.05). However, compared to SYC, the serum IgG at 14 days was significantly decreased in the SYC+SE group (P < 0.05) (Figure 4F). The serum IgM at 3 days in CON+SE was significantly decreased compared to that in IS, CON, and IS+SE group (P < 0.05) (Figure 4I). The serum IL-2 at 7 days in all groups challenged with *S. Enteritidis* was significantly higher than that in CON (P < 0.05) (Figure 4P). After the challenge with *S. Enteritidis*, the level of IL-6 at 14 days in IS+SE was significantly higher than those in the IS and SYC+SE groups (P < 0.05) (Figure 4O). The serum INF-γ at 3 days in SYC+SE was remarkably increased compared to that in the YS+SE group (Figure 4S), and the serum INF-γ at 7 days in SYC+SE was significantly higher than that in SYC group (P < 0.05) (Figure 4T). In addition, *S. Enteritidis* infection caused blue round particles in the lamina propria, which means that the infiltration of inflammatory cells is obvious. Se supplementation

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**TABLE 2** Effects of different selenium sources in diets on the performance of layers challenged by *S. Enteritidis*.

| Diets | SE | Egg production rate (%) | Egg mass (g/day/hen) | Mean weight of eggs (g) | Average feed intake (g) | Feed/egg ratio (g/g) |
|-------|----|-------------------------|----------------------|------------------------|------------------------|----------------------|
|       |    | 0–3 days | 4–7 days | 8–14 days | 0–3 days | 4–7 days | 8–14 days | 0–3 days | 4–7 days | 8–14 days | 0–3 days | 4–7 days | 8–14 days | 0–3 days | 4–7 days | 8–14 days | 0–3 days | 4–7 days | 8–14 days | 0–3 days | 4–7 days | 8–14 days |
| CON   | –  | 86.67±b | 85.83 | 83.81±b  | 51.08 | 50.90 | 48.78 | 58.91 | 59.27 | 58.18 | 114.90 | 100.30 | 98.19 | 2.26 | 1.98 | 2.02 |
| CON   | +  | 85.55±b | 83.33 | 83.81±b  | 50.49 | 48.37 | 48.41 | 58.93 | 58.05 | 57.77 | 111.60 | 103.30 | 100.20 | 2.22 | 2.14 | 2.07 |
| IS    | –  | 92.22a | 89.17 | 80.00a   | 53.45 | 51.96 | 46.07 | 57.99 | 58.26 | 57.59 | 96.89 | 101.20 | 98.29 | 1.82 | 1.95 | 2.14 |
| IS    | +  | 82.22a | 82.50 | 82.86±a  | 49.21 | 48.81 | 48.89 | 59.84 | 59.14 | 59.01 | 106.00 | 105.30 | 97.05 | 2.15 | 2.16 | 1.98 |
| YS    | –  | 90.74a | 87.69 | 87.83±a  | 52.55 | 50.94 | 50.39 | 57.91 | 58.12 | 57.31 | 105.53 | 98.43 | 101.80 | 2.01 | 1.94 | 2.02 |
| YS    | +  | 88.89±b | 83.33 | 85.71±b  | 52.22 | 49.24 | 50.06 | 58.75 | 59.06 | 58.40 | 105.60 | 105.80 | 101.20 | 2.02 | 2.16 | 2.02 |
| SYC   | –  | 91.97a | 91.88 | 93.92±a  | 53.31 | 52.53 | 53.87 | 57.96 | 57.16 | 57.35 | 112.20 | 99.44 | 108.30 | 2.10 | 1.89 | 2.01 |
| SYC   | +  | 83.33a | 84.17 | 83.81±b  | 48.39 | 48.90 | 47.99 | 58.09 | 58.12 | 57.30 | 98.89 | 100.70 | 100.50 | 2.05 | 2.06 | 2.10 |
| SEM   | 1.535 | 2.612 | 2.492 | 1.309 | 1.725 | 1.637 | 0.778 | 0.790 | 0.723 | 4.729 | 2.883 | 3.159 | 0.117 | 0.059 | 0.07 |
| SEM   | 1.335 | 2.612 | 2.492 | 1.309 | 1.725 | 1.637 | 0.778 | 0.790 | 0.723 | 4.729 | 2.883 | 3.159 | 0.117 | 0.059 | 0.07 |
| CON   | 86.11±a | 84.58 | 83.81±b | 50.78 | 49.63 | 48.60 | 58.92 | 58.66 | 57.97 | 113.22a | 101.79 | 99.19 | 2.24 | 2.06 | 2.05 |
| IS    | 87.22±a | 85.83 | 81.43±b | 51.33 | 50.39 | 47.89 | 58.92 | 58.70 | 58.30 | 101.44a | 103.25 | 97.67 | 1.99 | 2.05 | 2.06 |
| YS    | 89.82±a | 85.51 | 86.77±a | 52.39 | 50.09 | 50.22 | 58.33 | 58.59 | 57.85 | 105.54a | 102.13 | 101.52 | 2.02 | 2.05 | 2.02 |
| SYC   | 87.65±b | 88.82 | 88.87±a | 50.85 | 50.71 | 50.93 | 58.02 | 57.64 | 57.33 | 105.55ab | 100.05 | 104.38 | 2.08 | 1.98 | 2.06 |
| SEM   | 1.086 | 1.847 | 1.762 | 0.925 | 1.225 | 1.157 | 0.55 | 0.559 | 0.513 | 3.444 | 2.038 | 2.234 | 0.082 | 0.042 | 0.05 |
| SEM   | 0.768 | 1.306 | 1.246 | 0.654 | 0.863 | 0.818 | 0.389 | 0.395 | 0.362 | 2.365 | 1.441 | 1.580 | 0.058 | 0.030 | 0.035 |
| SE    | 0.148 | 0.611 | 0.042 | 0.600 | 0.934 | 0.184 | 0.590 | 0.504 | 0.611 | 0.133 | 0.739 | 0.204 | 0.171 | 0.509 | 0.955 |

