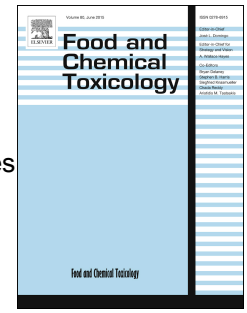

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Selenium-Enriched Yeast reduces caecal pathological injuries and intervenes changes of the diversity of caecal microbiota caused by Ochratoxin-A in Broilers

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Selenium-Enriched Yeast reduces caecal pathological injuries and intervenes changes of the diversity of caecal microbiota caused by Ochratoxin-A in Broilers

Abstract: We investigated the protective effect and mechanism of selenium-enriched yeast (SY) on caecal injury induced by ochratoxin A (OTA) in broilers. Eighty broiler chickens of 1-day-old with similar weight were randomly assigned to Control group, OTA group, SY group and OTA+SY group, and were intragastricaly administered with OTA and SY for 21 consecutive days. The results showed that SY could reduce the caecal pathological injuries and could inhibit oxidative stress caused by OTA exposure. The OTA+SY group showed a statistically significant (p<0.01) reduction in the level of MDA, IL-1β, IL-6 and IFN-γ, whereas the levels of GSH, SOD activity and IL-10 were significantly increased (p<0.01). By regulating TLR4/MYD88 signaling pathway, SY inhibited the expression of NF-κB, increased the expression of tight junction-related genes Claudin-1, Occludin and ZO-1, and antagonized the intestinal barrier injury caused by OTA exposure. Moreover, the microbial diversity analyses indicated that SY could intervene changes in the diversity of gut microbiota and the imbalance of gut microbiota caused by OTA. SY could relieve caecal pathological injuries, alleviate OTA-induced caecal oxidative stress and inflammatory response, increase the gut microbial diversity and protect broiler's intestinal barrier from injury.

Key Words: Selenium-Enriched Yeast; Caecal; Microbiota; Ochratoxin-A; Broilers

1. Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite secreted by several species of

Aspergillus and Penicillium(Greer&Brunet, 2008), and is widely present in grains, animal feeds, meat, eggs, vegetables, fruits, wine, beer, coffee, etc.(Koszegi&Poor, 2016; Smith et al., 2016). Due to its extensive distribution and strong toxicity, OTA has caused considerable economic losses for the livestock and poultry breeding, and creating challenges to meet the global demand for meat and dairy products, as well as threatened the food safety.

Mycotoxins display various toxicity mechanism to cause its deleterious effects when consumed by livestock. Toxic effects of OTA leads to a series of reactions in the body including nephrotoxicity, hepatotoxicity, cytotoxicity, genotoxicity, carcinogenicity, teratogenicity, immunotoxicity and intestinal toxicity(Zeferino et al., 2017). Some studies have shown that the nephrotoxicity of OTA may be related to oxidative stress, and can cause the formation of various ROS(Arbillaga et al., 2007; Petrik et al., 2003), such as superoxide anion (O^{2-}), hydroxyl radical (OH^{-}) and peroxide (ROO^{-}), which can induce immediate effects on kidney cells by decreasing the antioxidant capacity(Cavin et al., 2009; Di Giacomo et al., 2007). Studies have revealed that OTA activates the nuclear transcription factor NF-κB by activating the TLR4/MYD88 signaling pathway, stimulating the expression of inflammatory factors and promoting the occurrence of inflammatory responses(Xu et al., 2017). As OTA are introduced through food, the gastrointestinal tract is the first important target organs to play its toxic effect. It has been reported that OTA can cause the intestinal mucosal injury of chickens(Solcan et al., 2015), which then leads to inflammatory effects resulting in intestinal mucosal necrosis and exfoliation. OTA can interrupt the functioning of the digestive system by impairment of digestive enzymes, and contribute to food allergy(Varga et al., 2005). The cytotoxic effect of OTA has been further investigated in human colon adenocarcinoma cell (Caco-2) assays, where OTA changed the distribution of

specific tight junction proteins that maintained the integrity of cell membranes, resulting in an increase in cell permeability(Maresca et al., 2001; McLaughlin et al., 2004).

However, the diverse toxic effects of mycotoxins on their influence in colonization of the gut microbiota were rarely reported. Currently, a limited study illustrated the toxic effects of several mycotoxins on gut microbiota: aflatoxin B1(J. Wang et al., 2016; Yang et al., 2017), deoxynivalenol(Li et al., 2017; Saint-Cyr et al., 2013; Wache et al., 2009), fumonisin B1(Antonissen et al., 2015; Burel et al., 2013), zearalenone(Piotrowska et al., 2014), and ochratoxin A(Guo et al., 2014). These studies have provided us with important information that microbial communities are key targets for mycotoxins. It is a promising research direction for alteration of intestinal microbiota to reduce the toxicity of mycotoxins in animals.

Selenium (Se), one of the essential trace elements, having a strong antioxidant capacity is the main component of many antioxidant enzymes in the body(Schiavon et al., 2017). Studies by Kasaikina MV *et al.* have shown that dietary selenium can increase the expression of selenium-containing antioxidant proteins and the diversity of gut microbiota(Kasaikina et al., 2011). Selenium-enriched yeast (SY) is a high-quality organic Se source for animals, which has many advantages compared with inorganic sources, such as sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4). Se in the SY is stored in protein structure to improve the bioavailability(Rajashree et al., 2014). The total content of Se in SY is 1.4 to 2 times higher than that in inorganic compounds(Kubachka et al., 2017).

Currently, the effect of SY on the intestinal tract of animals, especially on the gut microbiota of broilers, is underreported. Therefore, this study will determine the beneficial and protective outcomes of SY on toxic effects in caeca and caecal flora of broilers caused by OTA, thereby

providing a theoretical reference for the prevention of OTA.

2 Experimental design and treatment

2.1 Animals and Treatment.

A total of eighty, white feather broilers of 1-day-old with the weight range being 49.14±1.57g were randomly assigned to four groups, with 20 in each group: Control group, OTA group (OTA: 50μg/kg/BW), SY group (SY: 0.4mg/kg/feed), SY+OTA group (0.4mg/kg/feed, OTA: 50μg/kg/BW). Dosages of OTA were based on those reported by Solcan et al. (2015), and SY doses were based on those reported by Bakhshalinejad et al. (2018) . The dosage was adjusted according to the change in weight of the broilers. At 8:00 every day, the SY solution was intragastrically administered, followed by OTA solution after 30 min interval. The indoor temperature was maintained at 30 ± 5 ℃, and the relative humidity 40 ± 5%. Light and darkness were alternated every 12 hours, and there was free access for food and drink throughout, which was continued for 21 days. After the end of the experiment, all birds were slaughtered by bisection of the cervical vessels. The caecal contents of 6 broilers in each group were aseptically frozen, and the remaining caeca were washed, placed in a frozen tube and stored in a -80 ℃ refrigerator.

Experimental procedures had been previously

approved by the Ethics Committee for Laboratory Animal Care (Animal Ethics Procedures and Guidelines of the People's Republic of China) for the use of Shenyang Agricultural University, China (Permit No. 264 SYXK<Liao>2011-0001,20, October, 2011)

2.2 Analysis of caecal histopathology

The caecal tissues of five chicken per group were excised and fixed in 10% neutral formalin, followed by dehydration in ethanol series of grading concentration, and subsequent clearing in

xylene and embedding in wax to make paraffin blocks. The tissues were cut into 4 μm-thick sections, stained with hematoxylin and eosin (H&E) (Servicebio, Wuhan, China), and examined by optical microscopy using a Leica DM750 microscope (Leica, Beijing, China) to assess histopathological damage.

