Dietary chitooligosaccharide supplementation alleviates intestinal barrier damage, and oxidative and immunological stress in lipopolysaccharide-challenged laying hens

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ABSTRACT This study aimed to investigate the effects of chitooligosaccharide (COS) on intestinal barrier, antioxidant capacity, and immunity of lipopolysaccharide (LPS)-challenged laying hens. A total of 360 Hy-line Brown laying hens (80-wk-old) were randomly divided into 5 groups with 6 replicates of 12 birds. Hens were fed a corn-soybean meal basal diet supplemented with different COS levels (0; 5; 10; 15; 20 mg/kg) for 8 wk. The results showed that 15 mg/kg COS administration elevated albumen height and Haugh unit (P < 0.05), and numerically optimized productive performance (P > 0.05), therefore, the dosage of 15 mg/kg was chosen for the subsequent experiment. Thereafter, 12 birds from non-supplemented group were randomly selected and assigned into 2 groups, and birds in each group were administered (1.5 mg/kg BW, i.p.) with saline (control group) or LPS (challenge group). Another 6 hens from 15 mg/kg COS-supplemented group were selected and injected with LPS in the same way. Compared with the control group, LPS-challenged birds exhibited elevated circulating diamine oxidase activity, and reduced jejunal villus height and ratio of villus height to crypt depth, and these indices were reversed to control levels by COS (P < 0.05). Also, LPS increased malondialdehyde accumulation and reduced several antioxidant enzyme activities in the intestinal mucosa (P < 0.05). Additionally, LPS increased jejunal secretory IgA and interferon-γ (IFN-γ), and ileal secretory IgA, IgM, and interleukin-1β (IL-1β) concentrations, whereas COS reduced jejunal IFN-γ and IL-1β, and ileal IgM levels (P < 0.05). Moreover, LPS down-regulated mRNA abundance of jejunal occludin and claudin 2, and upregulated expression of jejunal nuclear factor erythroid-2 related factor 2, superoxide dismutase 1, and IFN-γ as well as ileal IL-1β (P < 0.05). Besides, COS increased jejunal occludin and ileal claudin 2, nuclear factor erythroid-2 related factor 2, and heme oxygenase-1 expression, and decreased jejunal IFN-γ and IL-1β abundance (P < 0.05). These results suggested that COS could alleviate LPS-induced intestinal barrier impairment, and oxidative and immunological stress in laying hens.

Key words: chitooligosaccharide, intestinal health, laying hen, lipopolysaccharide, stress

INTRODUCTION

The chitooligosaccharide (COS) is a polysaccharide of D-glucosamine and N-acetyl-D-glucosamine degraded from chitin or chitosan involving physical, chemical, and enzymatic processes of incomplete deacetylation and depolymerization (Yin et al., 2009; Aam et al., 2010). Compared to the chitosan, COS has higher degree of deacetylation, lower levels of molecular weight and polymerization, and 3 functional reactive groups (i.e., amino/acetamido group, the hydroxyl groups, and glycosidic bond), contributing to the enhanced biological properties (Guan et al., 2019; Naveed et al., 2019). Accumulating data have reported that COS can exert immune function by mediating expression of cytokine genes to increase macrophage phagocytosis, lymphocyte proliferation, and natural killer cell activation under normal conditions (Bahar et al., 2012; Xing et al., 2017). Moreover, in cells challenged with inflammatory stress, COS has been found to be a promising regulator to alleviate the allergic reaction by inhibiting degranulation and cytokine generation as well as excessively stimulated state of neutrophils (Dou et al., 2007; Vo et al., 2011). Regarding antioxidant effects, COS has been identified to inhibit myeloperoxidase activity, decrease DNA and protein oxidative levels, and simultaneously increase radical scavenging ability and prevent apoptosis to
restore the viability of in vitro cells (Ngo et al., 2008; Xu et al., 2010). Further, several papers have revealed that COS can suppress the phosphorylation of mitogen-activated protein kinase and activate the nuclear factor erythroid-2 related factor 2 (NRF2) signaling and related genes to stabilize the redox state (Luo et al., 2019). For livestock, COS is considered to be a promising alternative to antibiotics for swine and poultry. In the swine production, especially during the weaning stage when piglets are sensible to stress from environment and diets, COS supplementation is able to promote growth performance and decrease the incidence of diarrhea by improving nutrient digestibility, intestinal morphology, fecal microbiota composition, and immune function (Chen et al., 2009; Wang et al., 2009; Thongsong et al., 2018). Likewise, dietary COS supplementation can improve growth performance, meat quality, and physiological conditions of broilers, as evidenced by the increased nutrient digestibility, and enhanced immunity and antioxidant capacity (Huang et al., 2005; Zhou et al., 2009; Li et al., 2019). With regard to laying hens, COS addition can improve hematomal parameters, egg quality, and productive performance in accordance with the previous studies (Meng et al., 2010; Yan et al., 2010).

