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Effects of dietary supplementation by modified palygorskite and essential oil/ palygorskite complex on growth performance and intestinal flora composition of broilers with diarrhea

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ABSTRACT With the development trend of the industry, it can be seen that the substitution of antibiotics and reduction of zinc oxiden is still the hot spot of the industry. Diarrhea and inflammation occur frequently during livestock and poultry production, which is difficult to control. This experiment aimed to explore the effects and mechanisms of dietary supplementation of modified palygorskite (**Mpal)** and essential oil/ palygorskite composite (**EO-PGS**) on disease resistance and intestinal inflammatory damage in diarrhea broiler. In this experiment, there were a total of 420 broilers of 10-day-old selected and divided into 7 groups (n = 60), which were the nondiarrhea group fed with basal diet (normal control, NC), the diarrhea group fed with basal diet (diarrhea control, **DC**), and the rest were the diarrhea test group (diarrhea), supplemented with 1 kg/t, 2 kg/t and 4 kg/t of essential oils/ palygorskite complex (EO-PGS 1kg/T, EO-PGS 2kg/T, EO-PGS 4kg/T) in the basal diet, respectively, and 2 kg/t, 4 kg/t modified palygorskite group (Mpal 2kg/T, Mpal 4kg/T) in the basal diets, respectively. The experiment lasted for 8 d. The results showed that compared to normal broilers, the diarrhea index of diarrhea broilers remained around 2.0 with persistent mild diarrhea during the test period. The duodenal epithelial cells were damaged and shed, goblet cells increased, inflammatory cells infiltrated, diffuse congestion and hemorrhage in lamina propria, the serum lipopolysaccharides (**LPS**) content, and malondialdehyde (**MDA**) content increased significantly ($P < 0.05$). The serum superoxide dismutase (**SOD**) activity and immunoglobulin-M (**IgM**) levels significantly decreased, while serum immunoglobulin-G (**IgG**) and complement 3 (**C3**) levels significantly increased ($P < 0.05$). The expression of inflammatory cytokines interleukin-1β (***IL-1β***), interleukin-6 (***IL-6***), tumor necrosis factor-α (***TNF-α***), and nuclear factor κB (***NF-κB***) in duodenal epithelial cells was significantly upregulated on d 5 ($P < 0.05$). The abundance of *Bacteroides* in the duodenum of diarrhea broilers was significantly decreased, while the abundance of *Proteobacteria* was significantly increased ($P < 0.05$). Feeding diets supplemented with EO-PSG and 4 kg/t Mpal increased the average weight of diarrhea broilers ($P < 0.05$), reduced diarrhea index, improved immunity by increasing serum IgG, IgM, C3 and complement 4 (**C4**) levels ($P < 0.05$), enhanced the activity of serum antioxidant enzyme glutathione peroxidase (GSH-PX) and SOD activity, reduced serum MDA content, serum LPS levels, and decreased the expression of proinflammatory factors in the duodenal epithelial cell on d 5 ($P < 0.05$), alleviated duodenal epithelial cell injury, hemorrhage, inflammation infiltration and intestinal injury of diarrhea broilers from d 5 to d 8. Meanwhile, supplemented with EO-PSG and Mpal in diets regulated the intestinal microbiota, significantly increased the abundance of *Bacteroidetes* and decreased the abundance of *Proteobacteria* at the phylum level ($P < 0.05$). Microbial richness and diversity of microbiota were significantly increased by feeding the diet supplemented with 2 kg/t EO-PGS. In the beta diversity of the intestinal flora of the diets supplemented with 4 kg/t Mpal and 2 kg/t EO-PGS, the microbial community composition could be relatively easily distinguished with NC and DC groups. As a result of LEfSe analysis, the diets supplemented with 2 kg/t EO-PGS *f_ Clostridiaceae* and *g_ Coprococcus* were enriched in the caecum of diarrhea broilers, and the diets supplemented with 4 kg/t Mpal *o_ Bacteroidales, f_ Rikenllaceae* and *g_ Peptococcus* were enriched in caecum of diarrhea broilers, between normal and diarrhea broilers ($P<0.05$). In conclusion, dietary supplementation with EO-PGS and Mpal could improve disease

Received August 9, 2024.
Accepted September 30, 2024.

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resistance and alleviate intestinal inflammatory damage in diarrhea broilers, but the effect of 2 kg/t Mpal was not significant. It was recommended that 2 kg/t EO-PGS or 4 kg/t Mpal be added to the broilers' diet according to the degree of diarrhea, and continuous feeding for more than 5 d.

Key words: modified palygorskite, EO-PGS, inflammatory factor, intestinal damage

2024 Poultry Science 103:104379
https://doi.org/10.1016/j.psj.2024.104379

INTRODUCTION

In the process of livestock and poultry breeding, diarrhea occurs more frequently, especially in poultry under high-temperature stress and cold winter temperatures, the body resistance is weakened (Tsiouris et al., 2015; Lauridsen, 2019; Gil et al., 2023). Common pathogens such as *Escherichia coli* (***E.coli***), *Proteus*, *Salmonella* and other gram-negative pathogens (**G-**), could release the LPS after its death or cell wall rupture. LPS will damage the intestinal tight junction structure, increase intestinal permeability, damage the colonic mucosa dysfunction (Al-Sadi et al., 2008; Zhang et al., 2011), increase moisture fecal content in feces and cause diarrhea (Madara and Stafford, 1989). Palygorskite (**Pal**), a clay mineral with a fibrous rod-like crystal structure with certain regular pores, had a large specific surface area (Serna and Vanscoyoc, 1979). Pal was used in the medical field as a gastrointestinal protection and antidiarrheal agent with a strong adsorptive property through ion exchange (DuPont et al., 1990). Pal was also commonly used as a feed additive for livestock and poultry farming (Wang et al., 2024).

Dietary supplementation of Pal could improve the intestinal integrity and barrier function of broilers and piglets (Zhang et al., 2013; Chen et al., 2016; Su et al., 2018), reduce plasma LPS, regulate the gut microbes, increase the growth performance and reduce diarrhea rate, (Zhang et al., 2013; Zha et al., 2023). Generally, the effect of ordinary Pal action was not as effective as MPal (Zhang et al., 2017; Su et al., 2018), which could achieve a small amount of high efficiency by increasing the adsorption capacity. As plant essential oils carvacrol and thymol have a variety of biological activities such as antioxidant (Salehi et al., 2018; Gunes-Bayir et al., 2022), immune-enhancing (Abo Ghanima et al., 2020; Chang et al., 2023), antibacterial (Gholami-Ahangaran et al., 2020), anti-inflammatory (Lu et al., 2014), etc, but essential oils are volatile and oxidized, and their bioactive compounds are degradable not resistant to high temperature, and it was difficult to add them directly in the feed (Si et al., 2006). Pal as a nano-sized molecular material contains a large number of pores and is resistant to high temperatures, acids and alkalis, so it is possible to load and fill plant essential oils into the pores made into a composite which effect was better than that of plant essential oils alone, and the amount added was smaller (Cheng et al., 2016; Lei et al., 2017; Tenci et al., 2017).

There are numerous applications of Pal in livestock and poultry breeding (Cheng et al., 2016; Zha et al., 2023; Chen et al., 2024), however, fewer studies have been conducted on specific inflammatory factors and intestinal damage in diarrhea broilers. In this experiment, the growth performance, diarrhea, antioxidant and immunity characteristics of diarrhea broilers were studied by adding different levels of Mpal and EO-PGS to the basal diet of diarrhea broilers with 10-day-old Xuefeng Ouzo chickens as the feeding objects, and the effects of endotoxin content, intestinal damage and inflammatory factors were detected. Meanwhile, 16s rRNA gene sequencing of intestinal microorganisms was carried out to explore the optimal dosage of Mpal and EO-PGS and their mechanism of diarrhea control in broilers, which provided a scientific basis for its application in broiler production.

MATERIALS AND METHODS

Ethics Statement

The procedures of all experiments and sample collection were conducted under the Chinese animal welfare guidelines and approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, (SYXK(Xiang) 2022-0007).

Source of Mpal and EO-PGS

EO-PGS and Mpal were provided by Jiangsu Sinitic Biological Technology Co., Ltd (Jiangsu, Xuyi, China). Mpal (Pal 80%, other minerals 20%), was determined by X-ray diffraction. The main components of EO-PGS were: Mpal >70%, essential oils (carvacrol and thymol as the main components) >15%, and the other minerals <15%. Nature Pal from Zhuzuishan Mine Area located in Xuyi County of Jiangsu Province, China.

Preparation of Mpal and EO-PGS

Preparation of Mpal: The natural pal was dried by light, crushed, doped, rolled and other processes, calcined at 450-500 °C, ground after cooling and heat preservation, and purified by the wet method: pulping is carried out in a solvent with the ethanol/water ratio of 6:4 in a vertical high-speed stirrer under the high-pressure condition of 30 MPa for 2 h, screening and impurity removal are carried out, the water content is filtered until the water content is 20 to 23%, and ethanol is removed in the strong drying process at 90-150 °C to obtain the Mpal finished product.

Preparation of EO-PGS: The Mpal, thymol and carvacrol plant essential oil complex were prepared in a

ratio of 17:3, first added the Mpal to the aqueous solution and stirred in a neutral high-speed mixer for 30 min to prepare a dispersed suspension, then dissolved the plant essential oil complex in absolute ethanol and added the suspension solution to the dispersed suspension stirred for 30 min, and then pressed it into a sheet with a thickness of 3 mm by a triple roller machine, entered the storage bin and stood for 12 h, and then sprayed the dissolved chitosan-citric acid aqueous solution at the same time by a double-shaft mixer Stirred for 30-120 min. Finally, after strong drying at 90-150°C, the EO-PGS product is obtained.

Animals and Experimental Design

The trial was undertaken at Hunan Yunfeifeng Agricultural Co., Ltd, Huaihua, Hunan Province, China. 420 Xuefeng black-bone broilers with similar weight at 10-days-old were divided into 7 groups (n = 60, 6 replicates of 10 broilers each). The nondiarrhea group was fed a basal diet, while the diarrhea groups were fed a basal diet and a basal diet supplemented with 1 kg/t, 2 kg/t, 4 kg/t EO-PGS, 2 kg/t and 4 kg/t Mpal and respectively marked as NC, DC, EO-PGS 1 kg/T, EO-PGS 2 kg/T, EO-PGS 4 kg/T, Mpal 2 kg/T, Mpal 4 kg/T groups for 8 d with free feeding and water. We formulated a basal diet that meets the nutrient requirements of broilers according to the China Agricultural Standard (Wen et al., 2004). Table 1 was shown the feed composition and nutrient levels. Before the experiment started, 800 one-day-old broilers were selected for the experiment without immunization and medication, the broilers were fed with a basal diet and the diarrhea broilers were continuously observed and recorded until 8 d of age (the number of diarrhea broilers was recorded, and the rate of diarrhea was counted), and the broilers without diarrhea and those with persistent diarrhea were divided into groups until 10 d of age.