2.3 Detection of parameters of caecal oxidative stress

In order to detect the antioxidant capacity of caecal tissues, tissue homogenate was prepared. BCA protein assay kit, SOD kit, micro reduced glutathione (GSH)

assay kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China) and Malondialdehyde （MDA） content assay kit (Beijing solarbio science & technology Co. Ltd, Beijing, China) were used to detect antioxidative functions following the manufacturers' instructions.

2.4 Detection of inflammatory factors

To detect the inflammatory changes in the caecal tissues, the tissue homogenates were subjected to ELISA and detected the levels of inflammatory factors, IL-1β, IL-6, IFN-γ and IL-10, using the ELISA kit (Jiangsu Enzyme Free Industrial Co., Ltd. China), following the manufacturers' instructions.

2.5 Detection of mRNA expression

Total RNA was extracted using the RNA extraction kit (Nanjing Noviacan Biotechnology Co. Ltd. China）. Real-time PCR was performed to detect mRNA expression of NF-κB ,TLR4 ,MyD88 , Caludin-1 ,Occludin and ZO-1. RNA was purified using the OD260/280 ratio, which was between 1.8 and 2.1. The experimental procedures were conducted on ice according to the instructions of kit. $2^{-\Delta\Delta}$CT method was used for Real-time PCR data analysis. All values were normalized using β-actin as a reference. Real-time PCR was conducted based on the procedure proposed by Yang et al. (2016). The PCR primers were commercially synthesized by Sangon Biotech Institute Co. Ltd.,

China. The PCR primers used in this study are shown in Table 1.

Table 1. The primers seqence of the target genes.

Primer	Seqence	Amplicon size (bp)
NF-κB-F	5'-TTCTCCACTTGGCGATCATTCACG-3'	1853
NF-κB-R	5'-GTCTGGCTGAGGTTGTTCTGGAAG-3'	
TLR4-F	5'-AGTCTGAAATTGCTGAGCTCAAAT-3'	3532
TLR4-R	5'-GCGACGTTAAGCCATGGAAG-3'	
MyD88-F	5'- CGAGCCACTCTGTTGCCATACC -3'	4335
MyD88-R	5'- CGAGCCACTCTGTTGCCATACC -3'	
Claudin-1-F	5'- GACCAGGTGAAGAAGATGCGGATG -3'	2578
Claudin-1-R	5'- CGAGCCACTCTGTTGCCATACC -3'	
Occludin-F	5'-ATCAACGACCGCCTCAATCA-3'	1975
Occludin-R	5'-CTTTGGTAGTCTGGGCTCCG-3'	
ZO-1-F	5'- GCCTACTGCTGCTCCTTACAACTC -3'	6762
ZO-1-R	5'- GCTGGATCTATATGCGGCGGTAAG -3'	
β-Actin-F	5'-AGGAGAAGCTGTGCTACGTC-3'	1736
β-Actin-R	5'-TACCACAGGACTCCATACCCAA-3'	

2.6 Western blot analysis

The caecal tissues were fully ground and lysed by RIPA high-efficiency lysis buffer (Beijing Solarbio Science & Technology Co Ltd, Beijing, China), and the total tissue proteins were extracted by high-speed centrifugation. The protein concentration was detected using the BCA protein assay kit (Beijing Solarbio Science & Technology Co. Ltd, Beijing, China), and the samples were prepared into the solution of 50 μg/20 μl for testing. Proteins were isolated using SDS-PAGE (SDS polyacrylamide gel electrophoresis), and transferred to the PVDF membrane at 150 mA for 85 min. Subsequently, blocking was conducted for 2 h with 1% BSA (Beijing Solarbio Science & Technology Co. Ltd, Beijing, China) blocking buffer, and then incubated for 2 h with corresponding antibodies of Claudin-1, TLR4, NF-κB and β-Actin, followed by incubation for 1 h with horseradish peroxidase (HRP)-coupled rabbit antibody. ECL luminescent reagent was used

for the detection. The image analysis system (Super ECL Plus, Applygen, Beijing, China) was used for the quantitative analysis of target protein expression.

2.7 Analysis of gut microbiota

Total DNA from caecal contents of broilers was extracted using the PowerSoil® DNA Isolation Kit (Mobio, USA). Simultaneously, the primers (F:5'-ACTCCTACGG-GAGGCAGCA-3'; R:5'-GGACTACHVGGGTWTCTAAT-3') were obtained according to the V3-V4 conserved region, and a sequencing joint was added to the end of the primer. The PCR amplification was performed, and the purification, quantification and homogenization of the product were conducted to form a sequencing library. The established library was first subjected to quality control, and the qualified library underwent sequencing using Illumina HiSeq 2500 (Illumina Inc., San Diego, CA, USA). The clustering was performed to obtain Operational Taxonomic Units (OTU) at 97% similarity level using UCLUST in QIIME (version 1.9.0) software, and the OTU was taxonomically annotated based on the Silva (bacteria) and UNITE (fungi) taxonomic databases. The Alpha diversity index of the sample was evaluated using Mothur (version v.1.30) software. For analyses of beta diversity, unweighted pair-group method with arithmetic means (UPGMA) and principal component analysis (PCA) were performed using QIIME software. All data was processed using the BMK Cloud online bioinformatics pipeline tool (Biomarker Technologies Corporation, Beijing, China).

2.8 Statistical analysis

Data were statistically analyzed using SPSS 21.0 software (IBM, Almon, NY, USA) and histogram was made using Graphpad prism 5.0 software. The results were expressed as mean ± standard deviation. One-way ANOVA was used to compare the differences of data among groups;

P value <0.05 indicated a difference, and *P* value <0.01 indicated a significant difference.

3 Results

3.1 Morphological and Histopathological changes

In the control group and SY treatment group, the morphological features of the caecum was normal, the intestinal glands and mucous membrane structure was complete, and the intestinal villi were arranged in an orderly manner, without any obvious abnormal changes. The caecum of broilers in OTA group was inflated, and there was a small amount of bleeding in lamina propria, and a small amount of intestinal mucosa was detached. After pretreatment with SY, the intestinal mucosal detachment of the broiler cecum was alleviated, and the lamina propria had no visible changes.