Lipopolysaccharide (LPS), a large glycolipid containing numerous sugars, is the primary component found in the outer leaflet of most gram-negative bacteria, and it has been widely applied to establish experimental models of bacterial infection on account of its efficiency in stimulating inflammatory responses in diverse species (Pi et al., 2014; Zhang et al., 2014; Geng et al., 2018). Also, LPS treatment can cause distorted histomorphological changes, accompanied by the downregulated expression of tight junction proteins that are closely associated with intestinal barrier function (Yi et al., 2014). It has been demonstrated that LPS administration can induce the excessive production of free radicals (Bhattacharyya et al., 2004), resulting in the oxidative damage and apoptosis of intestinal epithelial cells (Ozdemir et al., 2007). For laying hens reared under intensive husbandry environment where gram-negative bacteria are ubiquitous, they will inevitably inhale large amounts of endotoxins, such as LPS, which would lead to the prevalence of some diseases and impact the animal welfare issues (Zucker et al., 2000; Roque et al., 2015). According to the substantial papers, LPS challenge can induce immunological and oxidative stress, and severely impair the intestinal morphology and permeability, and it would interfere with metabolic process and organ functions, contributing to the inferior productive performance of laying hens (Jing et al., 2014; Geng et al., 2018; Liu et al., 2020). In commercial practice, dietary intervention can be a promising and potential method to resist against the stress responses induced by LPS or other stimuli for improving the performance of poultry and other livestock (Pi et al., 2014; Zhang et al., 2020).

Up to now, scarce published papers have concentrated on the ameliorative effects of COS on intestinal health of LPS-challenged laying hens. Considering the multiple benefits of COS and the living condition of laying hens, the current study was, therefore, conducted to evaluate whether COS administration could alleviate the intestinal barrier damage, and oxidative and immunological stress induced by LPS, for further application of COS in the poultry.

MATERIALS AND METHODS

Animals, Diets, and Treatment

This experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University, and the management of birds complied with an ethics committee-approved protocol established by the Jiangsu Provincial Department of Science and Technology (SYXK (SU) 2017-0007).

Two experiments were conducted in this study. Experiment one was designed to investigate the effects of different levels of COS on productive performance and egg quality of laying hens, in order to select the most suitable dosage of COS prior to the experiment two. A total of 360 Hy-line Brown laying hens (80-wk-old) were randomly assigned into 5 groups with 6 replicates of 12 birds. After a 2-wk preliminary experiment, hens were fed a corn-soybean meal basal diet from a same batch mixed with graded levels of COS (0; 5; 10; 15; 20 mg/kg) for an 8-wk trial. The COS was provided by Zhongkengongxin Biotechnology Co., Ltd. (Suzhou City, Jiangsu Province, P.R. China), with the purity of approximately 90% and average molecular weight of 1,000 to 2,000 Daltons. The composition and nutrient levels of the basal diet are presented in Table 1. The hens were reared in 3-level ladder cages (3 birds per cage, 40 × 40 × 35 cm) equipped with plastic floors and water nipples. During the experimental period, birds were supplied with mash feed and water ad libitum under a lighting program of 16 h illumination and 8 h darkness. Throughout the entire trial, the average temperature and humidity of chicken house were maintained at 18 to 25°C and 40 to 60%, respectively. Egg weight, egg production, and mortality of hens were recorded for calculating the indices of productive performance. Eggs were collected for the measurement of egg quality at the end of the 4th and 8th wk.

According to the results of experiment one, birds fed diets with 15 mg/kg COS (exhibiting the highest egg production rate, the lowest feed conversion ratio, and elevated albumen height and Haugh unit) were selected for experiment two, which aimed to evaluate the effects of COS supplementation on intestinal barrier, oxidative status, and immune function in laying hens challenged with LPS. At the end of experiment one, 6 birds from nonsupplemented group and 6 birds from 15 mg/kg COS-supplemented group were randomly selected and injected (i.p.) with 1.5 mg/kg BW of Escherichia coli LPS (serotype O111:B4, Sigma-Aldrich Inc., St. Louis, MO). Another 6 hens from non-supplemented treatment were injected (i.p.) with 1.5 mg/kg BW of 0.9% (wt/vol) sterile saline as the control group of experiment two. Feed was removed before sample collection.
Table 1. Composition and nutrient level of the basal diet (g/kg, as fed basis unless otherwise stated).

| Items      | Contents |
|------------|----------|
| Corn       | 640      |
| Soybean meal | 240     |
| Limestone  | 90       |
| Premix1    | 30       |
| Calculated nutrient levels | |
| Apparent metabolizable energy (MJ/kg) | 11.08 |
| Crude protein | 162      |
| Ether extract | 28       |
| Calcium    | 38       |
| Total phosphorus | 6.2     |
| Available phosphorus | 3.7   |
| Lysine     | 8.0      |
| Methionine | 3.6      |
| Total sulfur amino acids | 6.4   |