Table 1. Formulation and calculated composition of the basal diet (as-fed basis).

Ingredient	Content (%)	Calculated composition	Nutrient levels (%)[2]
Corn	59.39	ME/(MJ/kg)	13.58
Flour	1	CP(%)	21
Rice bran	1	Lys(%)	1.06
Soybean meal	20.5	Met(%)	0.45
Peanut meal	5	Ca (%)	0.9
Corn gluten meal	3	Available P (%)	0.37
Sprayed corn bran	2.5		
Palm Kernel Cake	1		
Hydrolyzed feather meal	1.8		
Duck fat	0.4		
Limestone	1.3		
Dicalcium phosphate	1.38		
Sodium chloride	0.2		
Lysine	0.35		
Methionine	0.18		
Premix[1]	1		
Total	100		

[1]The premix provided the following per kg of the diet: vitamin A 12,000 IU, vitamin D 33,000 IU, vitamin E 30 IU, vitamin K3 1.3 mg, vitamin B1 2.2 mg, vitamin B2 8 mg, vitamin B6 4 mg, vitamin B12 0.013 mg, nicotinic acid 40 mg, choline chloride 400 mg, D-pantothenic acid 10 mg, biotin 0.04 mg, folic acid 1 mg, Fe 80 mg, Cu 7.5 mg, Mn 110 mg, Zn 65 mg, I 1.1 mg, Se 0.3mg.

[2]The nutrition levels are all calculated values.

Sample Collection

Blood samples were collected from the wing vein in heparinized tubes on d 3, 5, 8, five samples per group were centrifuged at 3,000 r/min for 10 min and stored in 1.5 mL centrifuge tube at -20°C refrigerator for analysis of serum immunity and antioxidant indexes and LPS levels.

Slaughtered 5 broilers per group and separated the intestines, taken the chyme of caecum into a 1.5 mL sterilized centrifuge tube for determination of 16s rRNA gene sequencing of intestinal microorganisms on d8. Five duodenum Samples about 2 cm × 1.5 cm × 0.5 cm per group were collected and immersed in formalin solution fixative for duodenal morphology analysis, pathological damage and immunohistochemistry of IL-6 and TNF-α on d 3, 5, 8, five duodenum Samples per group for tests of expression of the inflammatory factors on d 5.

Performance and Diarrhea Index

During the experiment, all broilers were weighed daily and recorded repeatedly. The diarrhea index was scored daily using a 3-point scale as previously described (Hu et al., 2018). After the experiment, average weight and diarrhea index were calculated.

Detection of Serum Indices

The determination of GSH-Px/T-SOD/MDA/IgG/IgM/C3/C4/ LPS in serum was performed according to the ELISA kit instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). These kits were: Chicken glutathione peroxidase (**GPX**) ELISA KIT (Catalog_no: ml036972); Chicken superoxide dismutase1 (**SOD1**) ELISA KIT (Catalog_no: ml060830); Chicken malondialdehyde (**MDA**) ELISA KIT (Catalog_no: ml036969), Chicken Immunoglobulin G (**IgG**) ELISA KIT (Catalog_no: ml002792); Duck Immunoglobulin M (**IgM**) ELISA KIT (Catalog_no: ml061224); Chicken Complement Protein 3 (**C3**) ELISA KIT (Catalog_no: ml023598); Chicken Complement Protein 4 (**C4**) ELISA KIT (Catalog_no: YJ685690) and Chicken lipopolysaccharide (**LPS**) ELISA KIT (Catalog_no: ml059937), and the coefficient of variation CV is less than 10%.

Detection of Gene Expression Levels of Duodenal Inflammatory Cytokines

Tissue RNA was extracted with the trizol conventional method, and cDNA was generated by reverse

Table 2. Primers for quantitative real-time PCR.

Gene name	**Forward primer** -sequence (5′ to 3′)	**Reverse primer**- sequence (5′ to 3′)	Product length (bp)	GenBank No.
β-actin	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA	152	NM_205518.2
NF-kB	GTGTGAAGAAACGGGAACTG	GGCACGGTTGTCATAGATGG	203	NM_001396038.1
TNF-a	CTCCGCAGTACTCAGGACAGC	TCAGAGCATCAACGCAAAAGG	249	NM_204267.2
IL-1β	ACTGGGCATCAAGGGCTA	GGTAGAAGATGAAGCGGGTC	131	NM_204524.2
IL-6	AGGACGAGATGTGCAAGAAGTTC	TTGGGCAGGTTGAGGTTGTT	79	NM_204628.2

transcription of RNA with AG RNA (ex Pro Reagent Kit with AG21102 (AG, Hunan, China). The mRNA expression levels of *IL-1β, TNF-α, IL-6* and *NF-kB* in duodenal tissues were quantified by quantitative real-time polymerase chain reaction (qRT-PCR, Thermo Fisher, MA). The primer reference sequences and target genes were shown in Table 2 designed by Primer 5.0. Based on the cDNA template, 10 μL of reaction system was prepared: 5 μL of SYBR Green Premi×Pro Taq HS qPCR kit with AG11701 (AG, Hunan, China), 0.1 μL each of upstream and downstream primers, 3.8 μL of ddH2O, and 1 μL of the cDNA template. The reaction conditions were set at 50°C for 2 min predenaturation; 95°C for 10 min, 1 cycle; 95°C for 15 s, T_m°C for 1 min, 40 cycles. The $2^{-\Delta\Delta CT}$ method was used to normalize 3 replicates of each sample based on β-actin as the internal reference gene. Numerical values for the treatment group fed the basal diet were used as calibrators.

Detection of Intestinal Injury and Immunohistochemistry

Formalin-fixed intestinal tissues were processed, dehydrated, and embedded into the wax block, cut 3-μm-thick sections out and stained with heme staining method to make tissue sections, then used an optical microscope to record, photographed and observed according to the chiu intestinal injury histological score, the structure of the normal mucosal villi was smooth and intact, the color is moderate and uniform and without inflammatory infiltration; In the case of injury, the subepithelial space at villi tip of intestinal mucosa became wider and stripped from the lamina propria, and the intestinal mucosal epithelial cells were damaged and shed (black arrows), gradually as the intestinal mucosa injury worsens the goblet cells increase (blue arrows), inflammatory infiltration occurs (green arrows), diffuse hyperemia and hemorrhage in the lamina propria of the intestinal mucosa (red arrows). The degree of intestinal damage can be determined by the color of the arrows.

To obtain duodenal tissues, they were fixed with 4% paraformaldehyde solution for at least 24 h, then dewatered with gradient alcohol. The 3-mm-thick slice was taken, and stigmatized with hematoxylin-eosin staining (Liu-Fu et al., 2024). The primary antibody concentrations for immunohistochemistry of duodenal tissues were IL-6 (1:200) and TNF-α (1:1000). Tissue staining was observed under 3D Histech Quant Center 2.1 (3D Histech, Hungary) acquisition and analysis of images. Hematoxylin-stained nuclei were blue and DAB-positive expression was brownish-yellow. Duodenal intestinal tissue was observed and analysed. The depth and number of positives were scored using Quant center, the analysis software accompanying the 3D scanner, to quantify the brownish-yellow color of the DAB color development, and H-Score statistical methods as previously described (Liu-Fu et al., 2024).

Detection of Fecal 16s rRNA Gene Sequencing

A total of 0.2 to 0.5 g of sample was added to the centrifuge tube from which the lysate was extracted for grinding pretreatment (Tissuelyser-48 at 60 Hz). Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA). The quality of extracted DNA was determined by Nanodrop and 1.0% agarose gel electrophoresis and sent to Shanghai Paisano Bio-technology Co. for sequencing and analysis.

The bacterial 16s rRNA genes of the V3-V4 region were amplified by PCR using forward primer 338F (5′-ACTCCTACGGGGAGGCAGCA-3′) and reverse primer 806R (5′-GGACTACHVGGTWTCTAAT-3′). The protocol barcode in the forward primer was a 7 to 10 base oligonucleotide sequence used to separate different samples in the same library.

The sequence data were analyzed using the QIIME2 and R software packages (v3.2.0). The alpha diversity indices at the ASV level, such as Chao1 richness estimates, observed species, Shannon's diversity index, Simpson's index, Faith's PD, Pielou's evenness, and Good's cover, were computed using the ASV table in QIIME2 and optimally visualized as box plots. Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Jaccard metrics (Jaccard, 1908), Bray-Curtis metrics (Bray and Curtis, 1957) and UniFrac distance metrics (Lozupone and Knight, 2005; Lozupone et al., 2007) and visualized via principal coordinate analysis (**PCoA**), nonmetric multidimensional scaling (**NMDS**) and unweighted pair-group method with arithmetic means (**UPGMA**) hierarchical clustering (Ramette, 2007). The prominence of differences in microbiota structure between groups was assessed by PERMANOVA. LEfSe (Linear discriminant analysis effect size) analyses were conducted using default parameters (Segata et al., 2011) to examine the varying abundance of taxa across groups.

Statistical Analysis

Statistical analyses were conducted using SPSS statistical software (ver. 27.0, Spss Inc., Chicago, IL), all the data were analyzed by a one-way ANOVA, and differences among groups were tested using Duncan's multiple range tests. All experimental data were expressed using the mean ± standard error of the mean (S.E.M.), and differences were considered to be significant at $P < 0.05$.

RESULTS

Effects of EO-PGS and Mpal on Growth Performance and Diarrhea Index in Broilers

In order to examine the phenotypic properties of diets supplemented with EO-PGS and Mpal, we examined the average body weight and diarrhea index of broilers. The average weight results (Figure 1A) showed that the DC group was significantly lower than that of the NC group from d 1 to d 8 ($P < 0.05$). Except for the significant decrease in the average weight of the Mpal-added groups on d 2, the average weight increase trend was basically the same among the other groups. Compared with the DC group, there was no significant difference in the the average weight of the Mpal 2 kg/T-added group ($P > 0.05$). The average weight of Mpal 4 kg/T-added group was significantly higher than that of the DC group on d 4 to d 8 ($P < 0.05$), and lower than that of the NC group on d 6 to d 8 ($P < 0.05$). The average weight of EO-PGS-added groups was significantly higher than that of the NC group ($P < 0.05$), and there were differences among the EO-PGS-added groups, the average weight of EO-PGS 2 kg/T was at the intermediate level ($P > 0.05$).