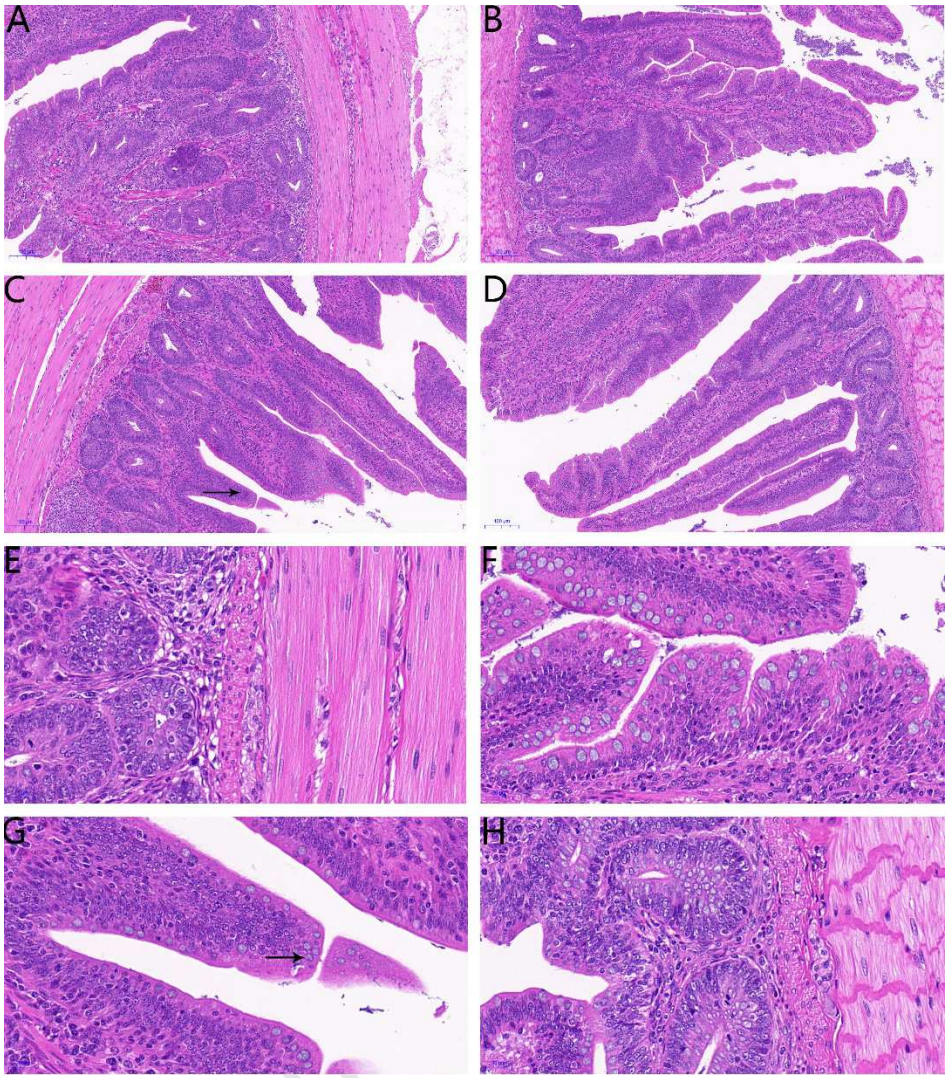


Fig. 1 Pathological changes in caeca were detected by tissue section H&E staining. Images were taken at magnifications of 100X （A,B,C,D） and 400X(E,F,G,H). (A) and (E)Control group , (B) and (F) SY (0.4mg/kg/feed) group, (C) and (G) OTA (50μg/kg/BW) group, and (D) and (H)OTA+SY (SY :0.4mg/kg/feed, OTA: 50μg/kg/BW) group. The arrow → indicates pathological damage in the caeca, such as a small amount of bleeding in lamina propria, and a small amount of intestinal mucosa was detached.

3.2 Detection of caecal oxidative indices

Antioxidant capacity and oxidative injury of OTA to caeca of broilers were evaluated through the detection of the oxidative indices, such as GSH content, SOD

activity and injury marker MDA content in the caecal tissues (Fig. 2). Compared with the control group, the caecal GSH content and SOD activity in the OTA group showed a highly significant decrease (p<0.01), indicating that OTA inhibited the antioxidant capacity of caecal tissues in broilers, whereas the caecal MDA content showed a highly significant increase (p<0.01), indicating that OTA could cause

oxidative stress in the caeca of the broilers. The addition of SY could reverse the results of GSH, MDA contents and SOD activity. Compared with the OTA group, the caecal GSH content and SOD activity in the OTA+SY group showed a highly significant increase (p<0.01), and the MDA content showed a highly significant decrease (p<0.01), indicating that SY could relieve the oxidative injury to the caeca in broilers.

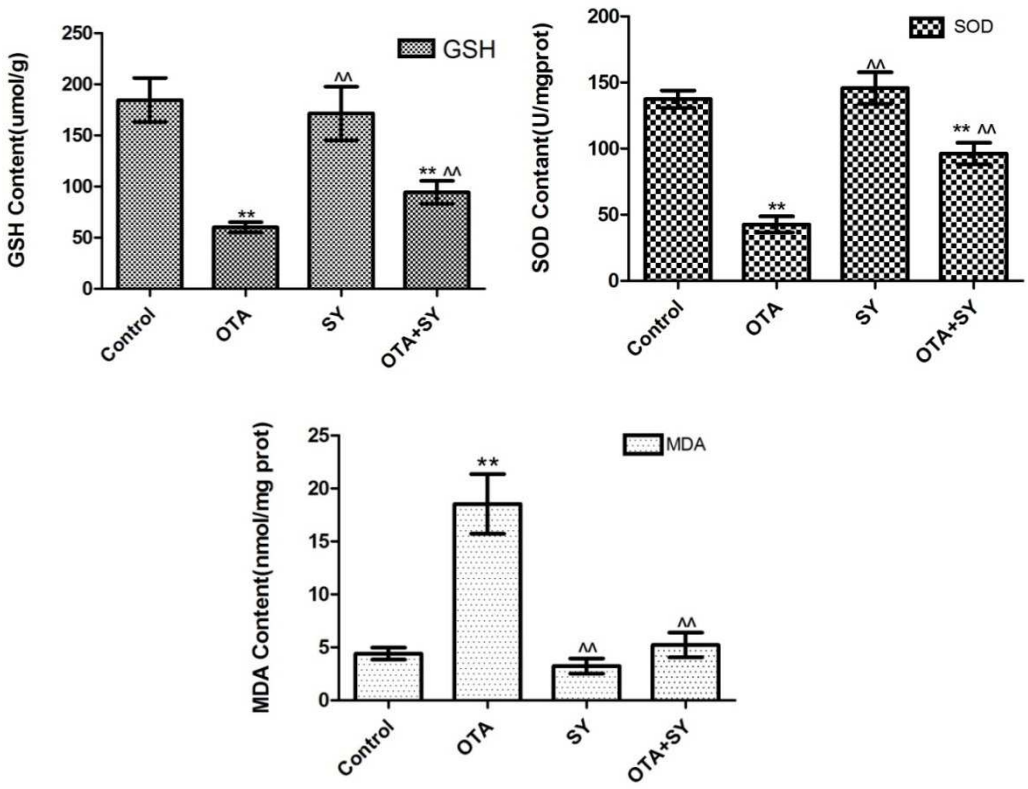


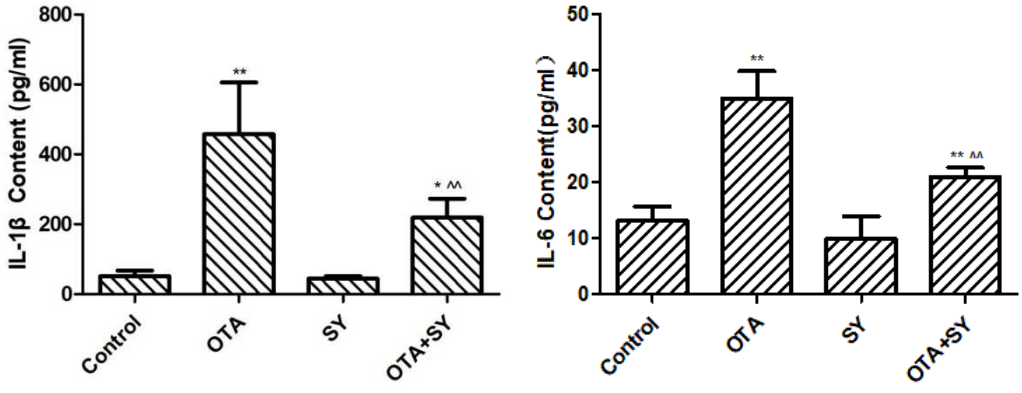
Fig. 2 Changes of GSH, MDA and SOD Contents

Note: ** indicates a extremely significant difference compared with the control group, $p<0.01$; ^^ indicates that it is associated with the OTA group. Compared with the extremely significant difference, $p<0.01$.