Different letters indicate statistically significant differences among different treatments (P < 0.05).

CON, basal diet; IS, sodium selenite; YS, yeast selenium; SYC, selenium-enriched yeast culture; SE, Salmonella Enteritidis; –, with physiological saline solution challenge; +, with SE challenge.
reduced the inflammatory cell infiltration in the lamina propria (Figure 5).

Effects of different SE sources on the small intestine morphology of laying hens challenged with *S. Enteritidis*

The histopathological changes of the small intestine are shown in Figure 6 to analyze the effects of Se supplementation on the intestinal morphology of layers after the *S. Enteritidis* challenge. Hematoxylin–eosin (H&E) staining suggested that the morphology of the duodenum, jejunum, and ileum was destroyed by the *S. Enteritidis* challenge, as revealed by crypt atrophy and the adhesion or fusion of villi, whereas Se supplementation could alleviate the degree of intestinal damage caused by the *S. Enteritidis* challenge, which was demonstrated by the increase in villus height and the ratio of villi and crypt while the crypt depth of the duodenum, jejunum, and ileum decreased (*P* < 0.05) (Figure 7).

Effects of different SE sources on the gut microbial composition of laying hens challenged with *S. Enteritidis*

High-throughput 16S rRNA gene sequencing was conducted to investigate whether Se supplementation would affect the gut microbial composition in laying hens challenged with *S. Enteritidis*. As shown in Figure 8, significant differences were observed in alpha diversity among different groups, including Ace and Sobs. Compared to IS, the Ace and Sobs in the YS and SYC groups were significantly increased (*P* < 0.05). The Ace and Sobs in the YS+SE group were significantly higher than that in the CON+SE group (*P* < 0.05).

A PCoA was conducted to evaluate the differences among different groups. Our results suggested that Se supplementation and *S. Enteritidis* infection would not alter the β diversity of the gut microbial composition (Figures 9A–F). The most abundant cecal microbiota composition among different groups was revealed by phylogenetic analysis. At the phylum level, *Bacteroidota*, *Firmicutes*, *Desulfovibrio*, *Proteobacteria*, *Campylobacter*, *Fusobacteria*, *Kang et al. 10.3389/fimmu.2022.928865*
Deferribacterota were dominant (Figure 9G). The predominant genera were Bacteroides, unclassified_o:Bacteroidales, Rikenellaceae_RC9_gut_group, norank_f:norank_o:Clostridia_UCG-014, Lactobacillus, Faecalibacterium, unclassified_f:Lachnospiraceae, Phascolarctobacterium, norank_f:norank_o:RF39, Desulfovibrio, Ruminococcus_torques_group, Alistipes, Parabacteroides, and so on (Figure 9H).

As shown in Figure 10, the specific bacterial taxa associated with different Se sources and S. Enteritidis treatments were identified using LEfSe (LDA score > 2.0). Se supplementation
FIGURE 4
Effects of dietary supplementation with different Se sources on the serum parameters of layers challenged with S. Enteritidis. CON, basal diet; IS, sodium selenite; YS, yeast selenium; SYC, selenium-enriched yeast culture; PS, challenged with physiological saline solution; SE, challenged with S. Enteritidis. The levels of serum IgA, IgG, IgM, IL-2, IL-6, IL-10, and INF-γ were measured among different periods and treatments (A–U). The data were presented as mean ± SEM. Significance was compared with every other group; *P < 0.05, **P < 0.01, ***P < 0.001.
increased the abundance of gut microbial composition before or after the challenge with *S. Enteritidis* (Figures 10A, B). Compared to the CON, the relative abundance of *Butyricimonas* and *Brachyspira* was significantly increased, and the relative abundance of *unclassified_f:Tannerellaceae*, *norank_f:UCG_010*, *norank_f:Barnesiellaceae*, *Clostridium_innocuum_group*, *Coprobacter*, *CAG_352*, *norank_f:norank_o:norank_c:Clostridia*, *Lachnospiraceae_UCG_002*, and *Bifidobacterium*, respectively, was significantly decreased in the CON+SE group (Figure 10C). The dominant bacteria of the IS group were *unclassified_o:Bacteroidales*, *Clostridium_sensu_stricto_1*, and *Paraprevotella*, while the dominant bacteria in the IS+SE group were *Shuttleworthia*, *Lachnospiraceae_UCG_002*, *unclassified_f:Paludibacteraceae*, *unclassified_p:Firmicutes*, and *unclassified_o:Erysipelotrichales* (Figure 10D). The dominant bacteria in the YS group were also *unclassified_o:Bacteroidales*, *unclassified_f:Tannerellaceae*, *Barnesiella*, *Alcaligenes*, *Ochrobactrum*, *Aquabacterium*, *Ralstonia*, and so on, while the dominant bacteria in the YS+SE group were *Shuttleworthia*, *norank_f:norank_o:norank_c:norank_p:WPS_2*, *unclassified_f:Barnesiellaceae*, *Lachnoclostridium*, and *Helicobacter* (Figure 10E). In addition, the dominant bacteria in the SYC group were *unclassified_f:Tannerellaceae*, *Megasphaera*, *unclassified_f:Eggerthellaceae*, *Shewanella*, *CHKC1002*, *Ochrobactrum*, *Arthrobacter*, and so on, while the dominant bacteria in the SYC+SE group were *Phascolarctobacterium*, *DEV114*, *Intestinimonas*, and *Tyzzerella* (Figure 10F).

**Effects of dietary supplementation with different SE sources on the difference of the gut microbiota and its correlation with the antioxidant and the immunity of laying hens challenged with *S. Enteritidis***

Spearman correlation was performed to predict the correlation among the intestinal microbial communities and the antioxidant and immunity of laying hens 14 days after the challenge with PS or *S. Enteritidis*. As shown in Figure 11A, at 14 days after the challenge with PS, *Lactobacillus* was negatively correlated with MDA and *Christensenellaceae_R-7_group* was positively correlated with IgA, but *Rikenellaceae_RC9_gut_group* was negatively correlated with IgA ($P < 0.05$). *Erysipelatoclostridium*, *Lachnoclostridium*, *Fournierella*, *Streptococcus*, *Fusobacterium*, *Barnesiella*, *Alistipes*, and *Faecalibacterium* were positively correlated with IgG ($P < 0.05$), while *Rikenellaceae_RC9_gut_group* was negatively correlated with IgG ($P < 0.05$). *Barnesiella* was positively correlated with IL-1β ($P < 0.05$), *Colidextribacter*, *Shuttleworthia*, and *Ruminococcus_torques_group* were positively correlated with IL-2 ($P < 0.05$), *Alloprevotella*, *Butyricicoccus*, and *Shuttleworthia* were positively correlated with IL-6 ($P < 0.05$), while *Parasutterella* and *NK4A214_group* were negatively correlated with INF-γ ($P < 0.05$).