As is shown in Figure 1B, the diarrhea index of diarrhea groups in the test was between 2.2 and 2.32 with mild diarrhea on d 0, whereas the diarrhea index of DC groups consistently remained around 2.0 or higher, and was significantly higher than that of the NC group on d 1 to d 8 ($P < 0.05$). Compared with the DC group, the diarrhea index of the EO-PGS-added groups and Mpal-added groups demonstrated a significant decreasing trend ($P < 0.05$), with the prolongation of the trial period from d 2 to d 8, and diarrhea index ranged from 1 to 0 which was between normal stool and soft stool. With the fluctuation of the diarrhea index of the NC group, there was no significant difference among them on d 4 and d 7 ($P > 0.05$).

Effects of EO-PGS and Mpal on Intestinal Morphology and Injury

The hematoxylin and eosin (**HE**) stained tissue section method was used to detect whether diet supplemented with EO-PGS and Mpal had effect on intestinal health of broilers. As shown in Figure 2, the duodenal mucosal villous epithelium in the NC group was undamaged with no obvious lesions. In the DC group, the duodenal mucosal epithelial cells were damaged and detached (black arrows), goblet cells were increased (blue arrows), inflammatory cell infiltrates (green arrows) and diffuse hyperemia and hemorrhage (red arrows) were seen in the lamina propria. In the EO-PGS groups, the duodenal villi there was no significant damage, and the number of glandular follicular cells was reduced, and diffuse hemorrhage and inflammatory cell infiltration were seen in the lamina propria with a decrease in the number of goblet cells and diffuse hemorrhages and inflammatory cell infiltration in the lamina propria. The mucosal structure of the EO-PGS 4kg/T and the EO-PGS 2 kg/T group returned to normal on d 5 and d 8, with no significant difference between and the NC group. There was a decrease in the number of diffuse hemorrhages and inflammatory cell infiltration in the lamina propria in the Mpal 4 kg/T group, with no significant difference between the Mpal 4 kg/T group and the NC group on d 8. The Mpal 2 kg/T group was not significantly with the DC group.

Effects of EO-PGS and Mpal on Serum Antioxidant Indexes

Detection of GSH-Px, SOD, MDA indicators in broiler chickens by serum antioxidant kit method

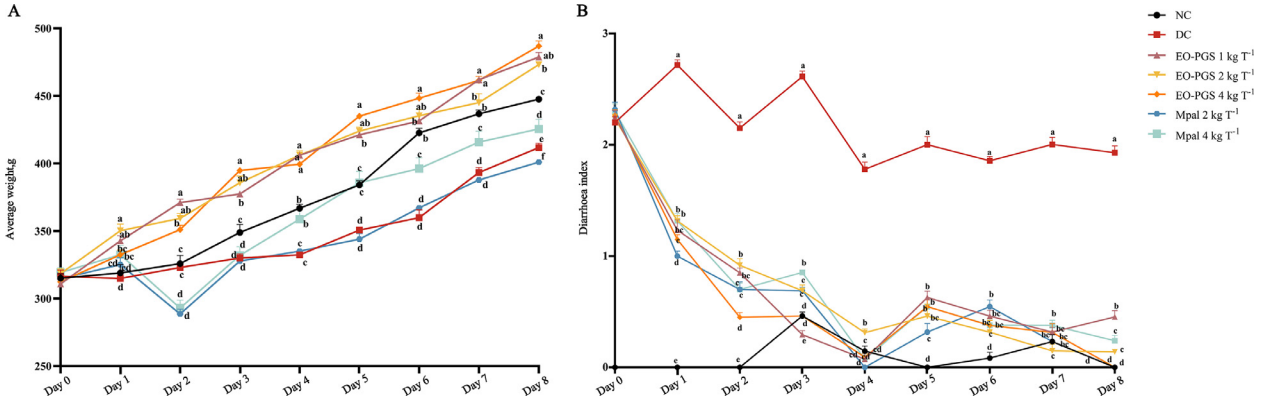

Figure 1. Effects of EO-PGS and Mpal on growth performance and diarrhea index in broilers(n=6)

(A), the effect of EO-PGS and Mpal on the average weight of diarrhea broilers; (B), the diarrhea index of all groups. Different lowercase letters (such as a, b) indicate that there are significant differences in the comparison of different groups at the same time ($P < 0.05$), the same as below.

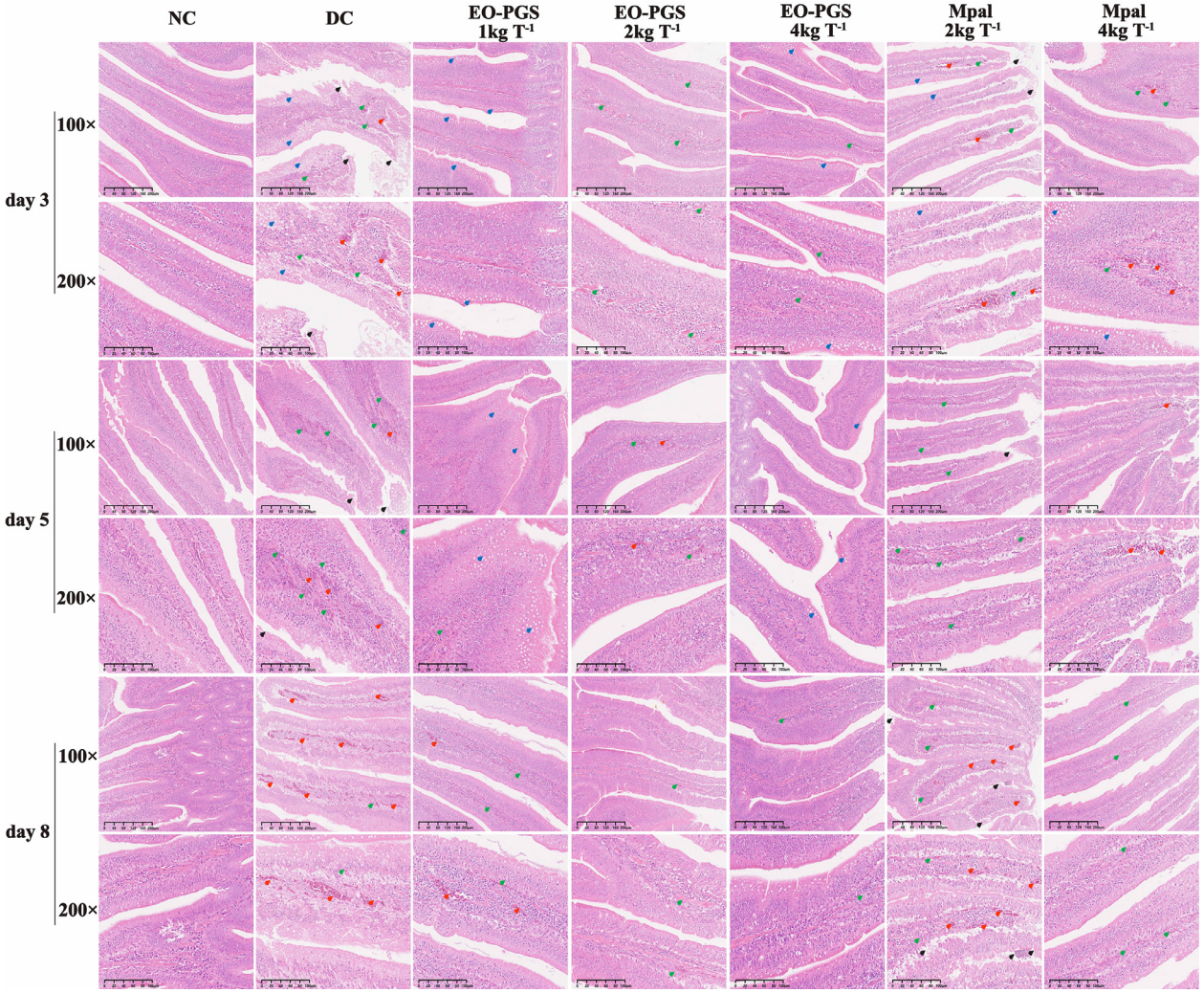

Figure 2. Effects of EO-PGS and Mpal on Intestinal morphology and damage of diarrhea broilers (n = 3).

(Figures 3A and 3B), there were no significant differences in GSH-Px and SOD activities of the DC group compared with the NC group on d 3 and d 8 ($P > 0.05$). In contrast to the DC group, the GSH-Px activity on d 3 and SOD activity on d 5 were considerably elevated in the EO-PGS-added group ($P < 0.05$), and GSH-Px activity on d 3 and d 5 was remarkably higher in the EO-PGS 4 kg/T group than in the NC group ($P < 0.05$). The GSH-Px activity in the Mpal 2 kg/T group and SOD activity in the Mpal-added group were substantially more than that in the DC group on d 5 ($P < 0.05$). However, there was no significant difference in GSH-Px and SOD activities in the EO-PGS 2 kg/T group compared to the Mpal 4 kg/T group on d 5 ($P > 0.05$). Concerning the MDA content analysis (Figure 3C), the results revealed that it was significantly higher in the DC group than in the NC group ($P < 0.05$). Compared with the DC group, MDA content was conspicuously reduced ($P < 0.05$) in all experimental groups except Mpal 4 kg/T group on d 5 and EO-PGS 1 kg/T group on d 8. The MDA content declined in the EO-PGS 4 kg/T group versus the DC group ($P < 0.05$) and was not appreciably dissimilar to that of the NC group on d 3 to 8 ($P > 0.05$). The MDA content in the EO-PGS groups was significantly lower than in the Mpal-added groups on d 5 ($P < 0.05$).