3.3 Detection of inflammatory factors

The levels of inflammatory factors, such as IL-1β, IL-6, IL-10 and IFN-γ were detected to study the effects of SY on OTA-induced inflammatory injury to caeca in broilers (Fig. 3). Compared with the control group, levels of IL-1β, IL-6 and IFN-γ in the OTA group showed a highly significant increase ($p<0.01$), while IL-10 level showed a highly significant decrease ($p<0.01$), indicating that OTA caused

inflammatory response of caecal tissues. In the OTA-SY group, the addition of SY reversed the levels of inflammatory mediators, IL-1β, IL-6, IL-10 and IFN-γ in caecum. Compared with the OTA group, levels of IL-1β, IL-6 and IFN-γ in the OTA+SY group were significantly decreased ($p<0.01$ or $p<0.05$), while IL-10 level showed a highly significant increase ($p<0.01$), suggesting that SY relieved OTA-induced inflammatory injury to caeca.



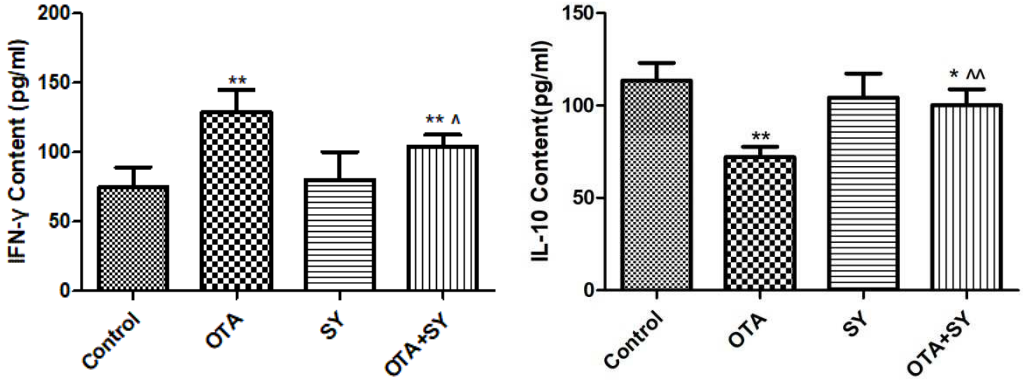


Fig. 3 Changes of IL-1β，IL-6，IL-10 and IFN-γ Contents

Note: * indicates a significant difference from the control group, $p<0.05$, ** indicates a extremely significant difference compared with the control group, $p<0.01$; ^ represents a significant difference compared with the OTA group, $p<0.05$, ^^ indicates that it is associated with the OTA group. Compared with the extremely significant difference, $p<0.01$.

3.4 Effects of SY and OTA on the mRNA of claudin-1, occludin, ZO-1, TLR4, MYD88 and NF-κB in appendix

3.4.1 mRNA expressions of caecal tight junction protein-related genes

To study the effects of SY on OTA-induced injury to the tight junction structure of the intestinal mucosa, expressions of caecal tight junction protein-related genes, claudin-1, occludin and ZO-1 were detected at the mRNA level. As shown in Fig. 4, compared with the control group, expressions of claudin-1, occludin and ZO-1 in the OTA group showed a highly significant decrease at the mRNA level ($p<0.01$). Compared with the OTA group, expressions of claudin-1 and occludin in the OTA+SY group showed a highly significant increase at the mRNA level ($p<0.01$), while there was no significant change in the mRNA expression of ZO-1.

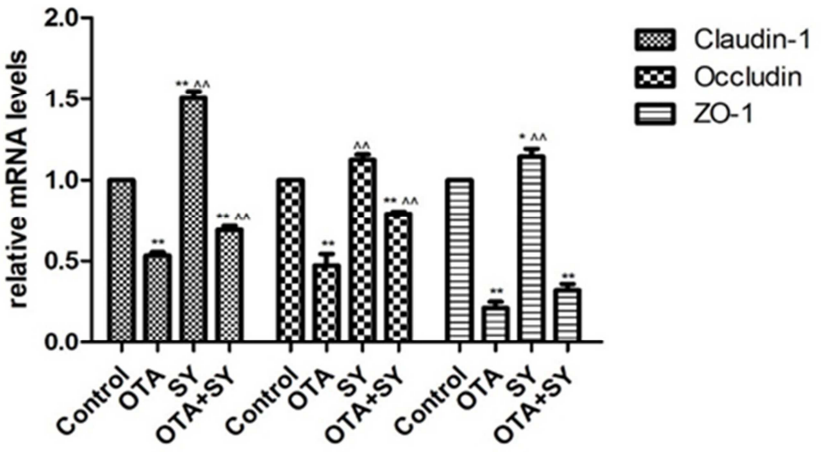


Fig. 4 TJ signaling gene expression

Note:* indicates a significant difference from the control group, $p<0.05$, ** indicates a extremely significant difference compared with the control group, $p<0.01$; ^^ indicates that it is associated with the OTA group. Compared with the extremely significant difference, $p<0.01$.

3.4.2 mRNA expressions of NF-κB pathway-related genes

The role of NF-κB signaling pathway in OTA-induced intestinal mucosal barrier injury were analyzed to detect the expression levels of NF-κB and NF-κB upstream genes, TLR4 and MYD88 (Fig. 5) at the mRNA level. Compared with the control group, expressions of TLR4, MYD88 and NF-κB in the OTA group showed a highly significant increase at the mRNA level ($p<0.01$). Compared with the OTA group,

expressions of MYD88 and NF-κB in the OTA+SY group showed a highly significant decrease ($p<0.01$), while the expression of TLR4 was significantly decreased at the mRNA level ($p<0.05$).

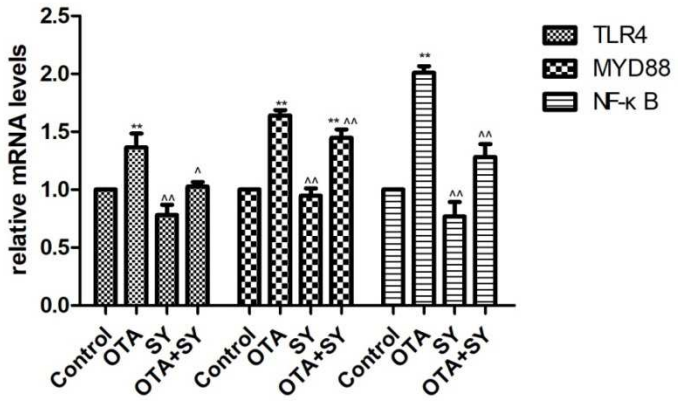


Fig. 5 NF-κB signaling gene expression

Note: ** indicates a extremely significant difference compared with the control group, $p<0.01$; ^ represents a significant difference compared with the OTA group, $p<0.05$, ^^ indicates that it is associated with the OTA group. Compared with the extremely significant difference, $p<0.01$.

3.5 Detection of protein expression

3.5.1 Expression levels of tight junction-related proteins

Effects of SY on OTA-induced injury to caecal tight junction structure was verified by detecting the expression level of claudin-1 protein in the caecal tissues (Fig. 6). There was a statistically significant decrease ($p<0.05$) in the expression level of claudin-1 protein in the OTA group compared with the control group, whereas the expression level of claudin-1 protein in the OTA+SY group was significantly increased ($p<0.05$) compared with the OTA group.