Analyzed nutrient levels

| Crude protein | 161 |
| Ether extract | 27  |
| Calcium       | 35  |
| Total phosphorus | 5.9 |

1Premix provided per kilogram of diet: transretinyl acetate, 10,000 IU; cholecalciferol, 3,000 IU; all-rac-α-tocopherol, 30 IU; menadione, 1 mg; thiamin, 1 mg; riboflavin, 6 mg; nicotinamide, 40 mg; choline chloride, 350 mg; calcium pantothenate, 10 mg; pyridoxine HCl, 3 mg; biotin, 0.11 mg; folic acid, 0.3 mg; cobalamin, 0.01 mg; Cu (copper sulfate), 8 mg; Fe (ferrous sulfate), 80 mg; Zn (zinc sulfate), 50 mg; Mn (manganese sulfate), 100 mg; I (calcium iodate), 1 mg; Se (sodium selenite), 0.3 mg; calcium, 6.25 g; phosphorus, 3 g; methionine, 1 g; sodium chloride, 3 g.

Sampling

After 4 h of injection, the blood sample of each bird was collected via wing venipuncture into non-heparinized tubes and clotted at room temperature (25°C) for about 2 h. The serum was then separated through centrifugation at 4,000 g for 15 min at 4°C, and frozen at −20°C for subsequent analysis. After the birds were euthanized by cervical dislocation and necropsied, approximate 2 cm mid-sections of the jejunum and ileum were taken and flushed with chilled phosphate-buffered saline solution, placed in the 10% formaldehyde reagent for tissue fixation. The remaining jejunal and ileal segments were opened longitudinally and chyme was rinsed off with phosphate-buffered saline solution. The intestinal mucosa was thereafter scratched with a sterile glass microscope slide, and collected into cryogenic tubes at −80°C for further determination.

Productive Performance and Egg Quality

In experiment one, egg weight, egg production, and mortality were recorded daily, and feed consumption was recorded weekly based on the replicate to calculate the average egg weight, average egg production, average egg mass, average daily feed intake, and feed conversion ratio.

At the end of the 4th and 8th wk of experiment one, 3 eggs from each replicate were randomly selected for egg quality determination. The eggshell breaking strength on the vertical axis was measured by eggshell strength gauge (Model-II, Robotmation, Japan). The albumin height, Haugh unit, and yolk color were tested on the egg multimeter (EMT-5200, Robotmation). The eggshell thickness was a mean value of measurements taken at 3 areas (equator, blunt, and sharp ends) of the egg using a dial pipe gauge.

Histological Examination

The fixed tissues were dehydrated, hyalinized, and embedded in paraffin, and cut into 5 μm slices. The intact slices were then selected, deparaffinized, rehydrated, and stained with hematoxylin-eosin for identification. Fifteen well-oriented villi and their corresponding crypts were selected to measure the villus height (distance from crypt opening to the end of villi) and crypt depth (distance from crypt villous junction to the base of crypt) under a Nikon ECLIPSE 80i light microscope equipped with an ocular micrometer (Nikon Corporation, Tokyo, Japan) at 40 × magnification.

Serum Diamine Oxidase Activity

The determination of serum diamine oxidase activity was carried out followed by the commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing City, Jiangsu Province, P.R. China), and the measurement procedures were totally in compliance with the protocol manual provided by the manufacturer.

Redox Status of Intestinal Mucosa

Intestinal mucosa samples were homogenized (1:4 or 1:9, wt/vol) with ice-cold sterile sodium chloride solution (154 mmol/L) using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH) for the supernatant, which was obtained by a centrifugation at 4,000 g for 15 min at 4°C. The levels of malondialdehyde (MDA), reduced form of glutathione, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) in the supernatant were measured with colorimetric kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's guidelines.

Intestinal Mucosal Immunoglobulin and Proinflammatory Cytokine Levels

The levels of secretory IgA (sIgA), IgM, IgG, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β) in the supernatant obtained from intestinal mucosal homogenates were quantified with chicken-specific sIgA, IgM, IgG, TNF-α, IFN-γ, and IL-1β ELISA kits (Nanjing Jiancheng Bioengineering Institute), respectively. The measurement procedures were strictly in accordance with the protocols of manufacturer.