As shown in Figure 11B, at 14 days after the challenge with *S. Enteritidis*, *Fusobacterium* was positively correlated with GSH-Px ($P < 0.05$) and *Ruminococcus_torques_group* and *Faecalibacterium* were negatively correlated with MDA. On
the contrary, Rikenellaceae_RC9_gut_group was positively correlated with MDA ($P < 0.05$). Parabacteroides was negatively correlated with IgA, and Lactobacillus was negatively correlated with IgG and IgM ($P < 0.05$). Campylobacter and Desulfovibrio were positively correlated with IL-1β ($P < 0.05$). Parasutterella and Phascolarcotobacterium were positively correlated with IL-2. On the contrary, Prevotellaceae_UCG-001 was negatively correlated with IL-2 ($P < 0.05$). Lachnoclostridium, GCA-900066575, Shuttleworthia, and Ruminococcus_torques_group were positively correlated with IL-6 ($P < 0.05$), while Fusobacterium and Phascolarcotobacterium were negatively correlated with IL-6 ($P < 0.05$).

**Discussion**

*S. Enteritidis* was one of the major factors that affected laying performance for a long time. Previous studies have shown that the *S. Enteritidis* infection of laying hens reduced their feed intake, egg production rate, and body weight (28), which may be related to the colonization of *Salmonella* in the gut (29), disrupting the composition of gut microbiota (30), which, in turn, destroyed the gut barrier function and induced inflammation (31). In addition, oxidative stress is often accompanied by inflammation. When the body was infected by external pathogens, it activated the immune system to clear the infection, and this progress also generated oxidative stress.
FIGURE 7
Effects of dietary supplementation with different Se sources on histomorphological measurements in the duodenum, jejunum, and ileum of hens challenged with *S. Enteritidis*. CON, basal diet; IS, sodium selenite; YS, yeast selenium; SYC, selenium-enriched yeast culture; PS, challenged with physiological saline solution; SE, challenged with *S. Enteritidis*. (A–I) The villus height, the crypt depth, and the villus/crypt ratio were measured randomly in each sample from different groups. The data were presented as mean ± SEM. Significance was compared with every other group; *P < 0.05, **P < 0.01, ***P < 0.001.

FIGURE 8
Effects of dietary supplementation with different Se sources on the alpha diversity of the cecal microbiota in layers challenged with *S. Enteritidis*. CON, basal diet; IS, sodium selenite; YS, yeast selenium; SYC, selenium-enriched yeast culture; CON+SE, IS+SE, YS+SE, and SYC+SE mean CON, IS, YS, and SYC challenged with *S. Enteritidis*, respectively. (A, E) Ace index of OUT level; (B, F) Chao index of OUT level; (C, G) Shannon index of OUT level; and (D, H) Sobs index of OUT level. The data were presented as means ± SEM. Significance was compared with every other group; *P < 0.05, **P < 0.01.
Oxidative stress is mainly manifested as a decrease in antioxidant capacity, such as a decrease in the concentration of antioxidant enzymes such as T-SOD and GSH-PX, an increase in the concentration of MDA, and a further increase in the degree of lipid peroxidation (33). Liu et al. reported that S. Enteritidis infection significantly increased the level of MDA in the serum of laying hens, further causing oxidative stress (10). Se is an essential trace element and involved in the composition of several metabolic enzymes, such as glutathione peroxidase (GSH-Px) and type I iodothyronine deiodinase (34, 35), and plays a critical role in the application of GSH in resisting the oxidation of host cells (34). A previous study reported that both organic and inorganic Se supplementation could alleviate the heat stress-induced oxidative stress of layers, including increasing the serum concentration of GSH-Px and decreasing the MDA content (15). Se supplementation could increase the effectiveness of immune function through increasing the T cell response, mainly improving IL-2 receptor expression, and prevented immune cells from damage induced by oxidative stress (32). In our study, the S. Enteritidis challenge obviously increased the level of MDA, IL-2, IL-6, IL-β, and INF-γ and decreased the level of GSH-Px, IgG, and IgM, further disrupting the intestinal barrier and the balance of the intestinal flora, while Se supplementation alleviated these changes. Therefore, Se supplementation has the potential to be used in alleviating Salmonella infection in the production practice of laying hens.

It was worth noting that Salmonella infection did not cause changes in the apparent quality and freshness of eggs (4). In the present study, we have found that $10^6$ CFU S. Enteritidis challenged for 3 days had no significant effect on the egg quality and laying performance of layers. Fan et al. reported that the dietary supplementation of $10^8$ CFU S. Enteritidis had no significant effect on the egg quality and production performance of layers, which was consistent with our study. However, it deposited in the tissues and organs of layers, infected the forming eggs, and increased the serum levels of ALT and...
AST (36). Although *Salmonella* does not affect the performance of birds, the infected *Salmonella* can continue to colonize the cecum and spread to other flocks as they grow (29). Thus, more attention should be paid to the detection of microorganisms in birds to prevent foodborne infections.

CD4 T cells play a critical role in immune protection by recruiting neutrophils, eosinophils, and basophils to the site of infection and responding to a full range of immune responses by producing cytokines and chemokines when the body was infected (37). Previous studies have reported that *Salmonella* infection activated the immune system of the host to conduct a series of immune responses (10, 38). Different cytokines that play important roles in regulating the body’s immune responses resist *Salmonella* infection. The invasion of *Salmonella* onto intestinal epithelial cells caused the secretion of pro-inflammatory cytokines such as IL-6, IL-8, and IFN-γ, which induced systemic inflammation by recruiting immune cells (39, 40). In the present study, systemic inflammation was observed after *S. Enteritidis* infection, including significantly decreased IgM and an increased number of CD4 T cells and the level of IL-1β, IL-2, and IL-6, while IS, YS, and SYC supplementation reversed those changes in IgM, CD4 T cells, and IL-1β. SYC also markedly increased the level of IgG compared to CON, IS, and YS. In addition, the level of IL-6 in SYC+SE was significantly higher than that in IS+SE, which is in line with a previous study suggesting that Se supplementation could increase the levels of IgM and IgG of birds, further increasing host immunity (41).

A specific antibody against *Salmonella* plays an important role in host resistance to *Salmonella* infection and directs the clearance of *Salmonella* infection (42). In the present study, we...
found that *S. Enteritidis* infection significantly increased the specific antibody against *Salmonella* in peripheral serum during the pre-middle period of infection. In line with this outcome, a previous study has reported that *Salmonella* infection can induce high levels of anti-*Salmonella*-specific antibodies in chickens (43). In addition, the present study found that dietary supplementation of organic selenium and inorganic selenium decreased the anti-*Salmonella*-specific antibodies to varying degrees in the middle and late stages of infection. The amount of a specific antibody produced is proportional to the antigen content in the body. Lower levels of a specific antibody in the peripheral serum in the middle and late stages of infection were found in *S. Enteritidis*-infected layers fed with different Se sources, indicating that Se either directly inhibited the growth of *Salmonella* and killed it or SE stimulated the production of anti-*Salmonella*-specific antibodies, further decreasing the load of *Salmonella* in layers. The result suggested that both organic and inorganic Se supplementations could protect against *Salmonella* infection by regulating specific humoral immunity.

As we all know, the small intestine is the main site of nutrient absorption and the body’s first barrier against external substances. It plays a critical role in maintaining gut homeostasis and keeping it healthy (44). Villus height and crypt depth—or the ratio of both (V/C)—were important indicators of intestinal function and maturity. An increase in villus height and V/C ratio indicated a healthy gut and better nutrient absorptive capacity. Conversely, with villus height becoming lower, the intestinal absorptive capacity becomes weaker (45–47). In the present study, *S. Enteritidis* infection significantly destroyed the villi and crypt of the small intestine, which were evidenced by crypt atrophy and villus adhesions. Interestingly, the addition of Se markedly increased the villus height and the ratio of villus and crypt and decreased the crypt depth of the small intestine of laying hens, which further alleviated the damages caused by *S. Enteritidis* infection. Thus, these results suggested that the integrity barrier of the duodenum, jejunum, and ileum of layers was destroyed by *S. Enteritidis* infection, whereas the supplementation of Se alleviated these changes through improving the immune response.

The intestinal flora constituted the intestinal microbial barrier, and a stable intestinal microbial barrier was essential in the digestion and absorption of nutrients and the maintenance of homeostasis in the intestinal environment (48). In the present study, both different organic Se supplementations (YS and SYC) and *S. Enteritidis* infection altered the gut microbial diversity, which was revealed by variations in α diversity, β diversity, and specific bacteria that occurred in different groups. In line with this outcome, a previous study has reported that, in 1-day-old chicks challenged with *Salmonella*, the diversity of the cecal microbiota was markedly decreased (49). Dietary Se supplementation also notably increased the α diversity and β diversity of microbiota in mice (50, 51). The changes of *Salmonella* to the gut microbial composition may be associated with the interaction between pathogen and commensal microbiota or the host mucosal immune response to pathogens or a combination of both of them (52). In addition, according to the LEfSe analyses, the microbial composition of layers was altered by both Se supplementation and *S. Enteritidis* infection. *S. Enteritidis* infection significantly decreased the relative abundance of microbial composition, which indicated that the gut homeostasis was disrupted and certain diseases may occur (53, 54), while Se supplementation reversed these negative effects. *S. Enteritidis* infection also significantly decreased the
abundance of Lachnospiraceae and Clostridium, which could utilize dietary carbohydrate and fiber metabolism to produce butyric acid, regulating both energy metabolism and the immune response of intestinal epithelial cells (55, 56). Butyric acid stimulated the intestinal cells to produce antimicrobial peptide substances that helped to resist the invasion and colonization of Salmonella, inhibiting the occurrence of intestinal inflammation and protecting intestinal health (57, 58). In addition, in the present study, YS and SYC supplementation markedly increased the abundance of Barnesiella. A previous study has reported that Barnesiella was able to clear the intestinal colonization of highly antibiotic-resistant bacteria (59). YS also increased the abundance of Bacteroidales, which was considered as an intestinal beneficial bacterial, which can increase immune function and improve intestinal health (60). Collectively, S. Enteritidis infection decreased the composition of intestinal microbiota, while Se supplementation could reverse these negative effects by increasing the relative abundance of microbes associated with anti-inflammation, further increasing intestinal homeostasis.

**Conclusion**

In conclusion, the present study suggested that selenium (Se) supplementation significantly increased egg production to resist the adverse effects caused by the S. Enteritidis challenge. These results also revealed that Se administration could alleviate the intestinal histopathologic damage caused by S. Enteritidis infection. In addition, S. Enteritidis infection significantly decreased the level of GSH-Px and IgM and increased the level of MDA, IL-1β, and Salmonella-specific antibody. However, Se addition reversed these outcomes. Moreover, yeast Se and selenium-enriched yeast culture supplementation maintained intestinal homeostasis through increasing the relative abundance of microbiota related to anti-inflammation, further alleviating the damage caused by S. Enteritidis infection.

**Data availability statement**

The data presented in the study are deposited in the NCBI repository, and the accession numbers can be found below: https://www.ncbi.nlm.nih.gov/sra/PRJNA849581.

**Ethics statement**

The animal study was reviewed and approved by the China Agricultural University Animal Care and Use Committee.

**Author contributions**

QM, JZ, CJ, SH, LZ, and ZW designed the study. WW conducted the experiments and collected the data. RK and WW performed the analysis of the experimental data. RK drafted the manuscript and finished the submission. RK, YL, JX and YH detected the samples. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.928865/full#supplementary-material
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