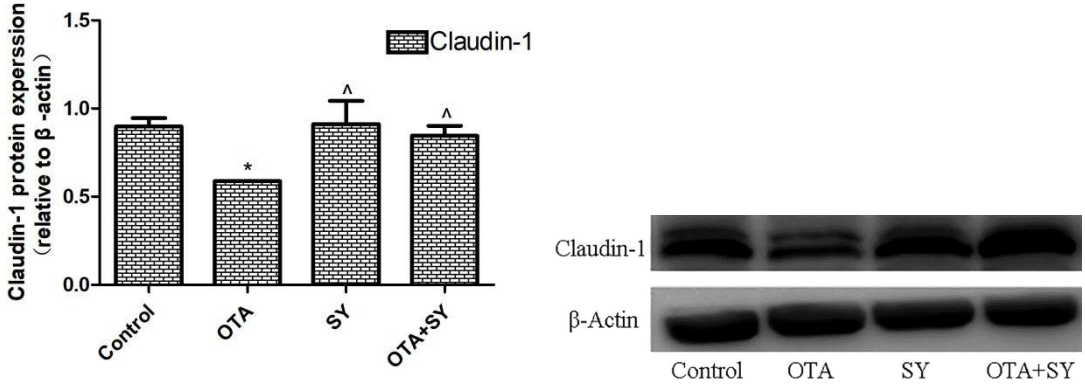


Fig.6 Claudin-1 preotein expression

Note:* indicates a significant difference from the control group, $p<0.05$; ^ represents a significant difference compared with the OTA group, $p<0.05$.

3.5.2 Expression levels of NF-κB-related proteins

To study the role of NF-κB signaling pathway in OTA-induced intestinal mucosal barrier injury, the expression level of NF-κB protein in caecal tissues was detected. Compared with the control group, expression levels of TLR4 and NF-κB protein in the OTA group showed a highly significant increase ($p<0.01$). Compared with the OTA group, the expression level of NF-κB protein in the OTA+SY group was significantly decreased ($p<0.05$), while there was no change in the expression level of TLR4 protein.

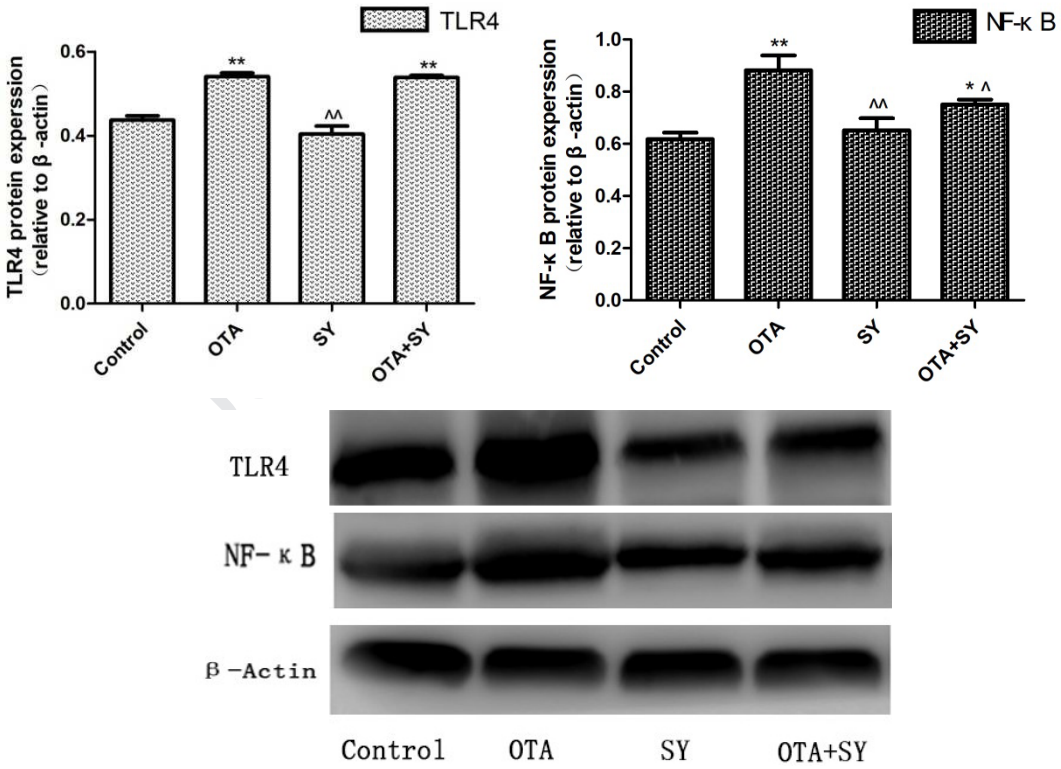


Fig. 7 TLR4 and 6 NF-κB preotein expression

Note:* indicates a significant difference from the control group, $p<0.05$, ** indicates a extremely significant difference compared with the control group, $p<0.01$; ^ represents a significant difference compared with the OTA group, $p<0.05$, ^^ indicates that it is associated with the OTA

group. Compared with the extremely significant difference, $p<0.01$.

3.6 Analysis of diversity of gut microbiota

A total of 24 intestine samples were collected during the sequencing experiment, and no exfoliation of samples was observed. Sequence analysis of intestine samples of broilers from different groups retrieved 1,469,730 pairs of reads. After the double-ended reads were spliced and filtered, a total of 1,233,457 clean tags were generated. Each sample produced at least 30,358 clean tags, with an average of 51,394. More than 80% of the sequences from the sample passed the quality test, demonstrating that the sequencing technique was effective.

3.6.1 Analysis of alpha diversity

Alpha diversity reflects the species richness and diversity of a single sample, which has many indices: Chao1, Ace, Shannon, Simpson. Chao1 and Ace index measure the species abundance, namely the number of species, and the greater the value, the more the number of species. As shown in Fig. 8A, compared with the

control group, the intestinal ACE index in the OTA group was significantly decreased ($p<0.05$). Compared with the OTA group, the ACE index in the OTA+SY group was significantly increased ($p<0.05$). In Fig. 8B, compared with the control group, the Chao index in the OTA group showed a highly significant decrease ($p<0.01$), indicating that OTA significantly reduced the number of microorganisms in the caecum, and SY ameliorated the effect of OTA and increased the number of intestinal microorganisms.

The Shannon and Simpson indices are used to measure species diversity and are influenced by species abundance and community evenness in the sample community. Under the same species abundance, the greater the evenness in the community, the larger the diversity in the community. The greater the Shannon index, the smaller the Simpson index, suggesting that the species diversity of the sample is higher. As shown in Fig. 8C, compared with the control group, the shannon index in the OTA group was significantly decreased (p<0.05). Compared with the OTA group, the shannon index in the OTA+SY group was significantly increased (p<0.05). In Fig. 8D , there was a same trend, but it had no significant difference.