Total RNA Isolation and PCR Analysis

The total RNA was extracted with TRIzol reagent (TaKaRa Biotechnology, Dalian City, Liaoning Province, P.R. China) from the intestinal mucosa sample. After the determination of RNA concentration, the
RNA of 1 μg was used for the synthesis of cDNA using PrimeScript RT reagent kit (TaKaRa Biotechnology). PCR was carried out to evaluate the mRNA abundance of following genes: zonula occludens 1, occludin (OCLN), claudin 1, claudin 2 (CLDN2), NRF2, heme oxygenase-1 (HO-1), superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPX1), heme oxygenase-1 (HO-1), interleukin-10 (IL-10), interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-2 (IL-2), interferon-γ (IFN-γ), and β-actin. The primer sequences were shown in Table 2. The cDNA samples were amplified using TB Green Premix Ex Taq kit (TaKaRa Biotechnology) based on ABI 7300 Real-Time PCR System (Applied Biosystems, Grand Island, NY). The quantitative real-time PCR reaction program was as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s (denaturation stage), 60°C for 30 s (annealing and extension stage), and 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s (melting stage). The gene expression level relative to reference gene was calculated using the 2−ΔΔCT method (Livak and Schmittgen, 2001).

Table 2. Sequences for real-time PCR primers.

| Items | Gene bank ID | Primer sequence, sense/antisense |
|-------|-------------|---------------------------------|
| ZO1   | XM_015278975.2 | TGTGACCCACAGCAAAAGGTG |
| OCLN  | NM_250128.1 | CCGTAAACCGCGACTTGAGT |
| CLDN1 | NM_001013611.2 | ATTGAGGCCGCTGTTGAGT |
| CLDN2 | NM_001277622.1 | GCAGTCACTCAATCTG |
| NRF2  | NM_250017.1 | CCGTTTTCGAGGGTGAGCA |
| HO-1  | NM_250344.1 | GTCGTTTGCAAGAAGACTCC |
| SOD1  | NM_250064.1 | GAGCGGCGCCAGGCIATTTC |
| GPX1  | NM_001277853.2 | AATGATCTATCGGCTGCGG |
| IFN-γ | NM_250149.1 | CTCGATGTCGTCCTGCAGTT |
| IL-1β | NM_250521.1 | TCCCGTTCTCATCCATCTTCTC |
| IL-4  | NM_00100709.1 | GACATCGGTGCCCTTAG |
| IL-10 | NM_00100414.2 | CAGACGACAGCAATCGTAC |
| β-actin | NM_250181.8 | TCCGGTGTATTCCTCATCTC |

1Abbreviations: CLDN1, claudin 1; CLDN2, claudin 2; HO-1, heme oxygenase-1; GPX1, glutathione peroxidase 1; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-10, interleukin-10; NRF2, nuclear factor erythroid-2 related factor 2; OCLN, occludin; SOD1, superoxide dismutase 1; ZO1, zonula occludens 1.

RESULTS

Production Performance and Egg Quality (Experiment 1)

Prior to LPS challenge, different levels of COS did not affect egg weight, egg production, egg mass, daily feed intake, and feed conversion ratio in comparison with the control group (Table 3, P > 0.05). However, laying hens fed basal diets with 15 mg/kg COS exhibited the highest numerical value of egg production rate and the lowest level of feed conversion ratio among treatments (P < 0.05).

At the end of the 4th wk of experiment one, COS supplementation linearly increased albumen height, Haugh unit, and yolk color (P < 0.05). Compared with the non-supplemented group, 15 mg/kg COS administration elevated albumen height and Haugh unit, and 20 mg/kg COS supplementation increased yolk color and Haugh shell strength.

Table 3. Effects of dietary chitooligosaccharide supplementation on productive performance and egg quality of laying hens (experiment 1).

| Items | Chitooligosaccharide level (mg/kg) | 0 | 5 | 10 | 15 | 20 | SEM | ANOVA | Linear | Quadratic |
|-------|----------------------------------|---|---|----|----|----|-----|-------|--------|----------|
| Egg weight (g) | 66.27 | 64.85 | 65.65 | 66.10 | 65.90 | 0.341 | 0.743 | 0.839 | 0.488 |
| Egg production (%) | 65.13 | 64.78 | 65.37 | 66.28 | 65.79 | 0.995 | 0.993 | 0.710 | 0.997 |
| Egg mass (g/hen/d) | 43.14 | 42.01 | 42.86 | 43.85 | 43.33 | 0.673 | 0.947 | 0.666 | 0.822 |
| Daily feed intake (g/hen/d) | 114.83 | 110.36 | 112.83 | 111.08 | 110.85 | 0.599 | 0.007 | 0.077 | 0.366 |
| Feed conversion ratio | 2.66 | 2.64 | 2.64 | 2.56 | 2.58 | 0.031 | 0.823 | 0.285 | 0.966 |
| 4w egg quality | | | | | | | | | |
| Albumen height (mm) | 6.77b | 7.06ab | 7.01ab | 7.99a | 7.59ab | 0.130 | 0.009 | 0.002 | 0.690 |
| Yolk color | 7.18b | 7.24ab | 7.28ab | 7.56ab | 7.86a | 0.078 | 0.021 | 0.002 | 0.207 |
| Haugh unit | 79.86c | 80.41bc | 80.53abc | 85.85a | 85.67ab | 0.736 | 0.003 | <0.001 | 0.451 |
| Eggshell strength (kg/cm²) | 3.01 | 3.43 | 3.01 | 3.32 | 3.09 | 0.096 | 0.538 | 0.925 | 0.482 |
| Eggshell thickness (μm) | 329.60 | 329.04 | 310.87 | 340.35 | 341.39 | 3.792 | 0.064 | 0.164 | 0.090 |

1SEM, standard errors of mean.
2Mean values within a row with different superscripts letters are significantly different at P < 0.05.
were observed among groups (P < 0.05). However, no significant difference of egg quality was observed at the end of the 8th wk among groups (P > 0.05).

Intestinal Barrier Function (Experiment 2)

As indicated in Table 4, compared with the control treatment, LPS injection increased the circulating diamine oxidase activity, which was normalized to the control value when supplementing COS in the basal diet (P < 0.05). The LPS-challenged hens exhibited the lower values of jejunal villus height and ratio of villus height to crypt depth than their control counterparts, and these 2 indices were both reversed to the control levels in COS-supplemented group (P < 0.05). However, jejunal crypt depth and ileal morphology did not differ among 3 groups (P > 0.05).

Compared with the control group (Table 7), LPS challenge down-regulated the mRNA abundance of jejunal mucosal OCLN and CLDN2 (P < 0.05), of which OCLN was totally reversed to the control level (P < 0.05) with COS administration, whereas CLDN2 was reduced to a certain extent, with the value being intermediate among 3 groups (P > 0.05). Besides, the supplemental COS increased ileal mucosal CLDN2 abundance to the control level in comparison with the LPS treatment (P < 0.05). No differences of zonula occludens 1 and claudin 1 mRNA levels in the intestinal mucosa were observed among groups (P > 0.05).

Intestinal Mucosal Redox State (Experiment 2)

Compared with birds receiving the basal diet (Table 5), laying hens in the LPS group exhibited the higher accumulation of MDA, and lower activities of SOD and GSH-Px in the jejunal mucosa, as well as lower activity of GSH-Px in the ileal mucosa (P < 0.05). In comparison with the LPS treatment, COS administration rendered the SOD and GSH-Px activities increased in the jejunal mucosa to some extent, though they did not reach the significant levels (P > 0.05). LPS challenge tended to increase the MDA content and reduce the SOD activity in the ileal mucosa, and both of them were normalized to the control levels with COS supplementation (P = 0.078, P = 0.055). Nevertheless, treatments did not alter the concentration of reduced form of glutathione in both jejunal and ileal mucosa (P > 0.05).

In comparison with hens from control group, birds subject to LPS challenge exhibited the higher mRNA abundance of NRF2 and SOD1 in the jejunal mucosa (Table 7, P < 0.05). The laying hens fed diets supplemented with COS showed the up-regulated mRNA levels of NRF2 and HO-1 in the ileal mucosa when compared with their LPS-challenged counterparts (P < 0.05). However, LPS and COS treatments did not affect mRNA abundance of glutathione peroxidase 1 in the intestine mucosa (P > 0.05).

Intestinal Mucosal Immunity (Experiment 2)

The increased concentrations of slgA and IFN-γ in the jejunal mucosa, and slgA, IgM, and IL-1β in the ileal mucosa were observed in the LPS-challenged treatment (Table 6, P < 0.05). In contrast, COS supplementation reduced the IFN-γ and IL-1β contents in the jejunal mucosa, and IgM level in the ileal mucosa of laying hens challenged with LPS (P < 0.05). Additionally, in comparison with the LPS group, dietary COS administration reduced intestinal mucosal slgA level to a certain

Table 4. Effects of dietary chitooligosaccharide supplementation on serum diamine oxidase level and intestinal morphology of lipopolysaccharide-challenged laying hens (experiment 2).

| Items         | CON   | LPS   | COS + LPS | SEM^1 | P-value |
|---------------|-------|-------|-----------|-------|---------|
| Serum Diamine oxidase (U/L) | 11.17^b | 15.13^a | 11.81^b | 0.639 | 0.014   |
| Jejunum Villus height (μm) | 1184.36 | 1016.58 | 1212.04^a | 31.446 | 0.012   |
| Crypt depth (μm) | 198.75 | 208.09 | 199.84 | 4.278 | 0.650   |
| Villus height: crypt depth | 6.08^a | 4.95^b | 6.24^a | 0.193 | 0.004   |
| Ileum Villus height (μm) | 777.19 | 762.10 | 831.55 | 14.624 | 0.122   |
| Crypt depth (μm) | 172.09 | 179.87 | 172.69 | 3.631 | 0.650   |
| Villus height: crypt depth | 4.56 | 4.32 | 4.95 | 0.119 | 0.079   |

Abbreviations: CON, nonchallenged laying hens fed a basal diet; LPS, LPS-challenged laying hens fed a basal diet; COS+LPS, LPS-challenged laying hens fed a basal diet supplemented with 15 mg/kg chitooligosaccharide.
^1SEM, standard errors of mean.
^ab Mean values within a row with different superscripts letters are significantly different at P < 0.05.

Table 5. Effects of dietary chitooligosaccharide supplementation on intestinal mucosal antioxidant capacity of lipopolysaccharide-challenged laying hens (experiment 2).

| Items^1 | CON   | LPS   | COS + LPS | SEM^2 | P-value |
|---------|-------|-------|-----------|-------|---------|
| Jejunum MDA (nmol/mg protein) | 0.33^b | 0.45^a | 0.41^b | 0.016 | 0.001  |
| GSH (mg/g protein) | 11.67 | 9.35 | 9.80 | 0.622 | 0.287   |
| SOD (U/mg protein) | 112.33^b | 103.25^a | 111.50^b | 1.647 | 0.034  |
| GSH-Px (U/mg protein) | 9.28^b | 6.94^a | 8.16^b | 0.383 | 0.034   |
| Ileal MDA (nmol/mg protein) | 0.27 | 0.34 | 0.26 | 0.016 | 0.078   |
| GSH (mg/g protein) | 9.89 | 9.97 | 9.08 | 0.460 | 0.712   |
| SOD (U/mg protein) | 107.03 | 102.59 | 108.70 | 1.103 | 0.055   |
| GSH-Px (U/mg protein) | 9.41^a | 7.62^b | 7.78^b | 0.258 | 0.002   |

Abbreviations: CON, nonchallenged laying hens fed a basal diet; LPS, LPS-challenged laying hens fed a basal diet; COS+LPS, LPS-challenged laying hens fed a basal diet supplemented with 15 mg/kg chitooligosaccharide.
^1Abbreviations: GSH, reduced form of glutathione; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
^2SEM, standard errors of mean.
^ab Mean values within a row with different superscripts letters are significantly different at P < 0.05.
Table 6. Effects of dietary chitooligosaccharide supplementation on concentrations of intestinal mucosal immunoglobulins and pro-inflammatory cytokines of lipopolysaccharide-challenged laying hens (experiment 2).

| Items | CON | LPS | COS+LPS | SEM | P-value |
|-------|-----|-----|---------|-----|---------|
| Jejunal mucosa | | | | | |
| sIgA (µg/mg protein) | 1.11 | 1.34 | 1.28 | 0.035 | 0.011 |
| IgM (µg/mg protein) | 1.09 | 1.32 | 1.31 | 0.047 | 0.075 |
| IgG (µg/mg protein) | 17.37 | 19.73 | 18.11 | 0.622 | 0.302 |
| TNF-α (pg/mg protein) | 4.55 | 4.76 | 4.35 | 0.188 | 0.697 |
| IFN-γ (pg/mg protein) | 4.64 | 7.11 | 4.70 | 0.449 | 0.025 |
| IL-1β (pg/mg protein) | 7.66 | 8.24 | 6.47 | 0.297 | 0.034 |
| Ileal mucosa | | | | | |
| sIgA (µg/mg protein) | 1.13 | 1.40 | 1.26 | 0.042 | 0.028 |
| IgM (µg/mg protein) | 1.18 | 1.94 | 1.47 | 0.085 | <0.001 |
| IgG (µg/mg protein) | 17.35 | 18.32 | 17.23 | 0.253 | 0.158 |
| TNF-α (pg/mg protein) | 4.32 | 4.65 | 4.06 | 0.223 | 0.593 |
| IFN-γ (pg/mg protein) | 4.65 | 5.20 | 4.81 | 0.230 | 0.638 |
| IL-1β (pg/mg protein) | 5.88 | 8.24 | 7.79 | 0.269 | <0.001 |

Abbreviations: CON, non-challenged laying hens fed a basal diet; LPS, LPS-challenged laying hens fed a basal diet; COS+LPS, LPS-challenged laying hens fed a basal diet supplemented with 15 mg/kg chitooligosaccharide.

1 Abbreviations: IFN-γ, interferon-γ; IL-1β, interleukin-1β; sIgA, secretory IgA; TNF-α, tumor necrosis factor-α.
2 SEM, standard errors of mean.
3 Mean values within a row with different superscripts letters are significantly different at P < 0.05.

Table 7. Effects of dietary chitooligosaccharide supplementation on mRNA abundance of intestinal mucosal genes of lipopolysaccharide-challenged laying hens (experiment 2).

| Items | CON | LPS | COS+LPS | SEM | P-value |
|-------|-----|-----|---------|-----|---------|
| Jejunal mucosa | | | | | |
| GPX1 | 1.00 | 1.37 | 1.30 | 0.077 | 0.106 |
| SOD1 | 1.00 | 1.35 | 1.42 | 0.064 | 0.007 |
| HO-1 | 1.00 | 1.32 | 1.32 | 0.064 | 0.006 |
| SO9 | 1.00 | 1.53 | 1.39 | 0.072 | 0.002 |
| GPX1 | 1.00 | 1.37 | 1.30 | 0.077 | 0.106 |
| IL-1β | 1.00 | 1.11 | 0.73 | 0.064 | 0.106 |
| IL-4 | 1.00 | 0.79 | 0.97 | 0.059 | 0.693 |
| IL-10 | 1.00 | 1.19 | 1.13 | 0.050 | 0.307 |
| Ileal mucosa | | | | | |
| GPX1 | 1.00 | 0.82 | 0.86 | 0.058 | 0.454 |
| SOD1 | 1.00 | 0.82 | 0.84 | 0.058 | 0.406 |
| HO-1 | 1.00 | 0.95 | 0.85 | 0.060 | 0.403 |
| SO9 | 1.00 | 0.99 | 0.99 | 0.062 | 0.010 |
| GPX1 | 1.00 | 0.97 | 1.03 | 0.077 | 0.946 |
| GPX1 | 1.00 | 1.16 | 1.15 | 0.066 | 0.579 |
| IL-1β | 1.00 | 0.86 | 0.94 | 0.049 | 0.514 |
| IL-4 | 1.00 | 1.14 | 1.08 | 0.069 | 0.030 |
| IL-4 | 1.00 | 0.94 | 1.21 | 0.054 | 0.028 |
| IL-10 | 1.00 | 0.68 | 1.21 | 0.063 | 0.094 |

Abbreviations: CON, nonchallenged laying hens fed a basal diet; LPS, LPS-challenged laying hens fed a basal diet; COS+LPS, LPS-challenged laying hens fed a basal diet supplemented with 15 mg/kg chitooligosaccharide.

1 Abbreviations: CLDN1, claudin 1; CLDN2, claudin 2; HO-1, heme oxygenase-1; GPX1, glutathione peroxidase 1; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-10, interleukin-10; NFR2, nuclear factor erythroid-2 related factor 2; SOD1, superoxide dismutase 1; OCLN, occludin; ZO1, zonula occludens 1.
2 SEM, standard errors of mean.
3 Mean values within a row with different superscripts letters are significantly different at P < 0.05.

**DISCUSSION**

A recent research has shown that supplementation of marine-derived polysaccharides that contain large amounts of chitin and chitosan can increase productive performance and egg quality of 62-wk-old layer hens, accompanied by the improvement of antioxidant capacity and intestinal morphology (Guo et al., 2020). As COS is the major degradation product of chitin/chitosan and possesses the enhanced absorption and diverse biological activities (Naveed et al., 2019), it is possible that supplemented COS can also exert beneficial effects on laying hens. Several studies have reported that COS treatment can optimize the productive performance and egg quality of laying hens at peak laying period (Meng et al., 2010; Yan et al., 2010). Our results of experiment one revealed that different levels of COS did not lead to the significant increase of productive performance, but still contribute to the numerical improvement, which was presented by the highest egg production rate and the lowest feed conversion ratio when supplemented with 15 mg/kg COS. With respect to the egg quality, we also found that eggs from layers fed diets with 15 mg/kg COS exhibited higher levels of albumen height and Haugh unit than other groups. Therefore, administration of 15 mg/kg COS was selected as the appropriate dosage to alleviate the intestinal stress stimulated by LPS in the subsequent experiment.

In experiment two, LPS challenge induced the elevation of serum diamine oxidase level, a sensitive marker reflecting intestinal mucosal integrity and permeability (Luk et al., 1980), and led to the reduction of villus height and ratio of villus height to crypt depth, as well as the downregulation of mRNA abundance of OCLN and CLDN2, both of which involved in the assembly of tight junctions for the maintenance of intestinal barrier (Gunzel and Yu, 2013; Zihni et al., 2016). Together, these results indicated that LPS could impair the intestinal barrier of laying hens in the current study. In weaned piglets which are easily subject to environmental and intestinal stresses, researchers have demonstrated that COS with lower molecular weight can improve the nutrient digestibility and growth performance through...
enhancing intestinal barrier function, as evidenced by the elongated villus height and increased ratio of villus height to crypt depth (Walsh et al., 2012; Thongsong et al., 2018). Another report has also showed that COS addition can reduce intestinal permeability by decreasing serum diamine oxidase, D-lactic acid and endotoxin levels, and simultaneously modulates antioxidant status and immune function, ultimately contributing to the lower incidence of diarrhea and improved growth performance of weaned pigs (Zhao et al., 2017).

Also, a previous study has indicated that COS supplementation can alleviate the intestinal histopathological injury in a piglet model challenged with *Escherichia coli* (Liu et al., 2017). For poultry, a published paper has reported that supplemental COS at a dose of 30 mg/kg can promote the growth performance and improve the intestinal barrier function, and up-regulate the expression of gene levels of tight junction proteins in broilers at an early age (Li et al., 2019). In consistent with these data, the present trial showed that COS administration alleviated the impaired intestinal barrier function induced by LPS, as supported by the decreased serum diamine oxidase activity, reversed intestinal morphology and upregulated gene levels of tight junction proteins.

The alteration of intestinal oxidative and inflammatory status often occurs with the modification of intestinal morphology and permeability (Wen et al., 2019; Xiong et al., 2020). Accumulating studies have identified that LPS administration can result in the oxidative stress both in vitro and in vivo (Liu et al., 2017; Wu et al., 2017; Sun et al., 2020). In the present study, we observed that LPS-challenged birds exhibited a higher concentration of intestinal mucosal MDA, an end product that formed in the process of lipid peroxidation (Janero, 1990), and lower activities SOD and GSH-Px, both of them participating in the antioxidant defence system and protecting the organisms against the excessive free radicals (Pisoschí and Pop, 2015). Meantime, the change of redox homeostasis is also accompanied with the alteration of associated genes. A primary component against oxidative stress in the cellular defense system is the activation of *NRF2*-antioxidant response element signaling pathway, where involves an array of genes that regulate the redox status, especially when organisms are exposed to oxidants (Nguyen et al., 2009).

The antioxidant gene *SOD1*, a copper and zinc-containing homodimer that is distributed mainly in the intracellular cytoplasm and control the expression of related SODs (Zelko et al., 2002), is of great importance to the antioxidant capacity of organisms. The current research showed that LPS injection could induce the upregulation of intestinal mucosal *NRF2* and *SOD1* abundance, indicating that antioxidant system has been activated to resist the LPS-induced oxidative stress. Simultaneously, from this study, the protection of COS on redox system of LPS-challenged laying hens was evidenced by the normalized levels of several antioxidants in the intestinal mucosa. In an in vitro study, Yang et al. (2006) found that COS can exert strong free radical scavenging activities. Also, several experiments performed on different lines of cells have effectively verified the powerful antioxidant property of COS against oxidative stress, which is supported by the inhibition of reactive oxygen species and malondialdehyde production, enhancement of antioxidant substances, as well as prevention of cell apoptosis (Xu et al., 2010; Lu et al., 2012). Further, it has been reported that COS pretreatment can mediate the activation of *NRF2* and phosphorylation of mitogen-activated protein kinase to attenuate the oxidative stress in hepatic cells (Luo et al., 2014). In line with these researches, the present study showed that supplemental COS could elevate the expression of the transcription factor *NRF2* and its target gene *HO-1* to exert antioxidant function, and this alteration may also be a possible explanation for the anti-inflammatory effects of COS in the laying hens (Hyung et al., 2016).

Oxidative stress has been proven to be closely related with the activation of inflammatory pathways, leading to a variety of modifications of physiological and pathological functions (Reuter et al., 2010). In chickens, 3 classes of immunoglobulins have been identified, including IgM, IgA, and IgG, of which IgM is the first antibody generated during a primary antibody response while IgG is the secondary antibody (Ratcliffe, 2006). With regard to IgA, it is presented as the monomer in the serum, but as the polymer (sIgA) in the intestinal mucosa. The intestinal sIgA serves as the first-line in protecting intestinal barrier from harmful toxins, antigens and microorganisms, and maintaining the balance of intestinal mucosal homeostasis (Mantis et al., 2011). In this research, higher concentrations of intestinal mucosal sIgA and IgM, as well as increased proinflammatory cytokine (IFN-γ and IL-1β) concentrations and paralleled gene expression were observed in the intestinal mucosa after receiving the LPS injection, which indicated that the inflammatory response had been induced by LPS administration under the current experimental condition. LPS-challenged birds fed COS exhibited the reduced levels of aforementioned immunoglobulins and proinflammatory cytokines when compared to their LPS-challenged counterparts, implying that 15 mg/kg COS could mitigate inflammatory stress in laying hens.

Several in vitro studies performed on the LPS-stimulated macrophage and epithelial cells have revealed that over-expression and elevated secretion of proinflammatory factors can be suppressed by COS pretreatment through related signaling pathways (Ma et al., 2011; Shi et al., 2019), indicating the promising therapeutic strategy of COS in the recovery of inflammatory damage. Consistently, in a study of mice model of sepsis induced by LPS, researchers have demonstrated that COS treatment can markedly decrease the overwhelming proinflammatory mediators (TNF-α and IL-1β) and attenuate the oxidative stress, which may partially contribute to the improved organ function and survival rate (Qiao et al., 2011), and a similar result was obtained in a piglet model challenged with LPS injection (Huang et al., 2016).

Collectively, our results suggested that dietary 15 mg/kg COS supplementation could improve the
productive performance under normal physiology conditions, and attenuate the intestinal barrier damage, and oxidative and immunological stress induced by LPS in laying hens. This finding will provide a guideline for the application of COS in laying hens.

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DISCLOSURES
The authors declare that there is no conflict of interest.

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