Effects of EO-PGS and Mpal on Serum Immunological Indexes

The content of IgG, IgM, C3 and C4 in broiler as immune-related indicators using the kit method, the serum levels of them in the DC group were not markedly different from those in the NC group on d 3 and d 8 ($P > 0.05$), the levels of IgG and C3 were prominently elevated in the DC group, and the IgM level was strikingly inferior to that of the NC group on d 5 ($P < 0.05$, Figures 4A–D). When a trend of increasing serum IgG levels was observed in all experimental groups compared to the DC group, serum IgG levels were significantly higher in the Mpal 4 kg/T group on d 3, and that in the EO-PGS 4 kg/T group and Mpal-added groups on d 5 were significantly increased ($P < 0.05$, Figure 4A). In comparison with the DC group, serum IgM levels in the experimental group had an upward trend from d 5 to d 8, with a remarkable surge in serum IgM levels in the EO-PGS 4 kg/T group on d 5, as well as in the EO-PGS

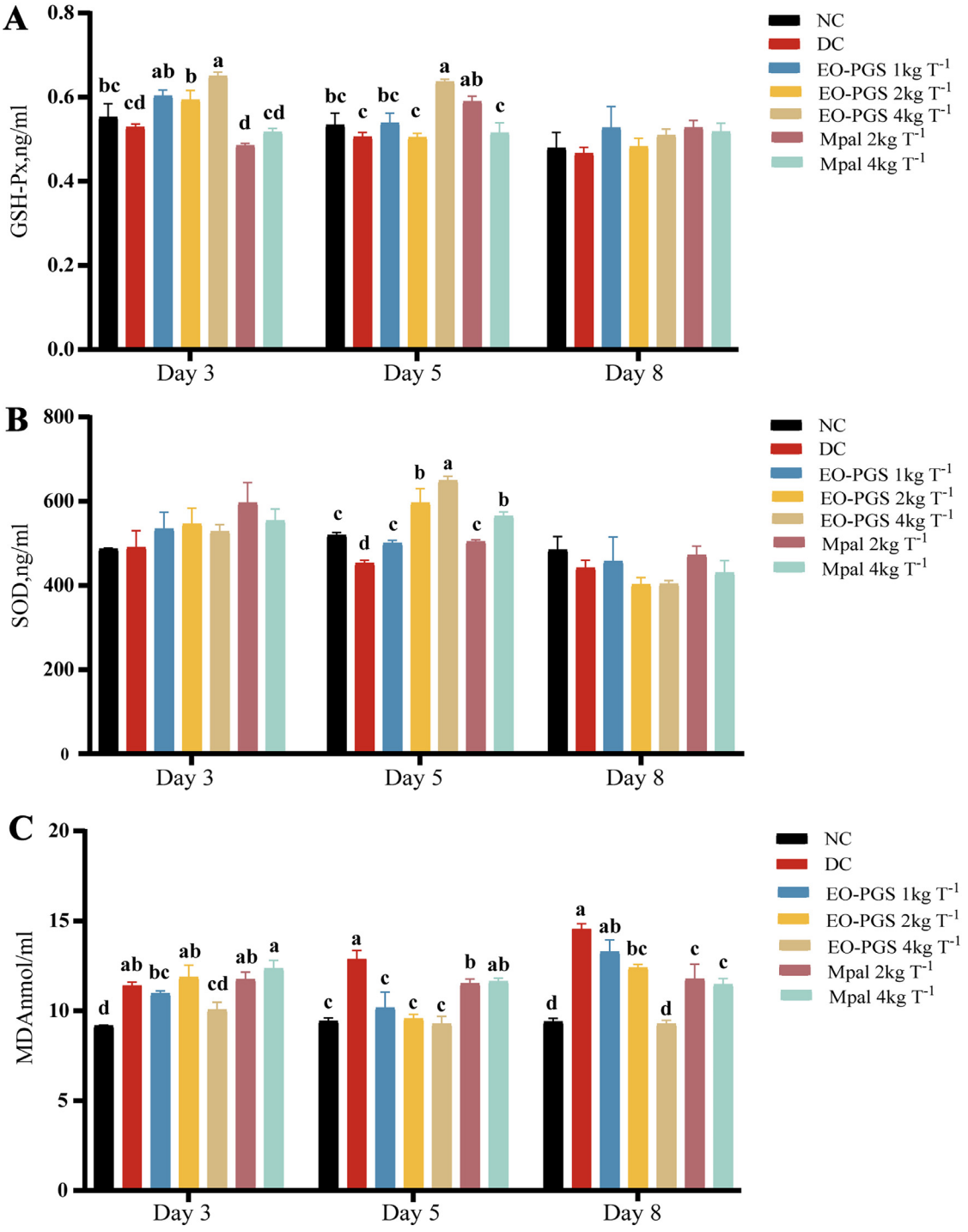

Figure 3. Effects of EO-PGS and Mpal on Serum antioxidant indexes of diarrhea broilers (n = 3). (A−C), the GSH-Px, SOD, MDA in the serum of diarrhea broilers.

2 kg/T and Mpal-added groups on d 8 ($P < 0.05$, Figure 4B). By contrast to the DC group, the serum C3 level in the EO-PGS-added group was considerably raised on d 3 ($P < 0.05$), and the serum C3 level in the experimental group appeared to be on the ascendant trend on d 5, with a pronounced heightening of the serum C3 level for both the EO-PGS 2 kg/T group and the EO-PGS 4 kg/T group ($P < 0.05$, Figure 4C). The serum C4 levels were significantly higher in the EO-PGS 2 kg/T and Mpal 4 kg/T groups on d 3 vs. the DC group ($P < 0.05$, Figure 4D).

Effects of EO-PGS and Mpal on Serum LPS Levels

Detection of serum LPS in broiler chickens reflected in Figure 5, the serum LPS content in the DC group was substantially higher than that in the NC group on d 3 to d 8 ($P < 0.05$), whereas that in the experimental group was remarkably weaker than that in the DC group on d 3 to d 8 ($P < 0.05$). The LPS content in the EO-PGS 2 kg/T group was the lowest on d 3 and d 8. The LPS content in the EO-PGS 2 kg/T group was significantly

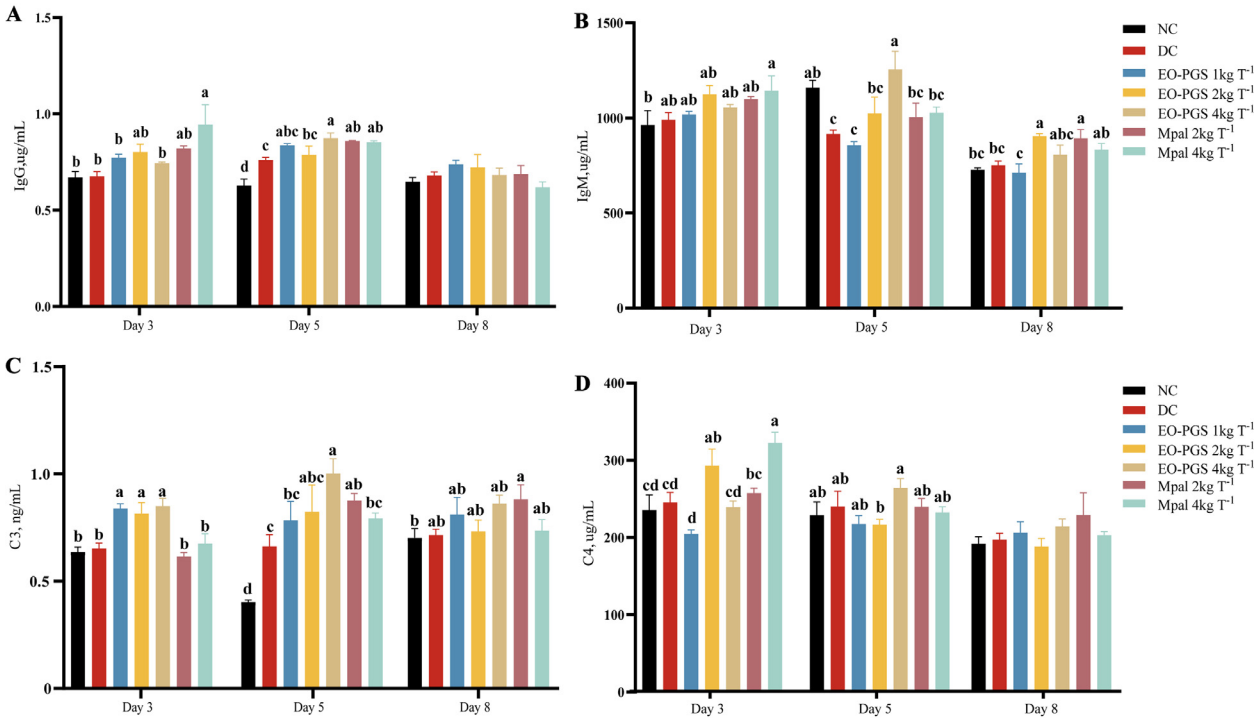

Figure 4. Effects of EO-PGS and Mpal on serum immunological indexes of diarrhea broilers (n = 3). (A), the IgG in the serum of diarrhea broilers; (B), the IgM in the serum of diarrhea broilers; (C), the C3 in the serum of diarrhea broilers; (D), the C4 in the serum of diarrhea broilers.

lower than that of the rest experimental groups on d 3 ($P < 0.05$).

Effects of EO-PGS and Mpal on Expression Levels of Duodenal Inflammatory Cytokines

Inflammatory cytokines in duodenal epithelial cells were detected using qPCR method (Figures 6A−D), indicating that the mRNA expression of *NF-kB, IL-1β, IL-6* and *TNF-α* in duodenum of broilers in the DC group was significantly higher than that in the NC control group on d 5 ($P < 0.05$). Compared to the DC group, the mRNA expression levels of *IL-1β, IL-6*, and *TNF-α* in the EO-PGS 2 kg/T group and EO-PGS 4 kg/T group were extremely reduced ($P < 0.05$). The mRNA expressions of *IL-1β* and *TNF-α* in the EO-PGS 4 kg/T group were not tremendously different from that of the NC group ($P > 0.05$). The mRNA expression of *IL-6* in the Mpal 2 kg/T group and the mRNA expression of *IL-1β* in the Mpal 4 kg/T group were significantly lower than that in the DC group ($P < 0.05$). The mRNA expression levels of *IL-1β* in the EO-PGS 2 kg/T group and the mRNA expression levels of *IL-1β* and *IL-6* in the EO-

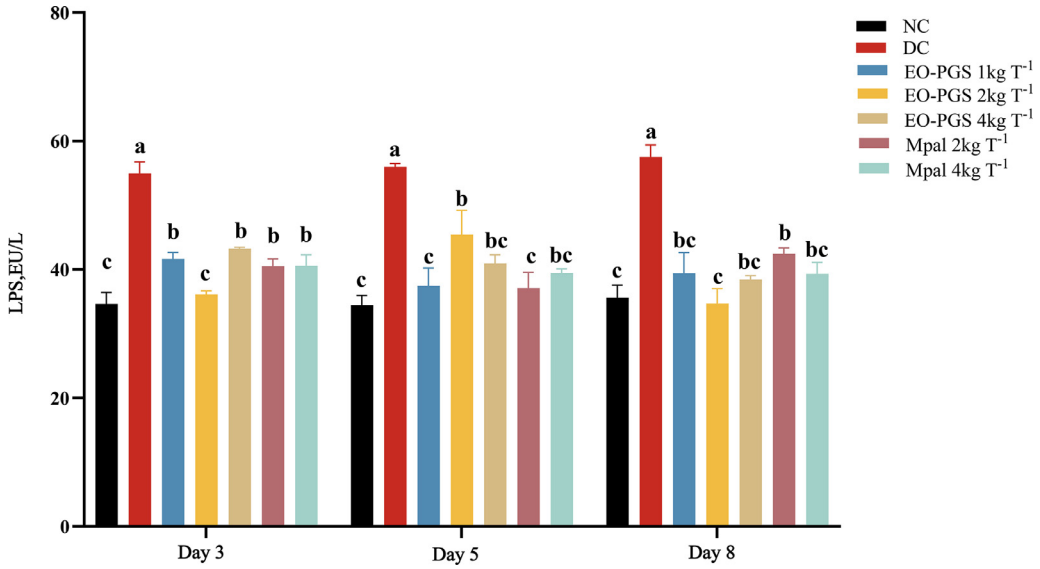

Figure 5. Effects of EO-PGS and Mpal on LPS levels in serum of diarrhea broilers (n = 3).

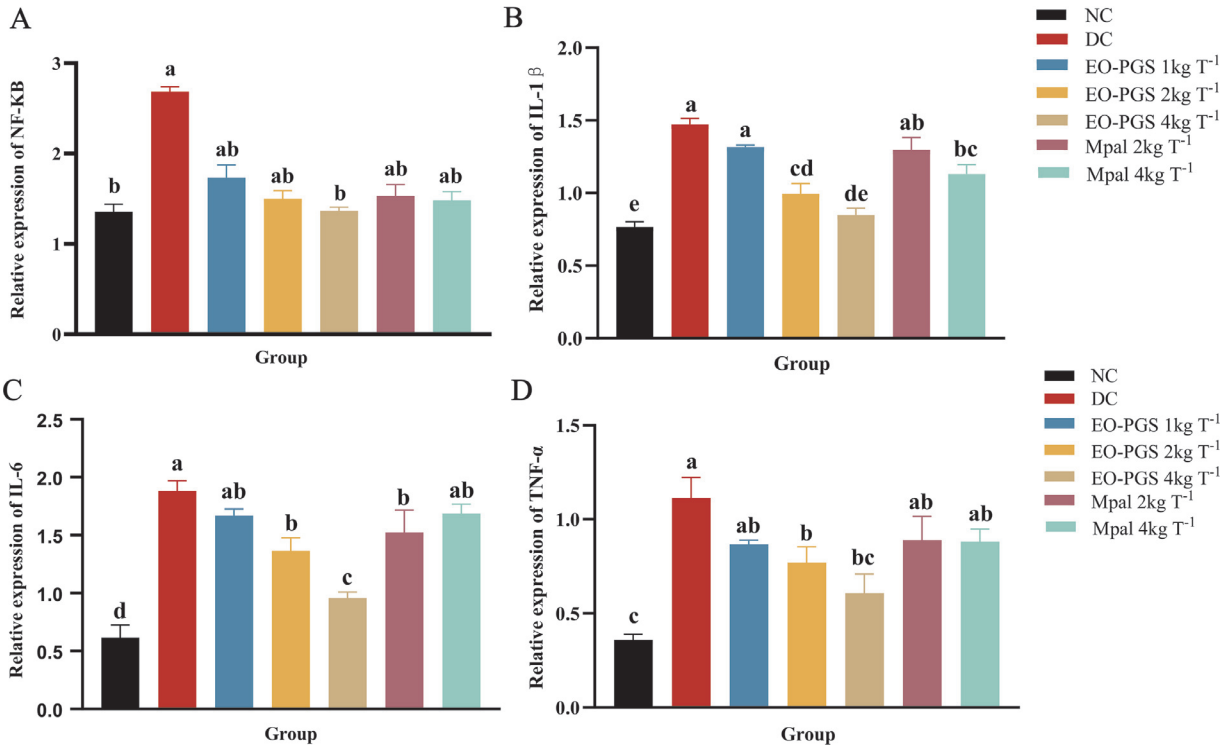

Figure 6. Effects of EO-PGS and Mpal on the mRNA expression of duodenal inflammatory cytokines in diarrhea broilers (n = 3). (A−D), the mRNA expression of *NF-Kb, IL-1β, IL-6, TNF-α* in the duodenum of diarrhea broilers.

PGS 4 kg/T group were drastically decreased than those in the Mpal-added groups ($P < 0.05$).

Effects of EO-PGS and Mpal on Immunohistochemistry of IL-6 and TNF-α

It was observed that the results of immunohistochemical detection (Figures 7A−C **and** 8A−C) demonstrated that the H-Score and positive cells of IL-6 and TNF-α in the DC group were remarkably enlargeder than those in the NC group ($P < 0.05$). H-score and positive cells of IL-6 tended to decrease on d 3 to 8 in the experimental group in contrast to the DC group. The H-Score and Positive cell of IL-6 in the Mpal 4 kg/T group were significantly lower than those in the DC group ($P < 0.05$), with no significant difference compared to the NC group on d 3 to d 5 ($P > 0.05$). Whereas the H-Score and Positive of IL-6 in the EO-PGS 2 kg/T group, the EO-PGS 4 kg/T group, and the Mpal 2 kg/T group were considerably lower than those in the DC group on d 8 ($P < 0.05$, Figures 7A−C). Compared with the DC group, the H-Score and Positive Cell of TNF-α in the Mpal-added groups on d 3 and Mpal 4 kg/T group and that of in the EO-PGS 1 kg/T group on d 5 and that of in the Mpal 2kg/T group on d 8 were significantly decreased ($P < 0.05$, Figures 8A−C). Therefore, it can be seen that protein expression levels were consistent with the trend of their mRNA expression of *IL-6* and *TNF-α* in the duodenal on d 5, and the expression on d 5 was higher than d 3 and d 8, indicating that the expression trend of inflammation in the diarrhea groups reached a peak at this time.

Effects of EO-PGS and Mpal on Cecal 16s rRNA Sequencing

The changes in the microbial content related to broiler chickens were examined using 16s rRNA sequencing, in order to explain whether the addition of different drug treatments has different impact. Compared with the DC group, the Simpson value, Shannon value and Pielou_e value of the EO-PGS 2 kg/T group were significantly increased, indicating that the microbial richness and alpha diversity of the microbiota were enhanced ($P < 0.05$) and the Chao1 value of the Mpal 4 kg/T group were increased, revealing that the microbial richness tended to increase, but there was no significant difference ($P > 0.05$). (Figure 9A). Principal co-ordinates analysis (**PCoA**) demonstrated that the microbial community composition between EO-PGS 2 kg/T NC, DC groups could be relatively easily distinguished, similar to Mpal 4 kg/T NC, DC groups (Figure 9B).

LEfSe analysis results (Figure 9C) showed that compared with NC and DC groups, *f_Clostridiaceae,* and *g_Coprococcus* were enriched in the caecum of diarrhea broilers in the EO-PGS 2 kg/T group ($P < 0.05$), and *o_Bacteroidales, f_Rikenllaceae* and *g_Peptococcus* were enriched in the caecum of diarrhea broilers in Mpal 4 kg/T group ($P < 0.05$).

At the phylum level (Figure 9D and Table 3), *Bacteroidete, Firmicutes,* and *Actinobacteria* were the dominant phyla in all groups, and the relative contents were 32.89 to 65.38%, 26.03 to 38.62%, and 2.95 to 10.73% respectively. Compared with the DC group, the relative abundance of *Bacteroidetes* and *Proteobacteria* in the remaining groups were significantly higher and lower

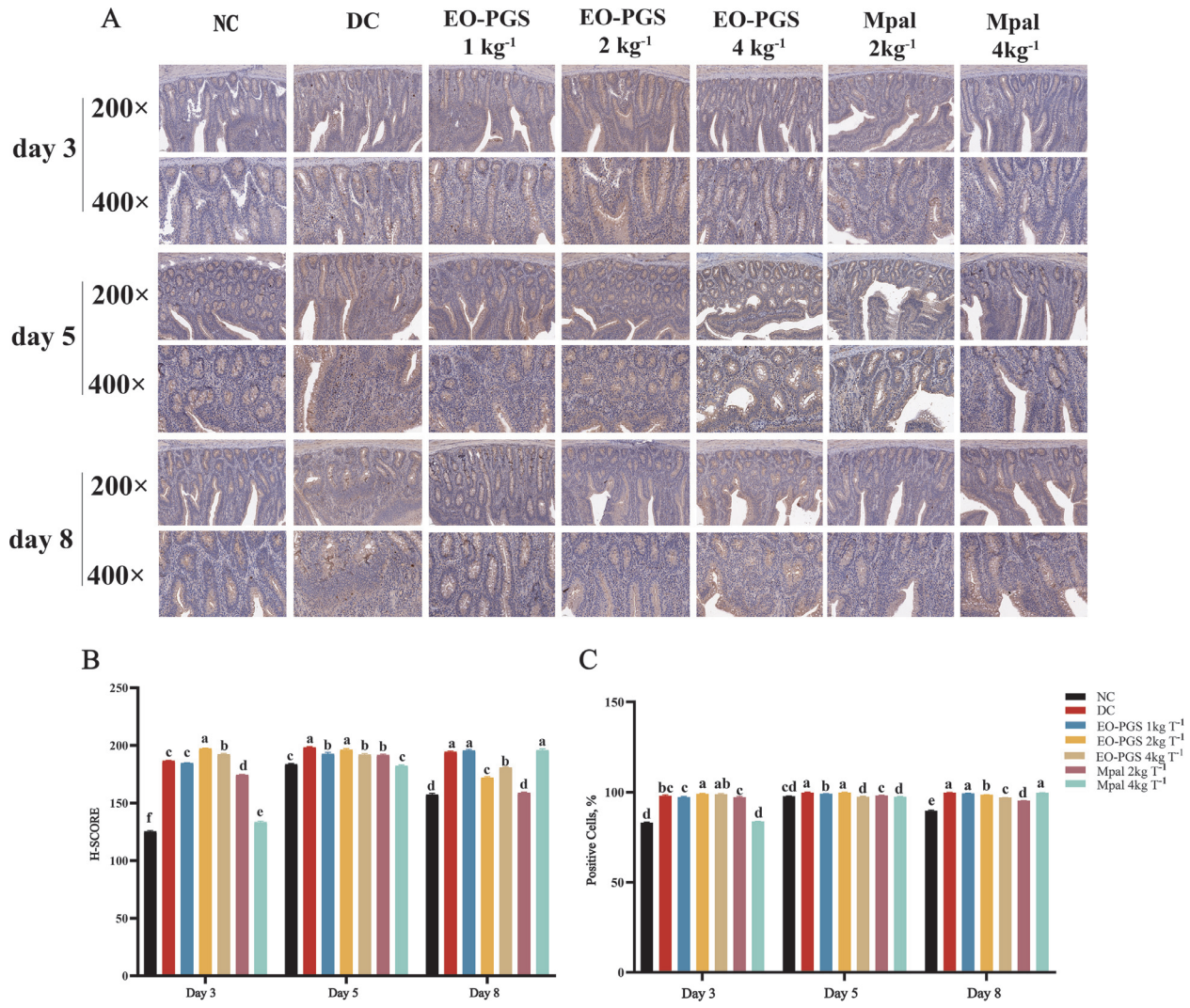

Figure 7. Changes in the immunohistochemistry of duodenal IL-6 in diarrhea broilers by EO-PGS and Mpal (n = 3). (A), IL-6 immunohistochemical staining image;(B), H-score results of quantitative IL-6 immunohistochemical analysis; (C) the result of positive cells of IL-6 immunohistochemical quantitative analysis.

respectively ($P < 0.05$), and there was no significant difference among the other groups ($P > 0.05$). At the genus level (Figure 9E and Table 4), the dominant genera were *Bacteroides* and *Ruminococcus*, with relative contents of 14.85 to 29.38% and 4.72 to 13.42%, respectively. Except for the NC and DC groups, the relative abundance of unnamed bacteria in the *Rikenellaceace* group was 1.71 to 7.79%. Compared with the DC group, the relative abundance of *Bacteroides* in the other groups tended to increase, but there was no significant difference ($P > 0.05$).