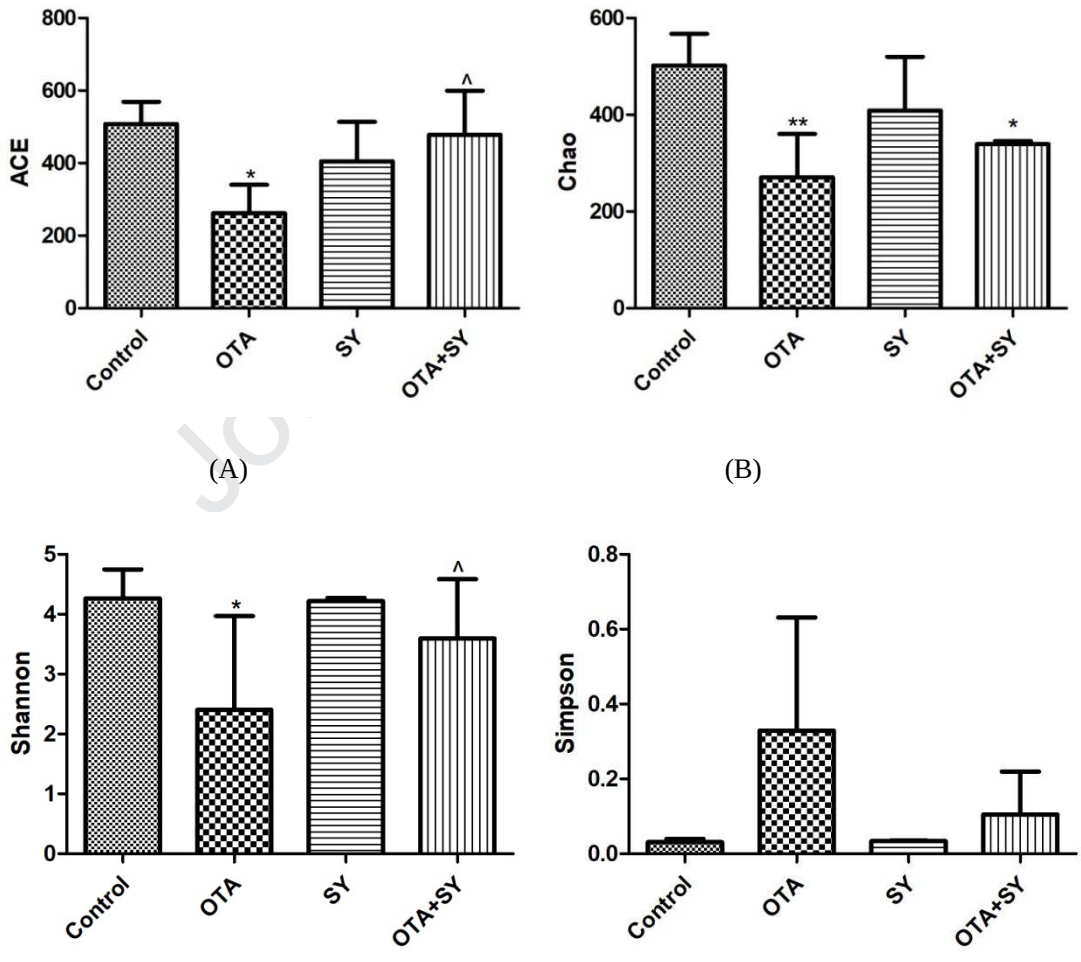


Fig. 8 α diversity index

Note:* indicates a significant difference from the control group, $p<0.05$, ** indicates a significant difference compared with the control group, $p<0.01$; ^ represents a significant difference compared with the OTA group, $p<0.05$.

3.6.2 Analysis of beta diversity

Principal component analysis (PCA) is a technique for analyzing and simplifying data sets. By decomposing the variance, the differences among multiple sets of data are reflected on a two-dimensional graph. By analyzing the OTU compositions of different samples, the difference and distance of the sample can be reflected. PCA uses variance decomposition to reflect the differences of multiple sets of data on a two-dimensional coordinate graph, and the coordinate axis takes two eigenvalues that can maximally reflect the variance. The closer the distance between the two samples, the more similar the compositions of the two samples. The PCA analysis chart was separately drawn using the R language tool, and the results of PCA analysis between the groups were shown in Fig. 9. There were intersecting regions between each group, which proved that certain similarity between the groups exist, and the differences between the groups were small. It was worth noting that, similar to the alpha diversity, the distance between the samples in the control group was far, indicating a greater difference. The effect of OTA shortened the distance between samples and minimized variation within groups, which was specifically reversed by SY, indicated by the increased distance between samples in the OTA+SY group. Fig. 9D demonstrated it more intuitively.

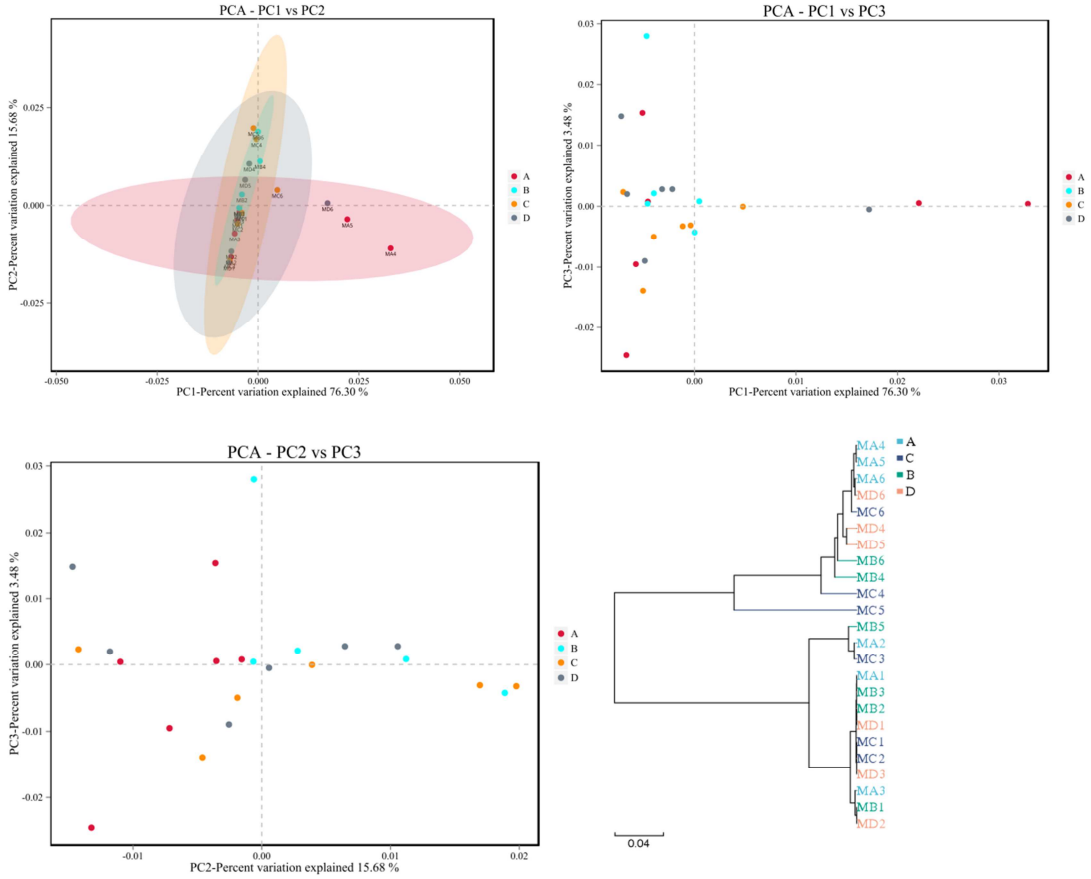


Fig. 9 Beta diversity analysis

Note:Each point represents a sample point. The ellipsoid around each point represents the uncertainty of each sample point based on the folding analysis. Points A, B, C and D in the figure represent the control group, the OTA group, the SY group and the OTA+SY group, respectively. The coordinates on the axis are for illustrative purposes only and are arbitrarily chosen, so there is no clear biological significance.

