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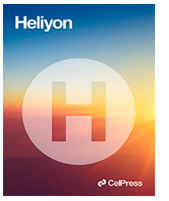

Research article

Effects of *Salmonella* Enteritidis infection on TLRs gene expression and microbial diversity in cecum of laying hens

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ABSTRACT

Salmonella Enteritidis (SE) is an important foodborne pathogen primarily causing human disease through contaminated food and water. In the current study, to assess the effect of *Salmonella* Enteritidis infection on the immune system and the microbial diversity of cecum and oviduct in chickens, twelve 24-week-old SE-negative White Leghorn layers were randomly selected and divided into 2 groups. Chickens in the challenge group were orally inoculated with SE, and chickens in the control group received an equal amount of sterilized Phosphate Buffered Saline solution. Serum and tissue samples (cecum, oviduct, ovary, liver, spleen, and pancreas) were collected at 7 days and 14 days post-infection (dpi). Quantitative PCR was used to detect the expression of Toll-like receptors (TLRs) in the cecum, oviduct and ovary. To understand the influence of SE infection on the microbial profile of the cecum and oviduct, microbial community composition of the cecal contents and oviducal contents were analyzed through 16S rRNA sequencing. Results showed that SE infection caused damage to the digestive organs, reproductive organs, and immune organs in laying hens. The expression of *TLR1a*, *TLR1b*, *TLR2*, *TLR4*, *TLR5*, *TLR7* and *TLR15* in the cecum were induced, and the content of IFN-γ, TNF-α, IL-2 and IL-18 in serum increased after SE infection. The composition of the microbial community significantly changed in cecal content, the dominant phylum of Firmicutes increased, and Bacteroidetes decreased significantly. In the oviduct, the microbial diversity became complicated, the dominant bacteria *Faecalibacterium* was significantly increased, and *Bacteroides* was significantly decreased. This study investigated the effects of SE infection in laying hens, including host innate immunity, the expression of TLRs, and changes in the composition of microbes in the cecum and reproductive tract. Our results may provide a scientific basis for the *Salmonella* Enteritidis control in chicken, the maintenance of oviduct function, and the guarantee of clean egg production.

1. Introduction

Salmonella Enteritidis (SE) is one of the primary pathogens causing gastroenteritis and food poisoning. Cases of Salmonellosis in

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chickens caused by SE are on the rise, hindering the development of the poultry industry, and threatening the health of humans and animals [1]. *Salmonella* has a variety of species, which makes it difficult to control infectious diseases. The genus *Salmonella* consists of more than 2600 serotypes posing a challenge to control and at least 100 serotypes are intestinal associated [2].

In chickens, *Salmonella* infection is initially recognized by Toll-like receptors (TLRs) [3]. TLRs are an essential class of innate immune pattern recognition receptors and play key roles in the immune response and defense against various pathogens [4,5]. After activation of a suitable ligand, TLRs begin to induce a series of reactions and eventually lead to the initiation of innate immune responses and the development of adaptive immune responses [6–8]. In chicken, 10 TLR genes have been identified, including *CHTLR1a* and *CHTLR1b*, *CHTLR2a* and *CHTLR2b*, *CHTLR3*, *CHTLR4*, *CHTLR5*, *CHTLR7*, *CHTLR15* and *CHTLR21*. These genes are primarily expressed by epithelial cells and immune cells [9]. TLRs have specific functions in chicken, and it has been reported that *CHTLR15* is unique to poultry [10]. Once activated by ligands, TLR signaling can induce expression of cytokines such as interleukin (IL)-2, IL-18, interferons (IFNs), and tumor necrosis factor (TNF) [11]. Cytokines are small molecular proteins synthesized and secreted by immune cells and certain non-immune cells after stimulation [12] with various functions such as immunity, hematopoiesis, cell growth, and repair of damaged tissue [13]. In addition, the cytokine gene expression is generally correlated with the relative abundance of gastrointestinal bacterial taxa [14].

The intestinal tract is the largest immune organ and is devoted to regulating host immunity, inhibiting colonization of pathogens, or improving intestinal barrier function [15,16]. The gut mucosal immune system consists of lymph nodes, lamina propria, and epithelial cells that constitute a protective barrier to the integrity of the intestine tract [17,18]. A beneficial microbial community is indispensable in maintaining normal physiological homeostasis [19–21]. Chickens infected with SE mounted a substantial immune response typified by high levels of antigen-specific antibodies (IgM, IgY, and IgA), strong T cell responses, and increased expression of genes in the spleen and an array of cytokines and chemokines in the gut [22]. Additionally, vertical transmission is an important route of SE spreading in poultry production and resulted in a significant loss of laying performance and egg quality [23,24]. At present, there are few studies that focus on the local immune response of the reproductive system to *Salmonella* infection in laying hens. We found that the microbial composition of the oviduct changes and became more complex after SE infection.

The purpose of this study is to investigate the changes in the host immune system, digestive system, and reproductive system after *Salmonella* Enteritidis infection in laying hens, to clarify a series of host defense mechanisms responding to SE infection, and provide a scientific basis for controlling the horizontal and vertical transmission of *Salmonella* Enteritidis.

2. Materials and methods

2.1. Inoculum preparation

The *Salmonella* Enteritidis strain (CVCC3377, purchased from China Veterinary Microbial Strain Management Center) was incubated in Nutrient Broth medium overnight at 37 °C under shaking condition at 180 rpm. The bacteria was washed with sterilized phosphate buffer saline (PBS), and serially diluted to a concentration of 3.0×10^9 cfu mL^{-1} to prepare the challenge dose.

2.2. Animal inoculation and sample collection

Twelve 24-week-old SE negative White Leghorn layers (Poultry Institute, Shandong Academy of Agricultural Sciences, Jinan, Shandong) were selected and equally divided into two groups. Each chicken in the challenged group (T) was orally challenged with 600 μL inoculant with 1.8×10^8 cfu SE. Each chicken in the control group (CK) was inoculated with an equal volume of PBS. These chickens were distributed into two separate isolators. Three chickens from challenged group were randomly selected and euthanized at 7 dpi (days post infection) and 14 dpi marked as T1 and T2 groups, respectively. The intestinal contents were collected to enumerate the number of SE in each sample by the plate count method. Briefly, the centrifuge tubes containing the contents of each section of the intestine were shaken well, diluted by 10-fold, and 0.1 mL suspensions were plated on SS agar (*Salmonella- Shigella* Agar) medium at 37 °C for 16–24 h. We selected SE colonies between 30 and 300 for counting, and calculated the amount of SE colonization in the contents. The number of SE per gram of content was used to quantify the SE colonization [25]. The cecum, oviduct, and ovary were collected and stored at −80 °C for further analysis. Blood was collected from the wing vein, and centrifuged to collect the serum for cytokine content measurement. The ovaries, oviducts, livers, spleens, pancreas, and jejuna were fixed in a 10% formaldehyde solution for histopathological examination. This study was approved by the Laboratory Animal Management and Use Committee of Shandong Agricultural University (Permit Number: SDAUA-2018-100). All methods were carried out in accordance with relevant guidelines and regulations. The study was carried out in compliance with ARRIVE guidelines.

2.3. Immune organ index

At 7 dpi and 14 dpi, three chickens were randomly selected from each group, and the thymus and spleen of each chicken were weighed to calculate the thymic and splenic index, respectively. Thymic index = (thymus weight/body weight) × 100%; Splenic index = (spleen weight/body weight) × 100% [26].

2.4. Measurement of cytokines content in serum

The content of IFN-γ, TNF-α, IL-2 and IL-18 in the serum was measured through ELISA kit (Shanghai Enzyme-linked Biotechnology,

Shanghai, China) according to the manufacturer's instructions.

2.5. Quantitative real-time PCR

Total RNA was extracted from the oviducts, ovaries, and ceca with TransZol Up (TransGen Biotech, Beijing, China) following the manufacturer's instructions. One μg total RNA was reverse transcribed into cDNA using Prime Script RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The cDNA was stored at −20 °C until further use. The expression levels of *TLR1a*, *TLR1b*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR15* and *TLR21* were quantified through real-time PCR with SYBR® Premix Ex Taq™ II (Takara, Dalian, China). Each reaction was carried out in triplicate in a 15 μL reaction, and β-actin was used as the endogenous control. The primers for TLRs were listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to estimate mRNA abundance [27].

2.6. Microbial community composition analysis

Cecal and oviducal contents from each chicken were collected and flash frozen in liquid nitrogen, and stored at −80 °C. DNA was isolated from each sample using the MOBIO Power Soil DNA Extraction kit (MOBIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 3 g of cecal and oviducal contents were ground in a mortar with the pestle in liquid nitrogen and then 0.3–0.5 g of cecal and oviducal contents were placed into a bead tube for extraction. Bead tubes were heated to 65 °C for 10 min, and then shaken horizontally for 2 min at maximum speed with the MOBIO vortex. DNA samples were stored at −20 °C.

The V3–V4 region of the 16S rRNA gene was amplified using the TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, Dalian, China). The following primers: V3–16S-Fw: CCTACGGGNGGCWGCAG and V4–16S-Rev: GACTACHVGGGTATCTAATCC were used. The amplification condition was 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 68 °C for 1 min, and kept at 4 °C [28,29]. The amplicon was sequenced using Illumina MiSeq by Shanghai Paisano Biological Technology Co., LTD.

The Quantitative Insights into Microbial Ecology version 2 (QIIME2) was used for quality filtering of DNA sequences, demultiplexing, taxonomic assignment, and calculating β-diversity. Sequences were clustered using USEARCH software (version 11, http://drive5.com/uparse/) to yield representative sequences of the operational taxonomic unit (OTU) with 99% similarity. Representative OTU sequences were compared to the Greengenes database using the RDP Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp; Michigan 4882, USA) for species annotation and relative abundance analysis [30]. Alpha and beta diversity and the significance of taxonomic differences between samples were estimated by QIIME 2. Linear discriminant analysis Effect Size (LEfSe) was performed to analyze the different microorganisms among all groups [31].

The data was deposited into National Genomics Data Center (https://ngdc.cncb.ac.cn) with accession number of CRA007489 and CRA007409.

2.7. Statistical analysis

The number of SE was logarithmically transformed. We evaluated OTUs, alpha and beta diversity between the two groups using unpaired *t*-test. One-way ANOVA was used to compare the difference of cytokine content and expression of TLR genes between two groups through SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Pearson correlation analysis was performed to analyze the expression of TLR genes and the abundance of gut microbial populations. $P < 0.05$ was considered significant.

Table 1
Primer Sequence for real-time PCR.

Gene	Gene ID	Primer Sequence (5′-3′)
β-actin	396526	F:GAGAAATTGTGCGTGACATCA
		R:CCTGAACCTCTCATTGCCA
TLR1A	426274	F:CAGACGAATTGTTCCAGAAT
		R:GTCCAGATACCTCAGTGATT
TLR1B	771173	F:CGCAGATGACAATGTAAGAA
		R:TGCCATACAACCTGAAGT
TLR2	769014	F:CAACACCTTCGCATTCAA
		R:TTGCCTGATGATGGAGAA
TLR3	422720	F:CTTATCTTTCAGACTGGTGTTT
		R:TGTCCAAGCCTGTTATGT
TLR4	417241	F:AATGACACGGACACTCTT
		R:CTTCTCAGCAGGCAATTC
TLR5	554217	F:CGGCAATAGTAGCAACAC
		R:CACAGTAAGAGAAGCGATTC
TLR7	418638	F:GCTGATTCCTACCAACCT
		R:GGCATTACCTGACAAGTTC
TLR15	421219	F:CTGCTCCATCGTAGAACT
		R:ATCGCTGTTACTTATGTCAAG
TLR21	415623	F:TGATGAAAGTTGGGTGTTG
		R:GTAGACAGCATCCACAATG

3. Results

3.1. SE colonization in the intestinal tract

At 7 dpi, SE colonization in the digestive tract showed that the number of SE in cecum of the challenged group was significantly higher than that in other sections of the intestine (6.96×10^6 CFU/g), followed by ileum (1.41×10^6 CFU/g respectively) ($P < 0.05$) (Table 2). At 14 dpi, the number of SE in cecum of the challenged group was the highest (1.59×10^3 CFU/g) across all sections of the intestine, and was significantly lower than that in the challenged group at 7 dpi ($P < 0.05$) (Table 2). No *Salmonella* was detected in the control group.

3.2. Immune organ index

At 7dpi, the thymus and spleen index of the challenged group were 2.49 and 1.66 respectively, which were higher than that of the control group (2.24 and 1.13 respectively), and the difference in spleen index was significant between the two groups ($P < 0.05$). At 14 dpi, the thymus and spleen index of the challenged groups were reduced compared with the control group (Table 3).

3.3. Serum cytokine responding to SE challenge

Compared with the control group, the contents of IL-2 and IL-18 in serum of the challenged group significantly increased by 131.56 pg mL^{-1} and 98.07 pg mL^{-1} ($P < 0.05$) at 7 dpi, respectively. The contents of TNF-α, IL-2 and IL-18 significantly increased by 29.65 pg mL^{-1}, 96.06 pg mL^{-1} and 126.18 pg mL^{-1} at 14 dpi ($P < 0.05$), respectively (Fig. 1).

3.4. Quantitative real-time PCR

The expression of *TLR1a*, *TLR1b*, *TLR2*, *TLR4*, *TLR5*, *TLR7*, and *TLR15* genes in the cecum were significantly increased in the challenged group at 7 dpi ($P < 0.05$), of which *TLR15* expression was 3.97 times that of the control group. At 14 dpi, *TLR4* and *TLR15* gene expression in the challenged group were significantly increased ($P < 0.05$), which were 1.42 and 2.23 times that in the control group, respectively. The expression levels of TLRs decreased at 14 dpi compared with 7 dpi. In the oviduct, the expression of *TLR1a*, *TLR5*, and *TLR7* increased significantly at 7 dpi ($P < 0.05$), which were 3.45, 3.52, and 3.13 times that in the control group, respectively. The expression level of TLRs in the challenged group was lower than that in the control group at 14 dpi. *TLR1b* and *TLR3* expressions were 0.3 and 0.22 times that in the control group, respectively ($P < 0.01$). The expression levels of *TLR4* and *TLR5* of the ovary at 7 dpi were higher than that in the control group ($P < 0.05$), which were 1.62 and 2.04 times that in the control group, respectively. The expression level of *TLR15* at 14 dpi was significantly higher than that of the control group ($P < 0.01$) (Fig. 2).

3.5. Microbiota composition of cecal and oviducal content

3.5.1. Microbial composition in cecum

Heatmap results indicated that SE infection caused dramatic changes in the cecal microbial composition (Fig. 3). The average relative abundance of Firmicutes was 76.19%, which was the dominant bacteria in the cecum contents. In addition, Bacteroidetes and Actinobacteria accounted for an average of 15.01% and 6.13%, respectively, followed by Proteobacteria (1.69%). The remaining categories combined with the unknown group accounted for 0.98%. After the infection, the proportion of Firmicutes in the cecum content was significantly increased, while the proportion of Bacteroides was reduced significantly (Fig. 3 a). At the genus level, *Anaerotruncus*, *Butyricicoccus*, *Parabacteroides*, and *Lactobacillus* accounted for a large proportion of the control group. The predominant

Table 2
The total counts of SE for each tissue of digestive system examined at different time points.

	7 d		14 d	
	CFU/g = mean number of colonies/100*1000/ quality of contents(A)	LOG (A+1)	CFU/g = mean number of colonies/100*1000/ quality of contents(A)	LOG (A+1)
esophagus	0.00	0.00	0.00	0.00
crop	227.27	2.36	1201.31	3.08
glandular stomach	3333.33	3.52	99.50	2.00
muscular stomach	144.93	2.16	0.00	0.00
duodenum	0.00	0.00	0.00	0.00
jejunum	0.00	0.00	1275.42*	3.11
ileum	140571.43	5.15	661.79*	2.82
cecum	6965174.13	6.84	1594.20*	3.20*
feces	741490.83	5.87	1504.30*	3.18

Note: "*" indicated $P < 0.05$.

Table 3
Comparison of thymus and spleen index between the two groups (n = 3).

DPI	7 d		14 d	
	thymus	spleen	thymus	spleen
CK	2.24 ± 0.17	1.13 ± 0.21	1.78 ± 0.16	1.08 ± 0.31
T	2.49 ± 0.27	1.66 ± 0.19*	1.67 ± 0.18	0.99 ± 0.16

Note: "*" indicated P < 0.05.

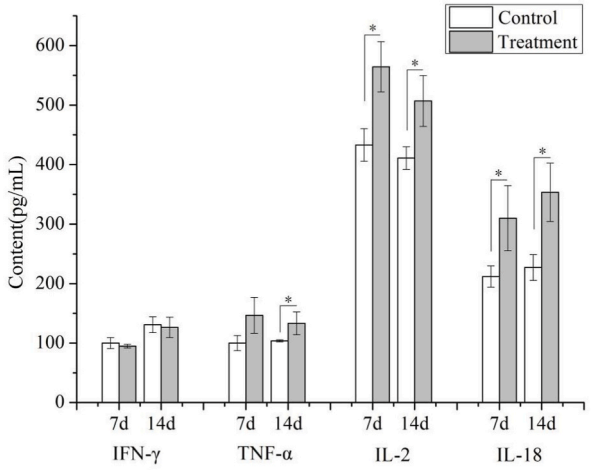


Fig. 1. Cytokine content in different groups.
Note: In this figure, "*" indicates significant within the 95% confidence interval ($P < 0.05$).

genera were *Blautia*, *SMB53*, *Faecalibacterium* and *Turicibacter* in the challenged group (Fig. 3 b).

3.5.2. Microbial composition in oviduct

The heatmap was used to identify differentially abundant taxonomic features of oviduct microbial composition at the phylum and genus levels (Fig. 4). At the phylum level, among the three groups, Proteobacteria accounted for 44.56% on average, followed by Firmicutes and Bacteroidetes (23.02% and 19.50% on average), and Actinobacteria accounted for an average of 5.03%. The remaining categories were below 1.00%, and the total proportion of mergers with unknown categories was 7.88% (Fig. 4 a). Therefore, Proteobacteria was the dominant strain in the contents of the oviduct. SE infection altered the microbial community in the oviduct, of which the Proteobacteria was significantly reduced while the Firmicutes was significantly increased ($P < 0.05$). At the genus level, the predominant genera in the control group were *Mycoplana*, *Phyllobacterium*, *Methylobaterium*, and *Erythrobacter*. In the challenged group, the predominant genera were *Sutterella*, *Pseudomonas*, and *Ruegeria* (Fig. 4 b).

3.5.3. Microbial diversity affected by SE infection in cecum

Venn diagrams were constructed to visualize differences and overlaps of OTUs between the control group and the challenged group (Fig. 5). The three groups shared 1444 OTUs. The number of unique OTUs was 4614 for CK, 2761 for T1 and 3222 for T2, indicating that SE infection lead to the number of unique OTUs in the challenged group decreased, especially at 7dpi. In pairwise sharing, the challenged group on the 7dpi and the 14dpi shared the most OTUs.

3.5.4. Principal component analysis

We performed PCA analysis on the composition of bacterial genera community structure in different groups. PCA analysis extracted two principal components, PC1 (80.7%) and PC2 (17.9%), and showed that the cecal bacterial genera community in CK was separated from that in T1 and T2, the similarity between T1 and T2 in the genus level was relatively high. Among the three groups, the differences between *Faecalibacterium*, *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, and *Rumenococcus* were greater than that of other genera (Fig. 6 a).

The PCA results of oviduct microbial composition showed that *Ochrobactrum*, *Bacteroides*, *Sediminibacterium*, *Acinetobacter*, *Agrobacterium*, *Cupriavidus*, *Blautia*, and *Lactobacillus* were different compared to other bacteria among the three groups. There was no overlap among the three groups, the control group and the challenged groups were separated (Fig. 6 b).

3.5.5. Effects of SE infection on probiotic and harmful bacteria in cecum contents

At 7 dpi, the relative abundance of *Bifidobacterium* and *Lactobacillus* in the challenged group was 0.031% and 1.030%, and in the

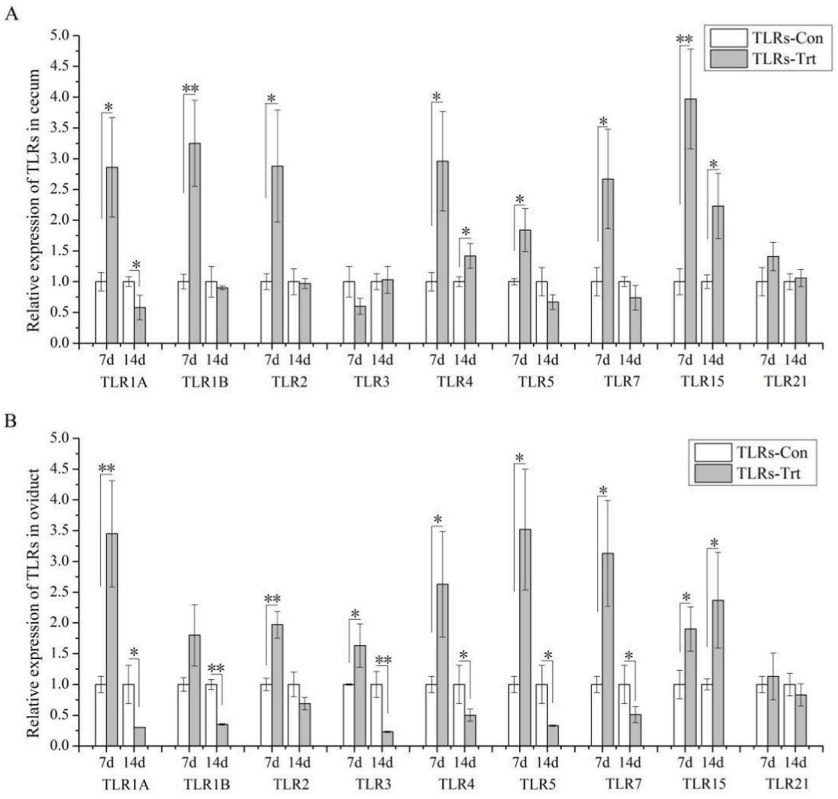


Fig. 2. Relative expression of TLRs mRNA in the cecum and oviduct.
Note: "*" indicated $P < 0.05$, and "**" indicated $P < 0.01$.

control group were 0.056% and 2.770%, respectively. Compared with the control group, the relative abundance of *Bifidobacterium* in the challenged group was reduced and the relative abundance of *Lactobacillus* was reduced significantly ($P < 0.05$). At 14 dpi, the relative abundance of *Bifidobacterium* and *Lactobacillus* increased significantly compared with that at 7dpi ($P < 0.05$), which was 0.036% and 1.046%, respectively (Fig. 7 a).

At 7 dpi, the relative abundance of *Enterobacteriaceae* and *Enterococcus* in the challenged group were 1.199% and 0.072%, respectively, which was significantly higher than that of the control group of 0.056% and 0.017% ($P < 0.05$). At 14 dpi, the relative abundance of *Enterobacteriaceae* and *Enterococcus* decreased compared with the 7dpi, which was 0.010% and 0%, respectively (Fig. 7 b).

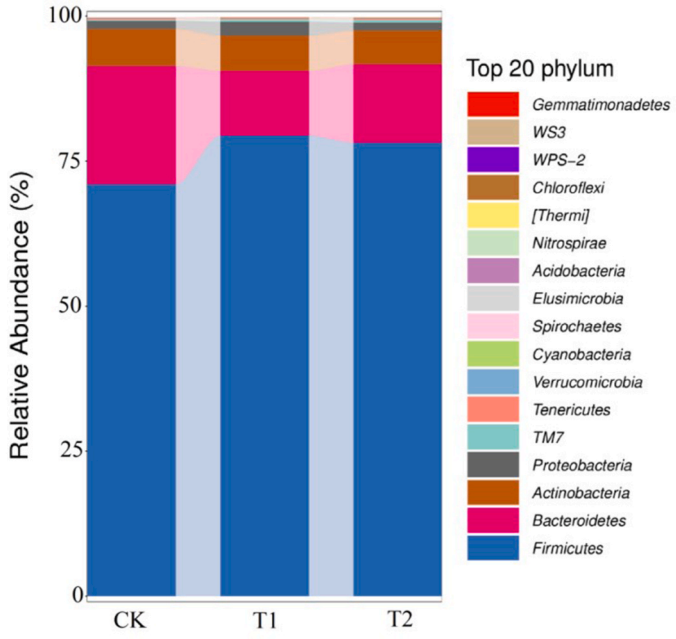
3.6. Relationship between TLRs expression and microbial abundance of the cecum

Pearson correlation analysis was performed between the expression of TLRs and the abundance of microbes in the cecum. The abundance of *Bifidobacterium* and *Lactobacillus* were negatively correlated with the expression of TLRs except for *TLR3*, expression *TLR4* and *TLR15* were strongly correlated with *Bifidobacterium* and *Lactobacillus*. The abundance of *Enterobacteriaceae* and *Enterococcus* were positively correlated with the expression of TLRs except for *TLR3*. *Enterobacteriaceae* were significantly correlated with the expression of *TLR1a* and *TLR5* ($P < 0.05$), and *Enterococci* were significantly correlated with *TLR1b* and *TLR2* ($P < 0.05$). TLRs expression was not correlated with the total number of OTUs (Table 4).

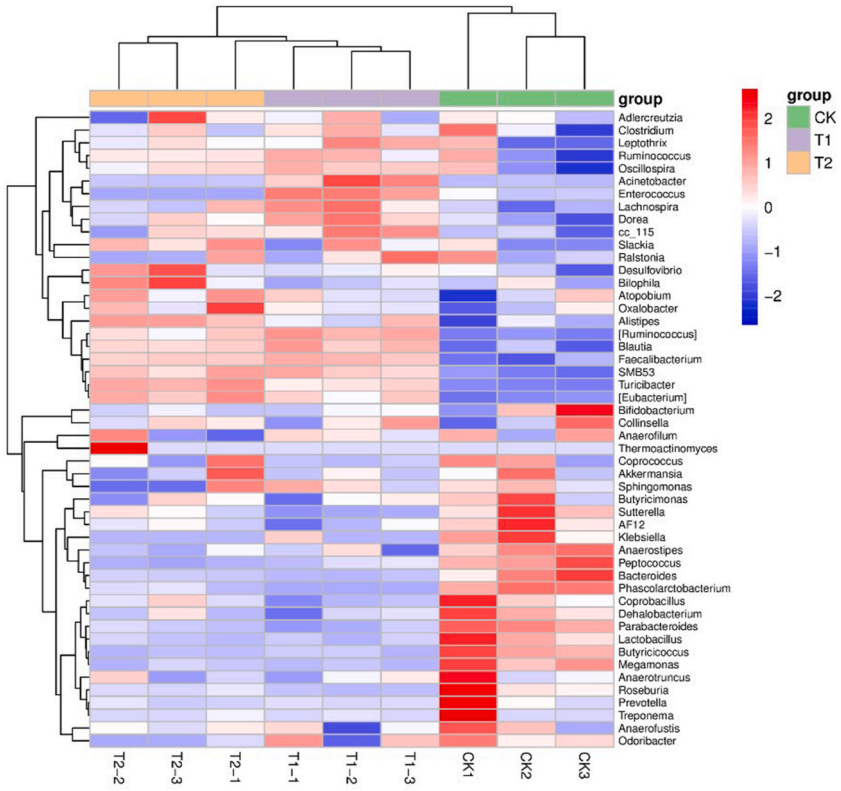
4. Discussion

Salmonella Enteritidis is recognized by pattern recognition receptors (PRRs) of the innate immune system. PRRs, such as TLRs and NLRs, are the first component of the immune system to detect the invasion of pathogens into the host, initiate the immune response, and establish a connection between innate immunity and adaptive immunity [32]. *Salmonella* Enteritidis stimulates the production of pro-inflammatory cytokines, and thus promotes pro-inflammatory functions in the host [24]. It has been reported SE infection can cause the expression of IL-6, IL-8, and INF-γ significantly upregulated in duck granulosa cells (dGCs) [33], increased the expression of the pro-inflammatory factor IL6 in the chicks [34]. Our data showed an increased trend of the immune index in the challenged group compared with the control group ($P < 0.05$), indicating that the infection of SE activated the innate system of the hens.

TLRs play a key role in immune response, recent researches have shown that SE infection affects the expression TLRs. Infection with



(a)



(b)

(caption on next page)

Fig. 3. Microbial composition of the cecal content.
Note: (a) Phylum level composition of the cecal content. (b) Genus level composition of the cecal content. CK: the control group, T1: the challenged group at 7 dpi, T2: the challenged group at 14dpi.

SE alters differently the expression of certain TLRs in various chicken tissues or cell types [35,36]. A study of the effects of *Salmonella* infection on toll-like receptor expression in the chicken vagina, reported a significant up regulation ($P < 0.05$) in the expression of TLR2-1, 4 and 15 in the vagina of aged 104-weeks-old SE infected birds, compared with the birds of the same age challenged with PBS [36]. Other studies also found SE infection has a tendency to up-regulate the expression of TLR2, TLR4, and TLR15 in chicken spleen [37,38]. In order to determine whether SE infection triggers the immune response mediated by TLRs in the chicken, we investigated the changes in the expression levels of TLRs. Our study observed that SE infection induced the expression of TLRs in cecum and oviduct, and the results proved that TLRs are closely correlated with the host's immune response. The findings confirm that when pathogens invade, TLRs recognize and induce a series of innate and adaptive immune responses, producing various cytokines and chemokines to interact with pathogens [39,40].

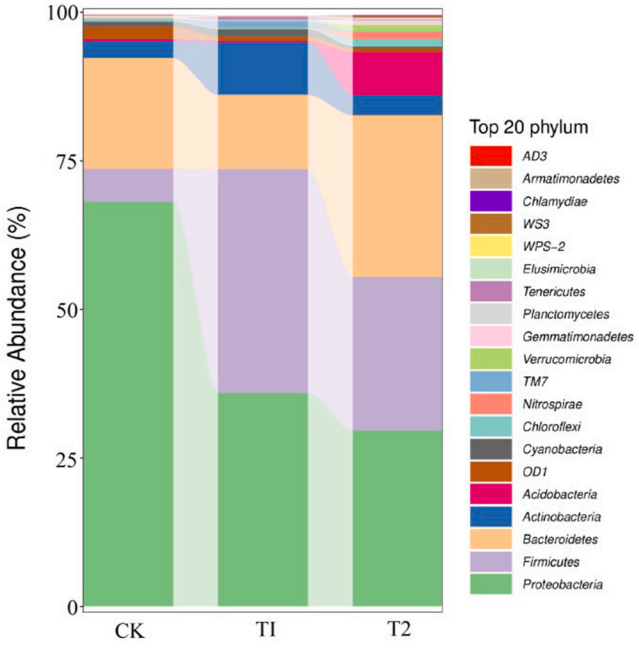
The gut microbiome is an essential regulator of several host pathways relevant to disease, including immune development and energy metabolism [41]. The microbiome affects immunity, metabolism, and developmental gene regulation [42–44]. The gut microbiota composition affects protein expression in the cecum of chickens [45]. *Bifidobacterium* and *Lactobacillus* are important beneficial microorganisms in the gut. They can inhibit the growth of harmful bacteria and resist infection by pathogenic bacteria. In contrast, *Enterobacteria* and *Enterococci* have strong pathogenicity. Growing evidence suggests that intestinal microbiota dysbiosis may involve the development of intestinal injuries such as intestinal structure, function and inflammatory disease [46]. Previous study has shown that abundances of *Faecalibacterium*, *Streptococcus*, and *Enterobacteriaceae* were significantly positively correlated with TLR4 gene expression [47,48]. We found *Enterobacteriaceae* and *Enterococcus* were strongly positively correlated with the expression of TLR genes, while the correlation between the abundances of *Bifidobacterium* and *Lactobacillus* and the *TLR* genes expression was negative. The result indicating that TLRs likely embody strong immune responses against pathogenic bacteria. Our results showed that colonization of the intestinal microbiota in chicken could trigger the host's immune response. Volf et al. (2017) [42] further demonstrated that the range of immunoglobulin expression was dependent on microbiota composition.

Pathogen infection has a certain effect on the total intestinal microbiota and further impacts the immune system [49]. After infected with *Salmonella* Enteritidis, the composition of the cecal microbiota in chicks was altered [50]. It was found that there was a significant reduction in the Shannon index in cecal contents estimated at 1dpi ($P < 0.01$). At 7dpi, the number of OTUs was significantly decreased in all subgroups relative to 1dpi ($P < 0.05$) [3]. In the present study, the number of OTUs in T1 and T2 was lower than that in the control group. Wu et al. (2021) [34] found that the microbial abundances of several dominant strains in chicken cecum content were great different. Bacteroidota, Firmicutes, Proteobacteria, and Actinobacteriota constituted the four dominant phyla in both groups of infected chicks. Firmicutes accounted for the largest proportion in three groups of samples, followed by Bacteroidetes and Actinobacteria, which is in line with previous studies [51,52]. As with other vertebrates, Firmicutes and Bacteroidetes are the predominant bacterial species in the microbial flora of the chicken cecum [53,54]. After challenged with SE, the abundance of the Firmicutes in the challenged group was higher than that of the control group, and the Bacteroidetes were significantly reduced. SE infection decreases the abundance of specific microbial genera and microbial diversity in the fecal material of laying hens and then causes dysbiosis [55]. Moreover, intestinal mucosal damage caused by SE infection leads to dysbiosis of the normal flora of the intestine, transforming intestinal flora from beneficial bacteria to pathogenic bacteria [56]. We found that the relative abundance of *Enterobacteriaceae* and *Enterococcus* in the challenged group increased significantly compared with the control group at 7dpi, indicating that SE infection changed the composition of the cecal microbiota.

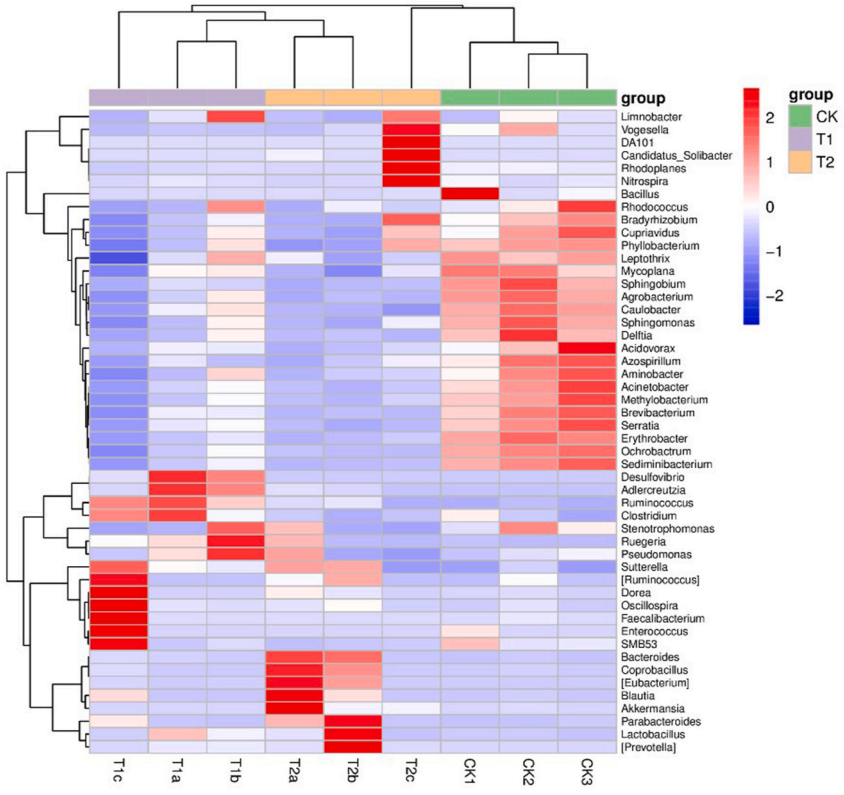
The composition of the microbial community in the oviducts has not been widely studied, however, it can provide theoretical support for *Salmonella* infection in eggs. The egg-laying capacity of the SE infected group was significantly lower than that of the non-infected group [25,26]. Bacterial dissemination to reproductive tissues (ovary and oviduct) in systemically infected hens caused the deposition of *Salmonella* Enteritidis inside the edible interior contents of eggs [52]. In layer chickens, Proteobacteria are most dominant up to 7 days of age; afterward, Firmicutes became abundant [16,46]. We found at 7dpi, Firmicutes was the dominant bacteria in the oviduct, and Bacteroidetes increased significantly at 14 dpi, became the second dominant bacteria in the oviduct. Our results indicated that infection *Salmonella* Enteritidis significantly altered the microbial community in the oviduct. Vertical transmission is an essential way in which *S.* Enteritidis spreads during the poultry production period [29]. The cloaca is the common opening to the digestive and reproductive tracts, where microorganisms can reach the cloaca and then potentially migrate into the reproductive organs [30]. In addition to systemic spread, SE can also access the oviduct through ascending infection from the cloaca [57], and this is one of the reasons why SE infection changed the composition of oviduct microbiota. Following colonization of the reproductive tract, *Salmonella* Enteritidis may be deposited into the albumen and yolk during egg formation, and the external shell surface may be contaminated during passage through the cloaca and vent [36]. Furthermore, SE infection-induced innate and adaptive immune responses may support its vertical transmission [58,59]. Another published work showed a similarity between the microbiomes of maternal hen feces, embryos, and chick ceca, implying vertical transmission of gut bacteria [60], so that the SE could transmit from chicken to eggs.

5. Conclusion

Salmonella Enteritidis infection triggered the host's innate and adaptive response, increased the expression of TLRs and cytokines



(a)



(b)

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Fig. 4. Microbial composition of the oviducal content.
Note: (a) Phylum level composition of the oviducal content. (b) Genus level composition of the oviducal content. CK: the control group, T1: the challenged group at 7 dpi, T2: the challenged group at 14dpi.

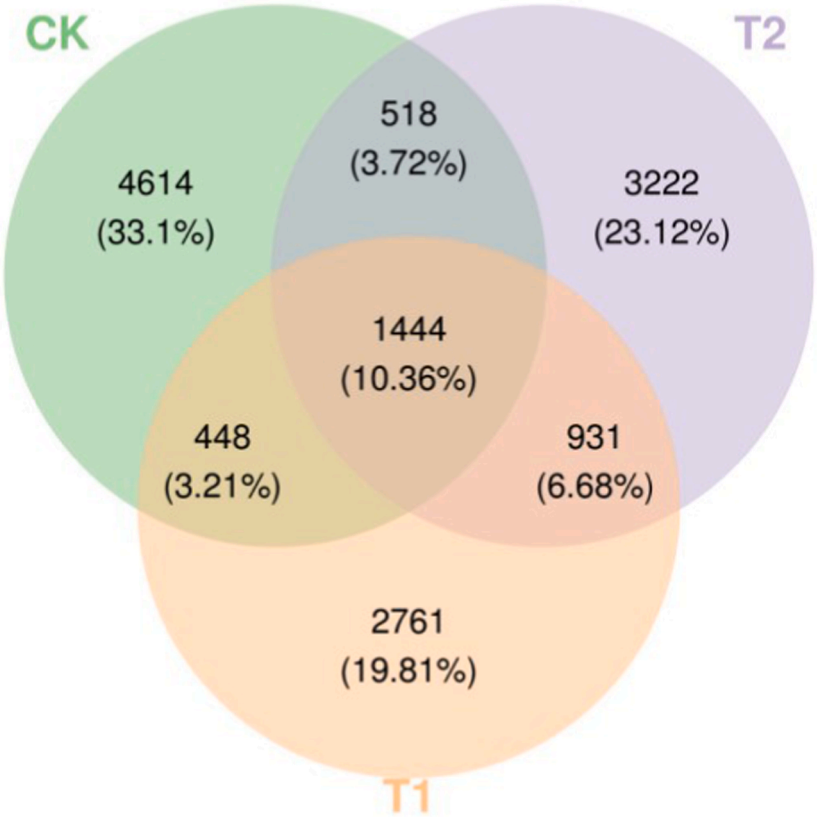


Fig. 5. Venn diagram of the sample ASV OTU.
Note: CK: the control group, T1: the challenged group at 7 dpi, T2: the challenged group at 14dpi.

contents, and changed the microbial diversity in the cecum and oviduct. These findings will lay the foundation for further study on reducing the vertical transmission of *Salmonella* Enteritidis, ensuring the production of clean eggs.

Ethics statement

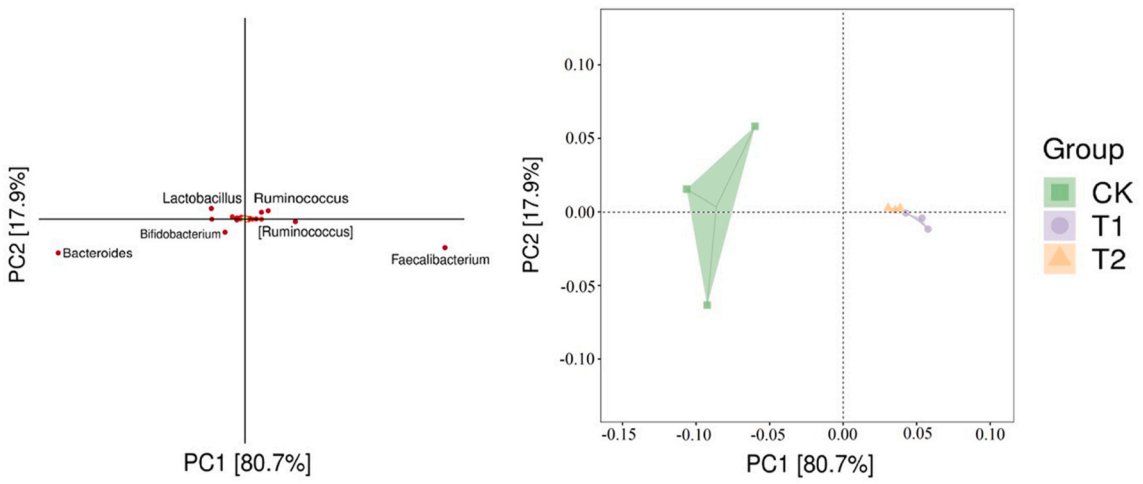
All animal procedures were approved by Shandong Agricultural University Animal Care and Use Committee. All methods were carried out in accordance with relevant guidelines and regulations. The study was carried out in compliance with ARRIVE guidelines.

Author contribution statement

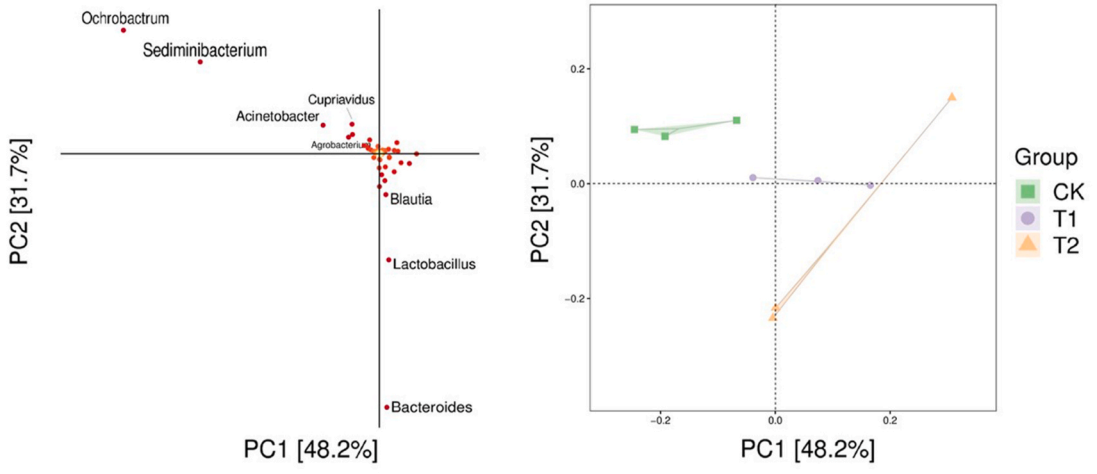
Liying Liu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Xianyao Li: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Xintong Jiang: Performed the experiments; Wrote the paper.
Xiao Zhang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Yuqing Sun: Analyzed and interpreted the data.
Zhongtao Sun: Contributed reagents, materials, analysis tools or data.
The final manuscript was reviewed and approved by all contributors.

Data availability statement

Data associated with this study has been deposited at National Genomics Data Center (https://ngdc.cncb.ac.cn) with accession number of CRA007489 and CRA007409.



(a)



(b)

Fig. 6. PCA analysis.
Note: (a) Species loading map analyzed by PCA. (b) Sample two-dimensional ranking chart (right) analyzed by PCA. CK: the control group, T1: the challenged group at 7 dpi, T2: the challenged group at 14dpi.

Additional information

No additional information is available for this paper.

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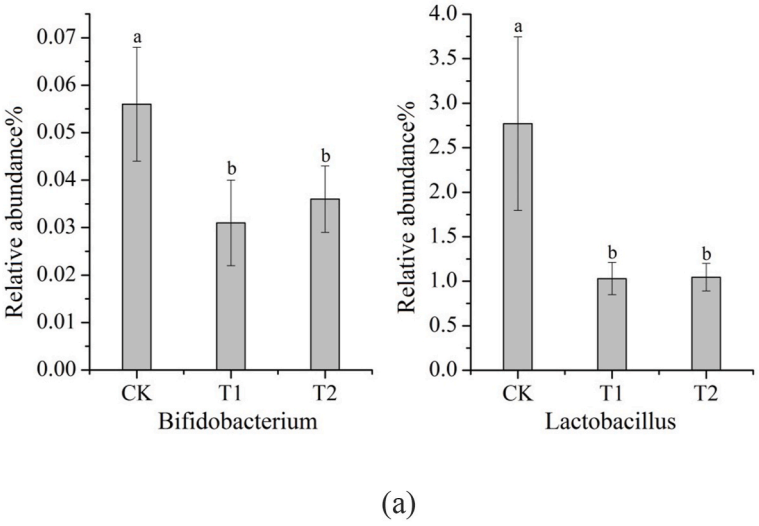
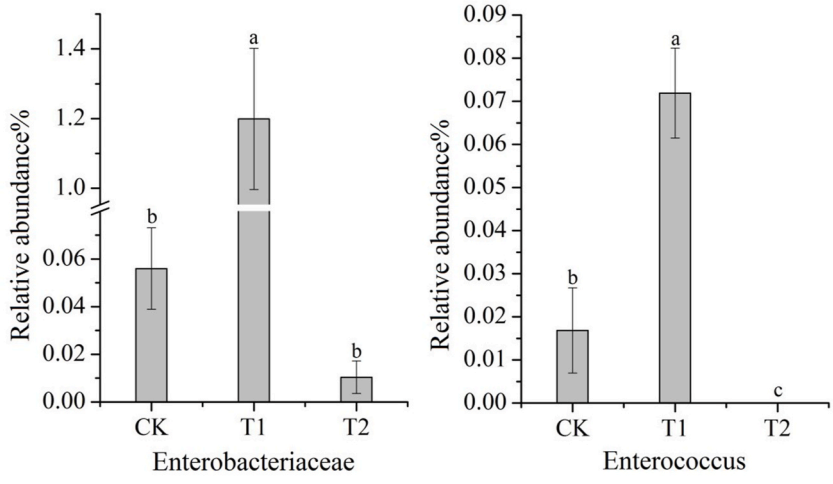


(a)



(b)

Fig. 7. Relative abundance of probiotic and harmful bacteria in cecum contents.
Note: (a) Relative abundance of probiotic bacteria in cecum contents. (b) Relative abundance of harmful bacteria in cecum contents. Different letters indicated $P < 0.05$. CK: the control group, T1: the challenged group at 7 dpi, T2: the challenged group at 14dpi.

Table 4
Pearson correlation coefficient between TLRs gene expression and cecal microbial community abundance.

	Pearson correlation coefficient				
	Bifidobacterium	Lactobacillus	Enterobacteriaceae	Enterococcus	OTU
TLR1A	-0.514	-0.35	0.999*	0.99	0.245
TLR1B	-0.626	-0.474	0.982	1**	0.111
TLR2	-0.644	-0.495	0.978	1*	0.087
TLR3	0.606	0.452	-0.987	-1	-0.136
TLR4	-0.795	-0.672	0.909	0.972	-0.131
TLR5	-0.423	-0.252	0.999*	0.971	0.344
TLR7	-0.556	-0.396	0.995	0.996	0.197
TLR15	-0.908	-0.817	0.796	0.897	-0.344
TLR21	-0.751	-0.619	0.935	0.986	-0.062

Note: The correlation is positively correlated with the absolute value of the correlation coefficient. "*" indicated $P < 0.05$, "**" indicated $P < 0.01$.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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