DISCUSSION

Broiler diarrhea is usually pathological diarrhea caused by the joint action of pathogenic microorganisms, often accompanied by enteritis, which reduces the growth performance of broilers (Zhang et al., 2022). In livestock and poultry farming, optimizing the prevention and control plan for diarrhea in broiler chickens is necessary, as the currently used dosage of ordinary Pal is too high (Zhang et al., 2017). In this study, we selected a low-dose optimized product to investigate the effect of diets supplemented with 1 to 4 kg/t of Mpal and EO-PGS on the intestinal health and disease resistance of broilers with natural diarrhea, and explored the optimal dosage and mechanism of action.

The present study showed that diarrhea broilers had a higher diarrhea index and lower average weight than normal broilers. Diets supplemented with Mpal and EO-PGS significantly reduced the diarrhea index, and dietary supplementation with 2 kg/t Mpal did not affect the average weight. However, adding 4 kg/t Mpal to the diet increased the average weight of diarrhea broilers, which was between that of diarrhea broilers and normal broilers, dietary EO-PGS supplementation increased the average weight of diarrhea broilers, which was higher than that of normal broilers. DuPont et al. (1990) found that Pal can effectively reduce the duration of unformed feces, promote fecal formation, and alleviate and treat diarrhea. In this study, the transition from mild diarrhea to soft stool occurred rapidly in broiler chickens supplemented with Mpal and EO-PGS, indicating that the

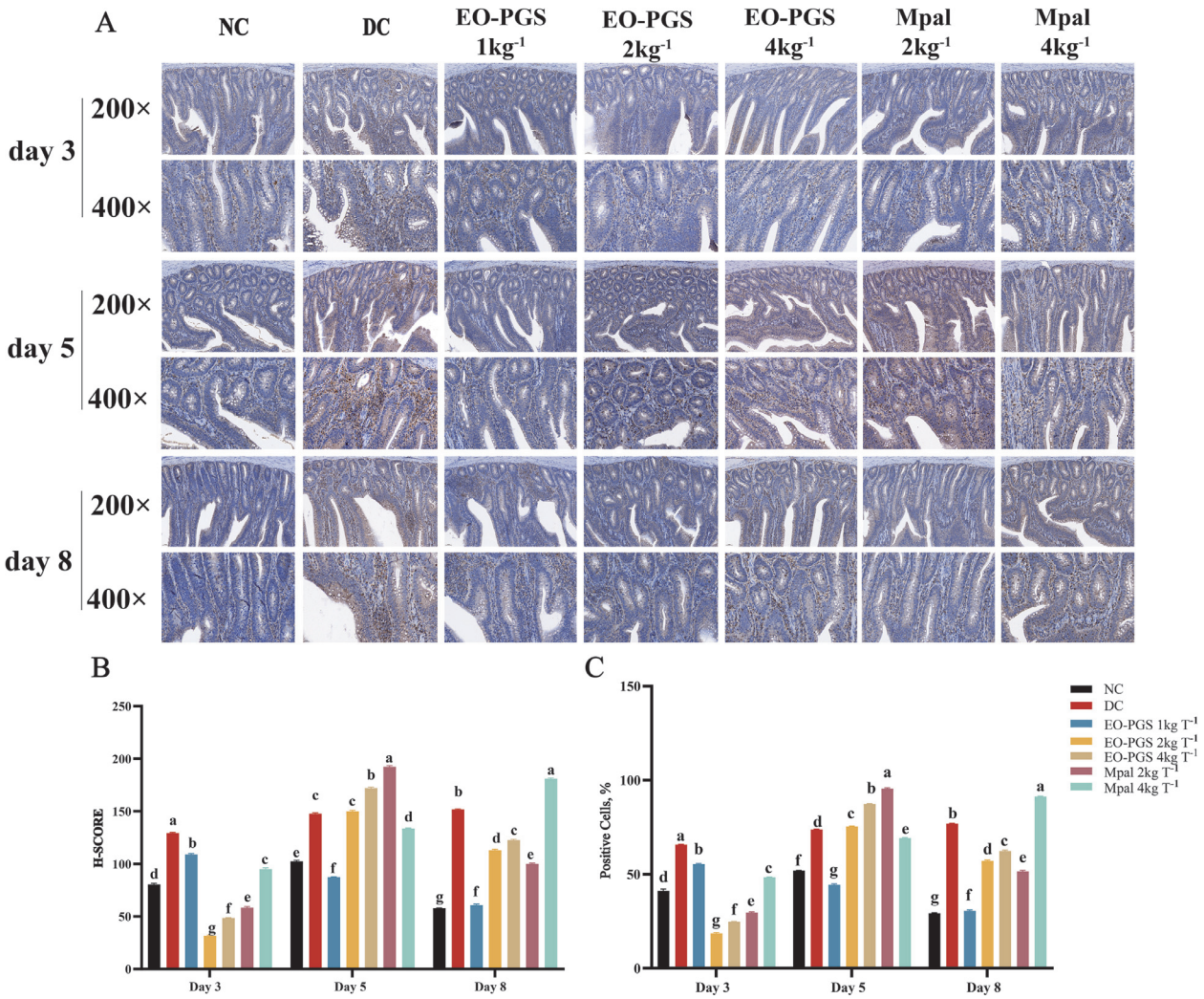

Figure 8. The immunohistochemical properties of EO-PGS and Mpal on duodenal TNF in diarrhea broilers (n = 3). (A), TNF-α immunohistochemical staining image;(B), H-score results of quantitative TNF-α immunohistochemical analysis;(C) the result of positive cells of TNF-α immunohistochemical quantitative analysis.

initial effect was mainly due to the effect of Mpal on apparent water absorption. The diarrhea index proceeded to decrease with time, between normal and soft stools, which may be related to the repair of intestinal damage. Combined with the results of duodenal intestinal sections, we found that the intestinal epithelial cells of diarrhea broilers were damaged and shed, goblet cells increased, inflammatory cells infiltrated, diffuse congestion and hemorrhage in lamina propria. The dietary addition of 2 kg/t Mpal did not improve intestinal damage. Dietary supplementation of 4 kg/t Mpal reduced diffuse hemorrhage and inflammatory cell infiltration in lamina propria and alleviated intestinal damage, and there was no significant difference from the NC group on d 8. Adding different levels of EO-PGS to the diet, the duodenal villi showed no significant damage. The number of goblet cells and, diffuse hemorrhage and inflammatory cell infiltration in the inherent layer were reduced, with the increase of dietary EO-PGS levels, intestinal injury was alleviated and intestinal structure recovered. The mucosal structure returned to normal on d 5 to d 8. It can be inferred that the intestinal damage in the diarrhea broiler may be related to average weight. Dietary supplementation of 2 kg/t Mpal reduced the diarrhea index, but had no significant effect on the alleviation of intestinal injury and average weight. Su et al (2018) previous studies showed that the addition of less than 2 kg/t of Mpal to the diet did not affect the average daily gain of broilers, which was similar to the results of this study. Ordinary Pal could promote average daily weight gain, but the additional amount needs to be 0.5%-1% (Chen et al., 2016; Cheng et al., 2016). Combined with the above studies, it is suggested that the average weight of broilers in diarrhea requires a higher dose of Mpal, while in this study 4 kg/t Mpal has an effect, and the additional amount is much lower than ordinary Pal.

Mpal alleviates intestinal damage probably through specially structured inorganic gels (Ruggeri et al., 2023), adheres to the gastrointestinal mucosa, effectively increases the barrier thickness, and adsorbs protons in gastric acid to release active substances such as Mg^{2+} and Al^{3+} to protect the gastrointestinal tract (Massaro et al., 2018). In addition, Pal might increase mucin 2 expression in the ileum of broilers (Chen et al., 2016). Accordingly, it was speculated that Pal could

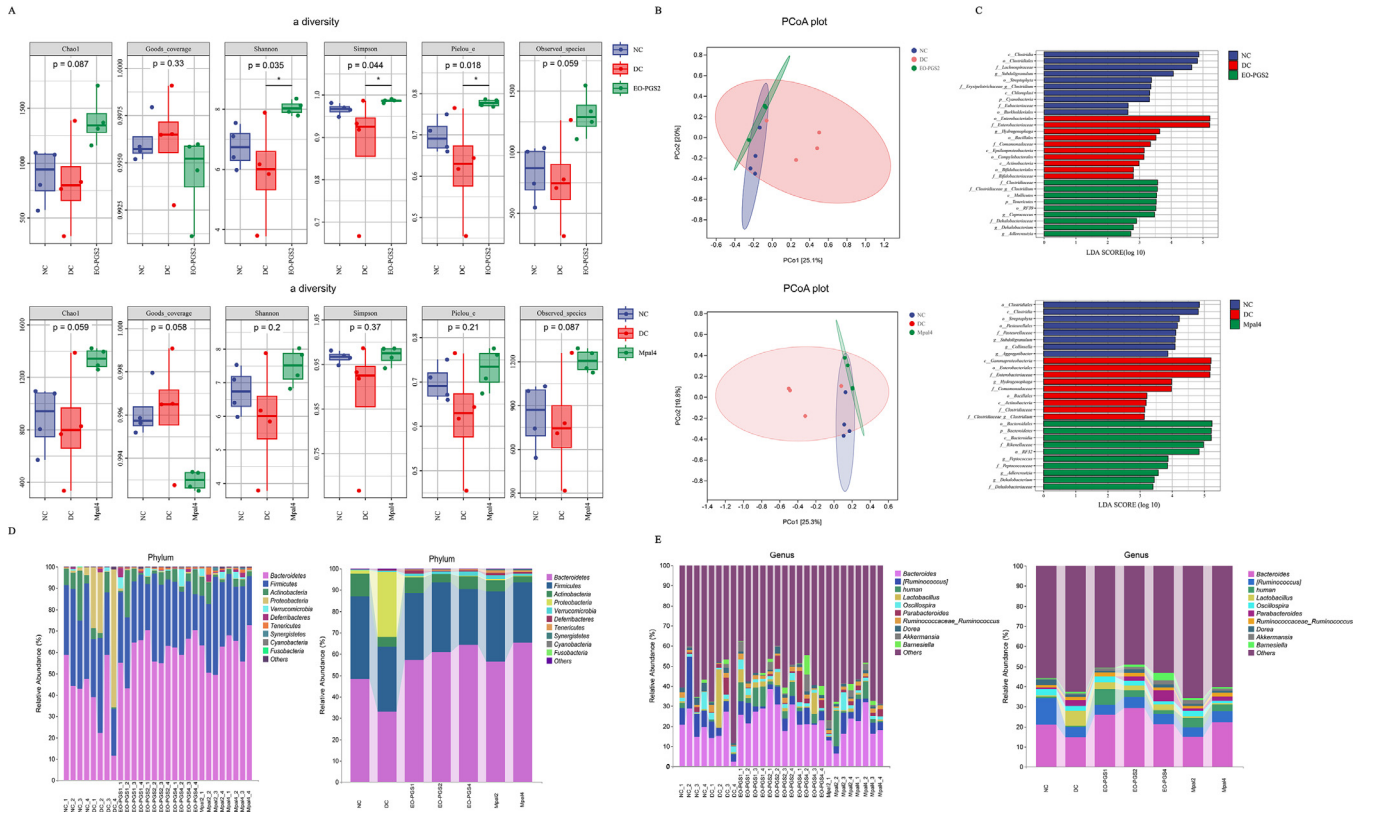

Figure 9. Effects of EO-PGS and Mpal on fecal microbiota composition of diarrhea broilers (n = 4). (A), alpha diversity; (B), beta diversity; (C), LEfSe analysis; (D), microbiota composition at phylum level; (E), microbiota composition at genus level. Date are shown as the mean ± SEM."*" represented ($P < 0.05$).

absorb liquid or exudate, contract protein tissue of intestinal mucosa injury site, have an astringent effect on the intestinal tract, and prevent further deterioration of injury, and it was related to additive dose. In this study, the addition of EO-PGS to the diet was more effective than Mpal in alleviating intestinal damage and increasing average weight in diarrheal broilers, possibly due to the combined effects of carvacrol and thymol with Mpal. Studies have shown that dietary supplementation of carvacrol and thymol improved intestinal morphology and function, helped to reduce cecal epithelial cell damage, reduced leukocyte infiltration with increased supplementation, improved cecal structural integrity, and promoted weight gain in broilers (Ibrahim et al, 2021; Hashemipour et al., 2013). However, to determine the actual effect of the product in broiler production, it is necessary to further explore the serum phase indexes related to intestinal inflammation damage and repair process. LPS, as a key indicator of inflammation, could destroy the morphological structure of the chicken intestine, change the expression of intestinal tight junction protein and increase intestinal permeability (Zhang et al., 2022; Feng et al., 2023; Tang et al., 2023). At the same time, serum IgG and IgM were 2 very important immunoreactive substances used to assess the immune status of broilers (Abdel-Moneim et al., 2022;

Table 3. Effects of EO-PGS and Mpal on the relative abundance of fecal microbiota composition at the phylum level (the top 10).

	EO-PGS and Mpal supplemental level/(kg/T)								
Items	NC	DC	EO-PGS 1	EO-PGS 2	EO-PGS 4	Mpal 2	Mpal 4	SEM	*P*-value
Bacteroidetes	48.33^{a}	32.89^{b}	57.08^{a}	60.87^{a}	64.38^{a}	56.39^{a}	65.38^{a}	2.67	0.004
Firmicutes	38.62	30.65	31.39	32.68	26.03	32.83	28.00	1.43	0.445
Actinobacteria	10.73	4.47	7.46	3.71	5.86	5.36	2.95	1.08	0.576
Proteobacteria	1.61^{b}	30.459^{a}	0.48^{b}	0.24^{b}	0.34^{b}	0.46^{b}	0.25^{b}	2.61	0.002
Verrucomicrobia	0.26	0.45	1.30	1.28	2.10	1.76	1.13	0.38	0.896
Deferribacteres	0.2	0.46	1.41	0.09	0.51	1.16	0.79	0.20	0.600
Tenericutes	0.02	0.17	0.48	0.73	0.49	1.24	0.84	0.13	0.195
Synergistetes	0.00	0.00	0	0.06	0.06	0.39	0.27	0.06	0.478
Cyanobacteria	0.01	0.00	0.01	0.00	0.00	0.04	0.01	0.00	0.126
Fusobacteria	0.03	0.01	0	0	0.00	0.00	0.00	0.00	0.108
Others	0.20	0.46	0.38	0.34	0.24	0.37	0.38	0.05	0.819

Different lowercase letters (such as a, b) indicate that there are significant differences in the comparison of different groups ($P < 0.05$), SEM, standard error of the means (n = 4), the same as below.

Table 4. Effects of EO-PGS and Mpal on the relative abundance of cecal microbiota composition at the genus level (the top 10).

Items	EO-PGS and Mpal supplemental level/(kg/T)							SEM	*P*-value
	NC	DC	EO-PGS 1	EO-PGS 2	EO-PGS 4	Mpal 2	Mpal 4		
Bacteroides	20.94	14.77	25.77	29.38	21.26	14.85	22.12	1.48	0.059
[Ruminococcus]	13.42	5.20	5.11	5.45	5.19	4.73	5.58	0.93	0.122
Human	0.69	0.39	7.79	3.29	1.71	4.7	3.34	0.82	0.197
Lactobacillus	0.43	7.72	3.34	2.41	2.88	0.72	0.50	1.08	0.609
Oscillospira	3.14	2.31	2.86	2.27	1.52	3.04	1.53	0.39	0.889
Parabacteroides	0.83	2.69	0.08	2.23	5.58	0.91	1.97	0.63	0.334
Ruminococcaceae_Ruminococcus	1.27	1.65	2.08	1.81	1.38	1.35	1.89	0.15	0.777
Dorea	2.8	1.36	0.84	1.62	1.60	1.12	0.95	0.18	0.061
Akkermansia	0.26	0.45	1.3	1.28	2.1	1.76	1.13	0.38	0.896
Barnesiella	0.44	0.74	0.24	1.15	3.59	1.1	0.77	0.39	0.314
Others	55.8	62.71	50.59	49.1	53.2	65.71	60.24	2.19	0.325

Mockett, 1986). In the present study, serum LPS levels of broilers were determined, and the serum LPS content in diarrhea broilers was significantly higher than that of normal broilers. Moreover, serum IgG and C3 levels of diarrhea broilers were significantly higher, serum IgM levels and SOD activity were significantly lower and serum on d 5, serum MDA content was significantly higher during the test period than that of normal broilers. It indicated that intestinal inflammatory injury occurred at this time, and oxidative stress and immunosuppression might occur. Previous research has also confirmed that *E.coli* can increase the serum LPS levels and decrease serum IgM levels in the broiler (Wu et al., 2021), and LPS stimulation significantly decreased serum IgM levels, and serum IgG levels had a tendency to increase (Shi et al., 2022). The present study showed that diets supplemented with Mpal and EO-PGS significantly reduced serum LPS levels in diarrhea broilers on d 3, and there was no significant difference in serum LPS content from normal broilers on d 5 to d 8. It suggests that dietary supplementation of Mpal and EO-PGS at different levels could regulate resistance to pathogens and LPS, reduce intestinal irritation and reduce intestinal permeability to normal levels. Similar studies have shown that Pal reduced plasma LPS levels in weaned piglets and broilers (Zhang et al., 2013; Zha et al., 2022). However, there was no difference in the effect of reducing serum LPS content between the supplementation groups in this study, suggesting that EO-PGS may not only reduce pathogenic stimulation in the intestine, but also promote the recovery of intestinal injury through the antioxidant, immune function and anti-inflammatory effects of carvacrol and thymol. Dietary supplementation of Mpal and EO-PGS resulted in a tendency for serum IgG and IgM to lessen and remain at high levels, Diets supplemented with 4 kg/t Mpal and 2 kg/t EO-PGS significantly declined serum C4 levels on d 3. Diets supplemented with 2 kg/t Mpal and 4 kg/t EO-PGS significantly increased serum C3 levels on d 5. C3 and C4 have systemic antibacterial defense in the blood and have a potential role in regulating intestinal barrier integrity (Sina et al., 2018), which was also reflected in this study, the addition of EO-PGS and Mpal improved immunity in broilers with diarrhea, dietary supplementation with EO-PGS and Mpal improved immunity in broilers with diarrhea. Several studies have confirmed that diets supplemented with 1 to 2% Pal can significantly increase IgM content in the ileum of broilers (Chen et al., 2016). Dietary supplementation with 0.75 kg/t-1 kg/t EO-PGS supplementation significantly increases serum IgG levels in laying hens (Cheng et al., 2022). This is similar to the results of this study, but there is a certain difference in the dosage of the additives. Therefore, we need to further examine the antioxidant capacity of this product.

Antioxidant capacity and immunity indicate the body's resistance to external environments, which is particularly important for the health of livestock and poultry. Serum SOD activity and GSH-Px activity are key antioxidant enzymes that eliminate reactive oxygen species in cells, while serum MDA is the final product of lipid peroxidation (Fan et al., 2015). Adding EO-PGS to the diet significantly increased the serum GSH-Px and serum SOD activity in broiler chickens with diarrhea, with the most significant effect observed at 4 kg/EO-PGS, followed by the same effect from adding 4 kg/t Mpal and 2 kg/t EO-PGS on d 3 and d 5. Dietary EO-PGS supplementation decreased MDA content, which was lower than that in the diet with Mpal on d 5. Dietary Mpal supplementation decreased MDA content and had the same effect as the dietary addition of 2 kg/t EO-PGS. The trend and duration of action of the addition of EO-PGS and Mpal on SOD activity and MDA were consistent with the results of intestinal biopsy, indicating that there was some association. Previous studies confirmed that Pal significantly increased T-SOD activity and decreased MDA content of jejunal mucosa in the jejunum, ileum and serum of broilers (Chen et al., 2016; Xu et al., 2018). Dietary Pal supplementation tended to increase SOD and decrease MDA in broilers breast and thigh, but not significantly (Cheng et al., 2016). These results indicated that Pal could improve the antioxidant capacity of different tissues in broilers. EO-PGS has a better antioxidant effect than Mpal at the same dose, which may be due to the synergistic effect of plant essential oils. Carvacrol could increase the SOD and GPx activities (Ismail et al., 2022), and the complex of thymol and carvacrol as the main components could increase the GSH-Px, T-SOD activities and decreased MDA levels of leg muscles

(Hashemipour et al., 2013), in which phenolic compounds are potent antioxidants whose activity is related to the position and number of hydroxyl groups in their specific chemical composition (Abd El-Hack et al., 2023), which can not only scavenge free radicals but also provide hydrogen to peroxyl radicals in the process of lipid oxidation to prevent the generation of peroxidation hydroxyl radicals, thereby blocking lipid peroxidation chain reactions, and can also regulate the GPx and SOD activities (Fraga, 2007; Quideau et al., 2011).The balance between antioxidant capacity and oxidative stress can regulate inflammatory response and play an important role in maintaining the process of homeostasis (Berger and Chioléro, 2007).

Inflammatory response is an important immune response that plays a key role in the containment of microorganisms and the repair of tissue damage (Broom and Kogut, 2018). Cytokines such as IL-1β, IL-6, TNF-α, and NF-κB produced by immune cells predominantly exert pro-inflammatory effects, triggering chronic intestinal inflammation and tissue damage, and promoting the ongoing progression of the disease (Neurath, 2024), and dominate in the early stages (Nakase et al., 2022). Wu et al. (2021) showed that *E. coli* O157 as a pathogen can significantly increase the serum LPS levels in broiler chickens. Intestinal LPS act as pathogen-associated molecular patterns (**PAMPs**) to initiate immune response and induce inflammatory response after binding to host Toll-like receptors (**TLRs**), up-regulate the expression levels of inflammatory factor-related genes such as intestinal *TLR4/NF-kB*, and increase the expression of intestinal *IL-1β, IL-6, TNF-α*, and *NF-κB*, and the serum levels of IL-6, and IL-1β in broilers (Cui et al., 2021; Shi et al., 2022). To relieve inflammatory bowel disease, downregulating or inhibiting the expression of major inflammatory cytokines is a common treatment strategy (Zhang et al., 2019; Tong et al., 2023). This study shows that the expression of inflammatory cytokines *IL-1β, IL-6, TNF-α*, and *NF-κB* in the duodenal epithelial cells of diarrhea broilers on d 5 was significantly upregulated, compared to normal broilers, indicating inflammatory damage in the intestines of diarrhea broilers. Dietary supplementation with 2 to 4 kg/t EO-PGS significantly down-regulated the expression of relevant inflammatory cytokines, which helped to restore them to the level of normal broilers. Dietary supplementation with 2 to 4 kg/t Mpal significantly down-regulated the expression levels of *IL-6* and *IL-1β* mRNA, respectively. The addition of 4 kg/t EO-PGS to the diet significantly reduced the expression levels of *IL-1β* and *IL-6* mRNA compared to the diet with Mpal, The addition of 2 kg/t EO-PGS to the diet significantly reduced the expression levels of *IL-1β* mRNA compared to the diet with 2 kg/t Mpal. Meanwhile, immunohistochemical results indicated that the expression levels of IL-6 and TNF-α proteins in the duodenal epithelial cells of diarrheic broilers were consistent with the mRNA expression trends. Therefore, adding MPal and EO-PGS to the diet can exert anti-inflammatory effects by downregulating the expression levels of inflammatory factors in the intestinal cells of diarrheic broilers, and for a certain period, the anti-inflammatory effect of EO-PGS may be superior to that of MPal.

Previous studies have shown that adding 5 kg/t Pal to the diet can reduce the concentration of TNF-α in the jejunum and ileum of *E. coli* challenged broilers, but the concentrations of IL-1β and IL-6 were not affected (Tan et al., 2024). Dietary supplementation with 5 to 10 kg/t Pal tends to reduce *IL-1β* mRNA expression in the jejunum and ileum of normal broilers, but not significantly (Chen et al., 2016). In addition to Mpal, EO-PGS also contains plant essential oils, and carvacrol can inhibit the secretion of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and NF-kB p65 in the small intestine caused by LPS, and affect the anti-inflammatory function of the TLRs/NF-kB pathway (Liu et al., 2019). Thymol and organic acid compounds downregulate the expression of *IL-1β* and *TNF-α* in the jejunum of broiler chickens (Bialkowski et al., 2023). EO-PGS combined with the adsorption of bacteria of Pal and the inhibited bacteria of essential oils to work synergistically to inhibit the expression of inflammatory cytokines, such as *TNF-α*, and *IL-6* alleviated intestinal inflammation (Xu et al., 2020). Combined with the results of LPS in this study, there was no significant change in serum LPS concentration from 3 to 8 d, which may be directly related to pathogenic bacteria and LPS concentration in the intestinal. To explore the mechanism of diarrhea, 16S rDNA diversity of intestinal microorganisms in cecum contents was sequenced.

Gut microbial composition interacting with host gene expression plays an important role in regulating host nutrient absorption, metabolism, immune system development and intestinal inflammation (Chu et al., 2016; Iweala and Nagler, 2019; Yin et al., 2023). Thus, this study conducted 16s rRNA sequencing, and the results indicated that the addition of 2 kg/t EO-PGS and 4 kg/t Mpal allowed for a relatively easy distinction between the cecal microbial community composition of diarrhea broilers and normal broilers. Dietary supplementation of 2 kg/t EO-PGS increased microbial richness and α diversity. Previous studies have shown that the natural flora of the broiler intestine is relatively constant under natural conditions, but there will be certain differences in the order of colonization and dominant flora in different growth stages such as *Firmicutes, Bacteroidetes, Proteobacteria, Actinobaceria*, etc., in the cecum of chicken (Videnska et al., 2014; Yang et al., 2020). Our study results showed that the dominant bacterial groups in the cecum of broilers were *Bacteroidetes, Firmicutes*, and *Actinobacteria* in all groups, broiler diarrhea corresponds to a significant decrease in the abundance of *Bacteroidetes* and a significant increase in the abundance of *Proteobacteria* in the cecum on d 8. Inflammatory states were more favorable for the growth of proteobacteria (Ni et al., 2017), accompanied by changes in the ratio of *Firmicutes* and *Proteobacteria* in the intestinal microbiota, with an increase in pathogenic bacteria such as *E.coli*, a decrease in *Clostridium leptum groups*(Matsuoka and Kanai, 2015). Previous studies

have confirmed that the diarrhea status of piglets is negatively correlated with the abundance of *Bacteroides* and positively correlated with the abundance of *Proteobacteria* (Zhang et al., 2020; Ren et al., 2022). This study shows that the addition of Mpal and EO-PGS to the diet significantly increases the relative abundance of *Bacteroidetes* in diarrheal broilers while significantly decreasing the relative abundance of *Proteobacteria*, with advantages at 4 kg/t Mpal and 2 kg/t EO-PGS. LEfSe analysis results showed that compared with diarrhea broilers and normal broilers, *f_ Clostridiaceae* and *g_ Coprococcus* were enriched in diarrhea broilers in the diet supplemented with 2 kg/t EO-PGS, while *o_ Bacteroidales, f_ Rikenllaceae* and *g_ Peptococcus* were enriched in diarrhea broilers in the diet supplemented with 4 kg/t Mpal, and *f_ Enterobacteriaceae, o_ Campylobacterales, c_ Gammaproteobacteria*, and *o_ Enterobacteriales* were enriched in diarrhea broilers. Research indicated that Pal can inhibit the colonization of *E. coli* and *Salmonella* in the cecum of broiler chickens (Chen et al., 2016; Tan et al., 2024). It was suggested that Pal may have adsorption and inhibition effects on *E. coli* and *Salmonella* in the caecum of broilers. Thymol, the major plant essential oil in EO-PGS, can regulate the intestine by changing the number of *Lactobacillus* and *Salmonella typhimurium* in the cecum of the broiler (Ibrahim et al., 2021). Thymol and carvacrol-based essential oil compounds have antimicrobial effects on *E.coli* and *Salmonella* (Du et al., 2015). Pal loaded with carvacrol has higher antibacterial activity against *E. coli* than carvacrol alone (Tenci et al., 2017). And the abundance of anaerobic bacteria, *Bacteroides* mainly promotes the metabolism of energy substances such as polysaccharides and lipids in nutrient metabolism, and can also inhibit inflammatory reactions and abscesses, and protect the intestine (Chu et al., 2016; Chen et al., 2020). *Rikenellaceae* was generally considered a beneficial bacterium, positively correlated with lipid metabolism in broilers (Chen et al., 2020). Its increased abundance can reduce the adverse effects associated with duck inflammatory bowel disease (Liu et al., 2023). These probiotics play a role in intestinal health. However, further research is needed on how specific flora regulates diarrhea.

CONCLUSIONS

In conclusion, dietary supplementation of Mpal and EO-PGS could improve intestinal barrier integrity, increase average body weight and reduce diarrhea by changing intestinal microbial composition, regulating intestinal flora, increasing Bacteroides abundance, reducing proteobacteria abundance, inhibiting pathogenic bacteria and LPS, reducing intestinal inflammation stimulation, and improving disease resistance. It was recommended that 4 kg/t Mpal would be added to broiler production to prevent diarrhea, and 2 kg/t EO-PGS be added to broilers with mild diarrhea for no less than 5 d.

ACKNOWLEDGMENTS

This study was funded by the Natural Science Foundation of Hunan Province-China (2020JJ4364), and the Science and Technology Innovation Program of Hunan Province (2024RC8258). Thanks to the support and help from the College of Animal Science and Technology, Hunan Agriculture University and Yuelushan Laboratory.

Author Contributions: Q. L. Yang, L. Yang, X. Y. Qu, and D. F. Xiao were responsible for the project design. Q. L. Yang and L. Yang performed the experiment, sample collection, data analysis and prepared the manuscript. X. Y. Qu, and D. F. Xiao coordinated the conduct of the experiment and sample collection, and revised the manuscript with Q. L. Yang. All authors approved the final manuscript.

DISCLOSURES

The authors declare no conflicts of interest.

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