3.6.3 Taxonomic analyses of gut microbiota

Taxonomy-based analyses of the bacterial community were conducted at the phylum level. As shown in Fig. 10, Firmicutes was the most abundant of the caecal microbiota among the four groups, with a relative abundance of 87%. Bacteroidetes was the second abundant phylum (5.8%),

followed by Tenericutes (4.7%), and finally Proteobacteria, Cyanobacteria and Actinobacteria, with the relative abundance of 1.1%, 0.9% and 0.1%, respectively. Compared with other three groups, the relative abundance of the Firmicutes in the SY+OTA group was lower. The relative abundance of Tenericutes in the control group was lower compared to the other three groups.

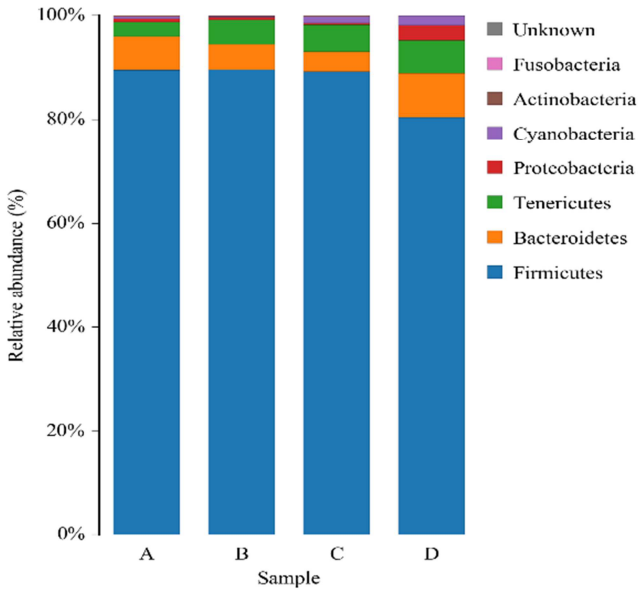


Fig.10 Phylum:Species distribution histogram

Note: The abscissa was the sample name; the ordinate was the percentage of relative abundance. Points A, B, C and D in the figure represent the control group, the OTA group, the SY group and the OTA+SY group, respectively.

Analysis was performed at the family level, as shown in Fig. 11. The most abundant families in the sample were Ruminococcaceae, Clostridiales_vadinBB60_group and Lachnospiraceae, with relative abundance exceeding 10%, followed by Rikenellaceae (5.9%), non-cultivable bacteria (o) Mollicutes RF9 (2.3%), Bacillaceae and Erysipelotrichaceae (1.1%). Intriguingly, the abundance of Clostridiales_vadinBB60_ group in the OTA group was decreased compared with the other three groups, but the abundance of Ruminococcaceae was significantly increased.

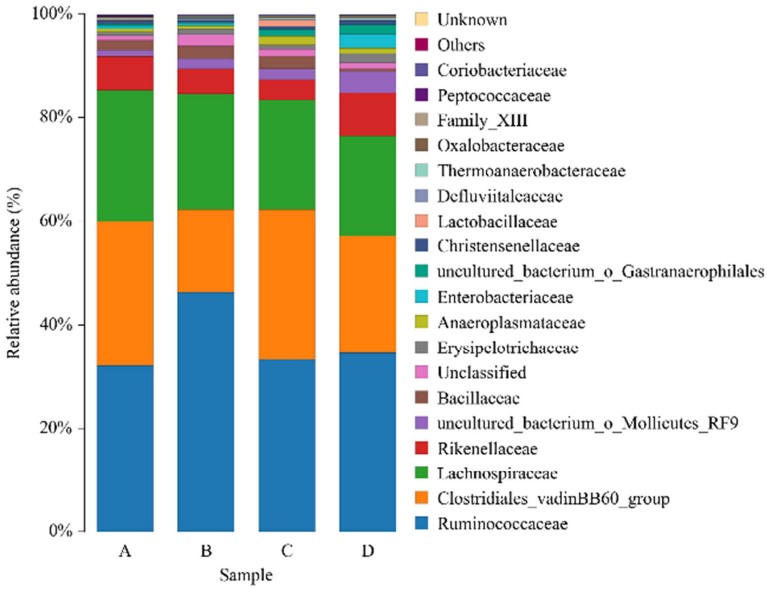


Fig.11 Family:Species distribution histogram

Note: The abscissa was the sample name; the ordinate was the percentage of relative abundance. Points A, B, C and D in the figure represent the control group, the OTA group, the SY group and the OTA+SY group, respectively.

Analysis was performed at the genus level, as shown in Fig. 12. The five most abundant genera in the sample were non-cultivable bacteria belonging to Clostridiales_vadinBB60_group, [Ruminococcus] torques group, Butyricicoccus, Ruminococcaceae and Alistipes, with relative abundance of 21.5%, 7.8%, 6.8%, 6.8% and 5.9%, respectively. Compared with other groups, the abundance of non-cultivable bacteria (f) Clostridiales_vadinBB60_group in OTA group was significantly decreased, and the abundance of Butyricicoccus was significantly increased.

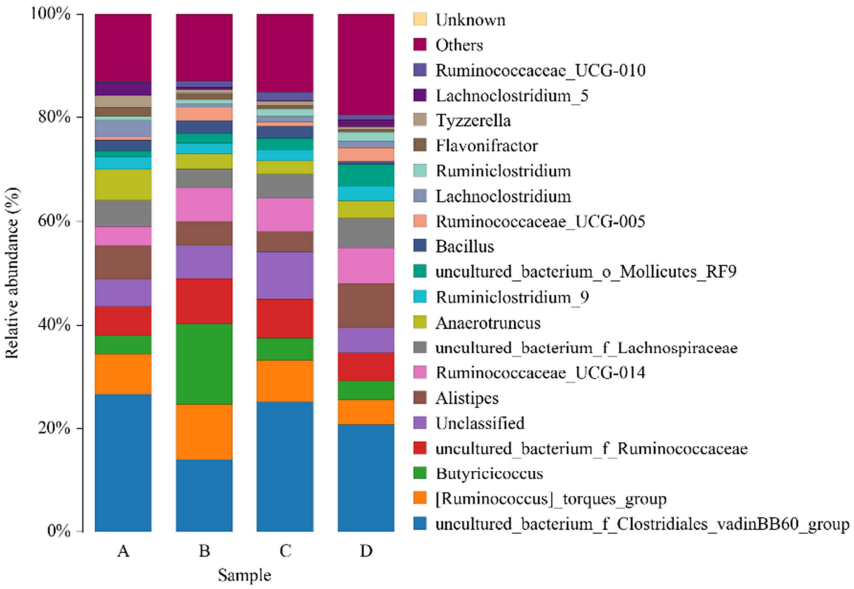


Fig.12 Genus:Species distribution histogram

Note: The abscissa was the sample name; the ordinate was the percentage of relative abundance

Points A, B, C and D in the figure represent the control group, the OTA group, the SY group and the OTA+SY group, respectively.

4. Discussion

Ochratoxin A is the most prevalent mycotoxin derived as a secondary metabolite from several species of Penicillium and Aspergillus. Due to their ubiquitous nature and existence as a gross contamination of crops and food products, OTA is posing serious health hazards to humans and animals(Koszegi&Poor, 2016). Recently, oxidative stress induced by OTA toxicity has gained considerable attention to researchers, and supplementation of livestock feed with SY as an organic form of Se in terms of its antioxidant capacity has reported beneficiary effects on animal health and performance. However, the results of the studies in this aspect did not show a uniform outcome, and data highlighting mechanism of action of SY on OTA-induced toxic injury in specific organs and tissues has not been extensively reported. This experiment used an *in vivo* exposure model to study the role of SY in OTA-induced intestinal barrier injury in white feather

broilers. The results showed that OTA caused intestinal barrier injury, which was associated with oxidative stress and activation of the NF-κB signaling pathway. Furthermore, we found that SY relieved OTA-induced oxidative stress and inhibited the activation of the inflammatory pathways, especially NF-κB pathway, which display crucial role in protecting the intestinal barrier.

Some previous studies have indicated that OTA exposure may lead to intestinal injury through inflammatory response and oxidative stress. On exploring the pathophysiology of OTA-induced acute kidney injury of rats, it was found that the reactive oxygen species ROS was increased by approximately 20%(Zhu et al., 2016), and the excess ROS level could cause serious injuries to biomolecules (nucleic acids,proteins and lipids), which could easily lead to lipid peroxidation(Schieber&Chandel, 2014). MDA was considered to be the most representative end product in the lipid peroxidation(Ho et al., 2013), hence in this study, MDA was used as the standard for measuring oxidative injury and intestinal cell injury. SOD and GSH, as ubiquitous antioxidant enzymes in animals play a significant role in the body to scavenge oxygen free radicals(Marin et al., 2018). SOD can reduce ROS to H_2O_2, which is subsequently reduced to H_2O and other substances by enzymes, such as GSH. Quantifying the activity of SOD and GSH serve as important indicators for assessing the antioxidant capacity of organisms(McCord&Fridovich, 2014). It has been reported that SY can decrease antioxidant and anti-inflammatory abilities of AFB1-induced mice(L. Liu et al., 2019). In the present study, the MDA content in the OTA group showed a significant increase, and activities of GSH and SOD were decreased significantly, suggesting that OTA induced oxidative injury to the broiler intestine, which was consistent with the results of Rasicd C *et al.(Rasic et al., 2019)*. The addition of SY reversed this phenomenon, which significantly reduced thecontent of MDA in broilers exposed to OTA injury. Moreover,

consistent with previous studies the activities of GSH and SOD also increased significantly in our study, indicating that SY could effectively protect the caecal tissues of broilers and could relieve OTA-induced oxidative injury.

The nuclear transcription factor NF-κB plays a key role in the inflammatory response and immune expression(Napetschnig&Wu, 2013). Studies have suggested that oxidative stress stimulates the TLR4/MYD88 signaling pathway to activate the nuclear transcription factor NF-κB, and the signal transduction generated can lead to inflammation(Xu et al., 2017). In addition, studies by Bi CL *et al.* have shown that Se can inhibit *Staphylococcus aureus*-induced inflammation by inhibiting the NF-κB signaling pathway(Bi et al., 2016). In the present study, expressions of NF-κB-related target genes were detected by RT-qPCR. The experimental results suggested that OTA promoted the activation of the NF-κB signaling pathway and inflammatory responses, whereas the activation of the NF-κB signaling pathway was inhibited after the addition of SY, which was consistent with the previous study results in which quercetin regulated OTA-induced oxidative stress in HepG2 cells and down-regulated the NF-κB(Ramyaa et al., 2014). These results clearly demonstrated that SY had a significant protective effect on OTA-induced intestinal inflammation.

It is well-known that the intestinal epithelial tight junction is an important barrier to maintain the intestinal epithelial permeability, which is greatly assisted with tight junction proteins that functions to prevent harmful substances from entering the body and maintain the tight junction structural integrity(Buckley&Turner, 2018). It has been reported that OTA can aggravate DON-induced injury to the intestinal tight junction barrier by activating NF-κB signaling pathway, suggesting that tight junction proteins are closely related to the activation of NF-κB signaling

pathway(Ying et al., 2019). Another study indicated that methotrexate can reduce the expressions of Claudin-1, Occludin and ZO-1 at the protein level by activating NF-κB signaling pathway, and eventually cause injury to the tight junction of the intestine(Beutheu Youmba et al., 2012). González-Quilen C *et al.* observed that grape seed proanthocyanidin could protect the tight junction structure by relieving oxidative stress and inflammatory responses(Gonzalez-Quilen et al., 2019). Liu F *et al.* demonstrated the heat stress effect of Se to porcine oxidative stress and improvement of intestinal epithelial barrier function(F. Liu&Cottrell, 2016). In this experiment, we have showed that compared with the OTA group, mRNA expression levels of Claudin-1, Occludin and ZO-1 along with Claudin-1 protein expression level was significantly increased in caeca of the broilers in the OTA+SY group. This finding was consistent with the above results that discussed oxidative stress, NF-κB and intestinal tight junction proteins.

As an important component of the intestinal barrier, the intestinal microbiota play an important role in maintaining the intestinal homeostasis and ensuring the healthy status of the intestinal tract. The diversity of the gut microbiota indicates the complexity of the species within the gut microbiota. However, mycotoxins, such as aflatoxin B1, ochratoxin A, zearalenone and fumonisin B1 can intervene the stability of the gut microbiota of animals(Robert et al., 2017). Aflatoxin B1 administered by oral gavage can reduce the alpha diversity of gut microbiota in broilers and increase the abundance of several harmful bacteria, such as Clostridium(Yang et al., 2017). OTA can increase the abundance of *Lactobacilliaceae* in the intestine of rats and reduce the alpha diversity of gut microbiota(Guo et al., 2014). In this experiment, the alpha diversity in the OTA group was significantly decreased compared with the control group, which was consistent with the results in which the alpha diversity of gut microbiota was decreased after long-term

exposure to OTA injury. The alpha diversity of gut microbiota in the OTA+SY group was significantly restored, which was consistent with the previously discussed results mentioning that Se could increase the diversity of gut microbiota. In terms of beta diversity, PCA confirmed that SY intervened the toxicity of OTA, and the OTA group was more concentrated compared with other groups, while the OTA+SY group was more dispersed, which was closer to the control group, indicating that SY could protect the diversity of gut microbiota and ensured the normal evolution of gut microbiota. Clostridiales, as an important butyric acid-producing bacterium, plays an essential role in maintaining intestinal homeostasis(Zhang et al., 2017). Studies have suggested that the addition of Se to feeds increases the abundance of beneficial Clostridiales(Kasaikina et al., 2011). Moreover, the OTA treatment reduced the abundance of Clostridiales, which was subsequently recuperated after supplemented with SY. However, the treatment with OTA increased the abundance of Ruminococcaceae, and the addition of SY could reduce its abundance. It is well-known that Ruminococcaceae has gained extensive attention as a beneficial intestinal bacterium, and studies have shown that Ruminococcaceae colonization increased in mice receiving oral nicotine(R. Wang et al., 2019). Therefore, the change in abundance of Ruminococcaceae indicates a disorder of gut microbiota. There are several pathogens in Butyricicoccus that can cause a variety of diseases when the disorder of gut microbiota occurs(Jalanka et al., 2018). In this experiment, SY inhibited the increased colonization of Butyricicoccus in the caecal gut microbiota induced by OTA, suggesting that SY could intervene changes in the diversity of gut microbiota and the structure of gut microbiota caused by OTA.

5. Conclusions

In conclusion, SY could successfully inhibit the oxidative injury of caecal tissues in broilers

caused by OTA exposure. By regulating TLR4/MYD88, SY inhibited the NF-κB signaling pathway and antagonized OTA-induced intestinal TJ injury. In addition, SY could also intervene the changes of gut microbiota caused by OTA by increasing the diversity of gut microbiota and reducing the abundance of harmful bacteria.

Author Contributions: S.Y wrote the paper; S.Y, M.L and P.Lconceived and designed the experiments; S.L. performed the experiments; K.L and C.Z. analyzed the data; L.L. analyzed the data and contributed materials; Miao Long and P.L. supervised;

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: