Effect of benzoic acid, *Enterococcus faecium*, and essential oil complex on intestinal microbiota of laying hens under coccidia and *Clostridium perfringens* challenge

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ABSTRACT The objective of this study was to investigate whether dietary supplementation with benzoic acid, *Enterococcus faecium*, and essential oil complex (**BEC**) could help laying hens recover from coccidia and *Clostridium perfringens* type A challenge. A total of 60 (35-wk-old) Lohmann-laying hens were randomly assigned to 3 experimental groups (10 replicates with 2 hens per replicate): I) control group (**CON**), II) challenge group (**CC**), and III) BEC group (2,000 mg/kg BEC). The total experimental period was 8 wk. The results shown that the challenge layers had lower egg-laying rate and average daily feed intake (**ADFI**) ($P < 0.05$), and addition of BEC after challenge increased egg-laying rate ($P < 0.05$). The content of propionic acid (**PA**) and butyric acid (**BA**) in short-chain fatty acid (**SCFA**) was significantly decreased by challenge ($P < 0.05$). CC and BEC groups had lower villus height to crypt depth ratio (**V/C**) and higher pathological scores in duodenum ($P < 0.05$), whereas the BEC group had lower pathological scores in jejunum when compared with the CC group ($P < 0.05$). The challenge increased the concentration of proinflammatory cytokines (IL-1β and IL-6) ($P < 0.05$). An increase in the abundance of *Bacteroidoes* (genus), *Bacteroidaceae* (family), *Bacteroidoes* sp. *Marseille P3166* (species), *Bacteroidoes caecicola* (species) was observed in the CC group, whereas the BEC group had higher abundance of *Bacteroides caecigallinarum* (species). The genera *Faecalibacterium* and *Asterolplasma* were positively correlated with egg-laying rate ($r = 0.57$, 0.60; $P < 0.01$); and the genera *Bacteroides* and *Romboutsia* were negatively correlated with egg-laying rate ($r = -0.58$, -0.74; $P < 0.01$). The genera *Bacteroides, Lactobacillus*, and *Rombutzia* were positively correlated with jejunal mucosa proinflammatory factor IL-1β level ($r = 0.61$, 0.60, 0.59; $P < 0.01$), which were negatively correlated with genera *Rikenbacteriaceae RC9, Faecalibacterium*, and *Olsenlla* ($r = -0.56$, -0.57, -0.61; $P < 0.01$). There genera UCG.005 was positively correlated with proinflammatory factor IL-6 level in jejunal mucosa ($r = 0.58$; $P < 0.01$), which was negatively correlated with *Rikenbacteriaceae RC9* ($r = -0.62$; $P < 0.01$). The experiment results revealed that the addition of BEC to the diet restored the production performance of the laying hens. In addition, supplementation of 2,000 mg/kg BEC modulated gut health by reducing gut damage scores and modulating microbial composition, thereby promoting recovery of laying hens after coccidia and *Clostridium perfringens* challenge.

Key words: benzoic acid, intestinal health, proinflammatory factor, modulating microbial composition

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INTRODUCTION

For decades, the necrotic enteritis (**NE**) induced by *Clostridium* (*C.*) *perfringens* and coccidia had caused great economic loss in the global poultry industry (Wade and Keyburn, 2015). NE is one of the most important enteric diseases in poultry as it impaired performance and food safety, in which coccidia and *C. perfringens* are 2 important factors of this disease (Biggs, 1985). In general, *C. perfringens* is a commensal bacterium in the intestine, which does not develop disease under normal circumstances. The pathogenic process of the bacteria requires inducers, such as special dietary composition, immune suppression, intestinal machinery damage, and intestinal flora disturbance, etc. Coccidia can parasitize in the intestinal epithelium, thereby causing intestinal mucosa destruction and immune response,

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creating conditions for the colonization of *C. perfringens*, and continuously promoting the development of NE (Immerseel et al., 2008). Adding antibiotics to the diet is an effective method to control NE in poultry. However, some studies have shown that with the prohibition of the use of antibiotics, the incidence of NE caused by *C. perfringens* of broilers has increased, which could lead to the production performance have a sharp decline. And the studies have shown that the diarrhea and mortality of piglets due to *Escherichia coli* infection has increased (Mark et al., 2003; Dahiya et al., 2006). Thereby, the development of new antibiotic alternatives is essential for the treatment of NE. In recent years, organic acids, essential oils (**EOs**), probiotics, and other feed additives have been widely used in promoting livestock production performance and protecting intestinal health. Kaya et al. (2014) shown that the addition of organic acids has a positive effect on the performance and egg quality of laying hens. Further research shown that an appropriate amount of benzoic acid improved egg quality and intestinal morphology, and promoted the gastrointestinal health of laying hens (Gong et al., 2021). Several studies have demonstrated that EOs may improve laying performance and health status by its anti-inflammatory, anthelmintic, antimicrobial, and antioxidant properties as well as stimulation of digestive secretions and immune modulation. And it reported that Eos, such as thymol and carvacrol, can destroy the structure of coccidia oocysts and inhibit the growth of both gram-positive and gram-negative bacteria (Remmal et al., 2011; Achahbar et al., 2012; Du et al., 2015). In addition, previous studies have shown that *Enterococcus faecium* (***E. faecium***) significantly improved production performance and nutrient digestibility of laying hens and breeders (Park et al., 2016; Zhao et al., 2019; Wang et al., 2021a). And Placha et al. (2010) believed that *E. faecium* as a probiotic could neutralize the negative effects of EOs on the intestinal integrity of laying hens. Our previous study found that the dietary supplementation of BEC could effectively alleviate the stress of laying hens challenged by coccidia and *C. perfringens* (Zhang et al., 2022). Then whether higher level of BEC can recover its stress damage, further research is needed. Therefore, the purpose of this study was to investigate whether the dietary supplementation with the high level of BEC help laying hens recover more quickly from coccidia and *C. perfringens* challenged.

MATERIALS AND METHODS

Chemicals, Pathogen, and Bacterial Strain

The commercial BEC product used contained 70% of benzoic acid (99.5% of purity), 5% of EO (thymol:carvacrol = 1:1), 5% of *Enterococcus faecium* (**EF**, 2×10^8 CFU/kg diet), and 20% of its own carrier (50% silica and 50% dextrin) from DSM (DSM Nutritional Products Inc., Shanghai, China).

The avian coccidiosis quadrivalent live vaccine (provided by the Foshan Standard Biotech Co., Ltd., Guangdong, China), containing *Eimeria tenella, Eimeria poisonous, Eimeria acerola*, and *Eimeria giant*. The chicken *C. perfringens* was purchased from the China Veterinary Drug Administration (CVCC2030). After activation, it was inoculated into a sterile thioglycolate liquid medium at a volume ratio of 2% and cultured in a sterile incubator at 37°C for 24 h.

Experimental Birds, Management, and Diets

At 35 wk of ages, a total of 60 Lohmann gray hens were randomly assigned to 3 experimental groups including 10 replicates with 2 hens per replicate. I) Control group, basal diet; II) challenge group, from the sixth week (d 42−48), the hens form the challenge group and BEC group were treated with 80-fold anticoccidia vaccine (55,000 coccidia sporangia/mL/hen) via oral gavage and 40 mL of *C. perfringens* (2.5×10^{10} CFU/mL) via mix into feed individually; III) BEC group (dietary supplementation of 2,000 mg/kg BEC complex after challenge). The CON group sterile phosphate-buffered saline was administered instead. The challenge was performed at every day and lasted a week. All hens were housed individually in an environmentally controlled room where temperature was maintained at approximately 20°C to 22°C and artificial light by a daily lighting schedule of 16 h light and 8 h dark (challenged and unchallenged were kept in 2 separate room with the same facilities and equipment). Hens were given free access to water and a complete feeding mixture in mash form, the experimental diets meet the National Research Council (Kim, 1994) requirements, as shown in Supplementary Table 1.

Productive Performance and Sample Collection

After the challenge, egg numbers in each replicate were recorded from d 49 to d 62. Egg production was calculated as the average production per day.

On d 63, 8 hens/treatment were randomly selected to slaughter and collect samples. The hens were sacrificed by cervical dislocation. The middle of the duodenum and jejunum segments (about 3 cm) was fixed in 4% paraformaldehyde for mucosal morphology. Then, the intestine tissues (duodenum, jejunal) and cecum chyme were taken and then stored at −80°C till gene expression analysis.

Intestinal Morphology Analysis

Duodenum and jejunal mucosa morphology were analyzed as described previously (Yang et al., 2020; Gong et al., 2021). Briefly, following fixing in 10% paraformaldehyde, the intestinal segments were embedded in paraffin and sectioned (the section thickness was 3 μm), then stained with hematoxylin and eosin. The middle of duodenum, jejunum, and ileum (1 × 2 cm) under an optical microscope to collect images (Microscope:

NIKON Eclipse ci, imaging system: NIKON digital sight DS-FI2, MADE in Japan). Observed the villus height and crypt depth, calculated the V/C.

Macroscopic Lesion Scoring of Small Intestines

Lesions in the small intestine (duodenum, jejunum) were scored as described by Keyburn et al. (2006) as follows: 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = small focal necrosis or ulceration (1−5 foci); 3 = focal necrosis or ulceration (6−15 foci); 4 = focal necrosis or ulceration (16 or more foci); 5 = patches of necrosis 2- to 3-cm long; 6 = diffuse necrosis typical of field cases. Lesion scores of 2 or more were classified as necrotic enteritis positive.

Real-Time PCR for Jejunal Barrier-Related mRNA Expression

Total RNA of jejunum mucosa was extracted with TRIzol reagent (TaKaRa, Dalian, China) on basis of the manufacturer's instructions. The concentration of RNA was measured by using DU 640 UV spectrophotometer detection (Beckman Coulter Inc., Fullerton, CA). cDNA was synthesized by using primeScript RT reagent kit (Takara).

The primers of genes (*Claudin-1, Claudin-2, zonula occudens (**ZO**)-1, ZO-2, Occludin, Mucin-1, Mucin-2*), listed in Supplementary Table 2, were purchased from TaKaRa Biotechnology (Dalian Co., Ltd., Dalian, China), and the real-time PCR was performed using the SYBR Premix Ex Tap (Takara). The PCRs were run on an Applied Biosystems 7900HT Real-Time PCR system (Applied Biosystems, CA). The house keeping gene (β-actin) was chosen to correct for variance in the amount of RNA input in the reaction. The relative mRNA expression compared to the house keeping gene was obtained with previous methods (Wang et al., 2021a,b).

The Levels of Intestinal Inflammatory Factor

Enzyme-linked immunosorbent assay (**ELISA**) test kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) were utilized to analyze the cytokine levels in jejunum, including interleukin (**IL**)-1β (**IL-1β**), IL-4, IL-6, and tumor necrosis factor alpha (**TNF-α**) following the previous method (Wang et al., 2021b).

Gut Microbiota Analysis and Short-Chain Fatty Acids Quantification in Cecum

Microbial profile in the cecum digesta (n = 8) was evaluated by the sequencing and clustering of 16S rRNA gene with high-throughput pyrosequencing, the sequencing and bioinformatics analysis were performed by Novogene Bioinformatics Technology Co. (Tianjin, China), and the method were used as recently described by Yang et al. (2020). SCFA (n = 8) including acetate, propionate, valerate, and butyrate in the cecum content were also analyzed using Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) following previous protocols (Wang et al., 2021b).

Statistical Analysis

All data were analyzed by one-way ANOVA using GLM procedure of SAS 9.0 software (SAS Institute, Cary, NC) and GraphPad Prism (GraphPad Inc., La Jolla, CA). For the microbiota, data were analyzed by Wilcox rank sum test, differences among treatments were considered significant at $P < 0.05$ or extremely significant at $P < 0.01$. Beta diversity based on the weighted UniFrac distance matrices were calculated with QIIME (Version 1.7.0) and Cluster analysis was preceded by principal coordinate analysis (**PCoA**). Differentially represented bacterial taxa between different samples were analyzed using the linear discriminant analysis effect size (**LEfSe**). The results were expressed as the mean and SEM.

RESULTS

Production Performance and Cecum SCFA

As shown in Figure 1B, after challenged the layers had lower egg-laying rate (**ELR**), ADFI compared with the CON group ($P < 0.05$). Dietary supplementation with BEC after challenge was shown to alleviate ELR than challenged ones ($P < 0.05$). The CC and BEC group had lower cecum content concentration of the main SCFA (propionate, butyrate) than those observed in the CON group (Figure 1C; $P < 0.05$).

Intestinal Lesions Score and Intestinal Morphology

The CC and BEC groups had higher pathological scores in duodenum, whereas the BEC group had lower pathological scores in jejunum when compared with the CC group (Figure 2A; $P < 0.05$).

The layers in CC group had lower V/C ratio, and the layers fed BEC diet had higher crypt depth in jejunum than CON group; but had no effect on crypt depth and V/C ratio when compared with the challenged ones (Figure 2B; $P < 0.05$). No effect of BEC supplementation after challenge was observed on villus height in duodenum and jejunum, and crypt depth in jejunum ($P > 0.05$).

Jejunal Inflammatory Cytokine Levels and Intestinal Barrier Function

The challenge upregulated the concentration of proinflammatory cytokines (IL-1β and IL-6) (Figure 2C; $P < 0.05$), whereas the BEC addition after challenge didn't influence the proinflammatory cytokines levels and intestinal barrier function-related gene expression (*Mucin-1,*

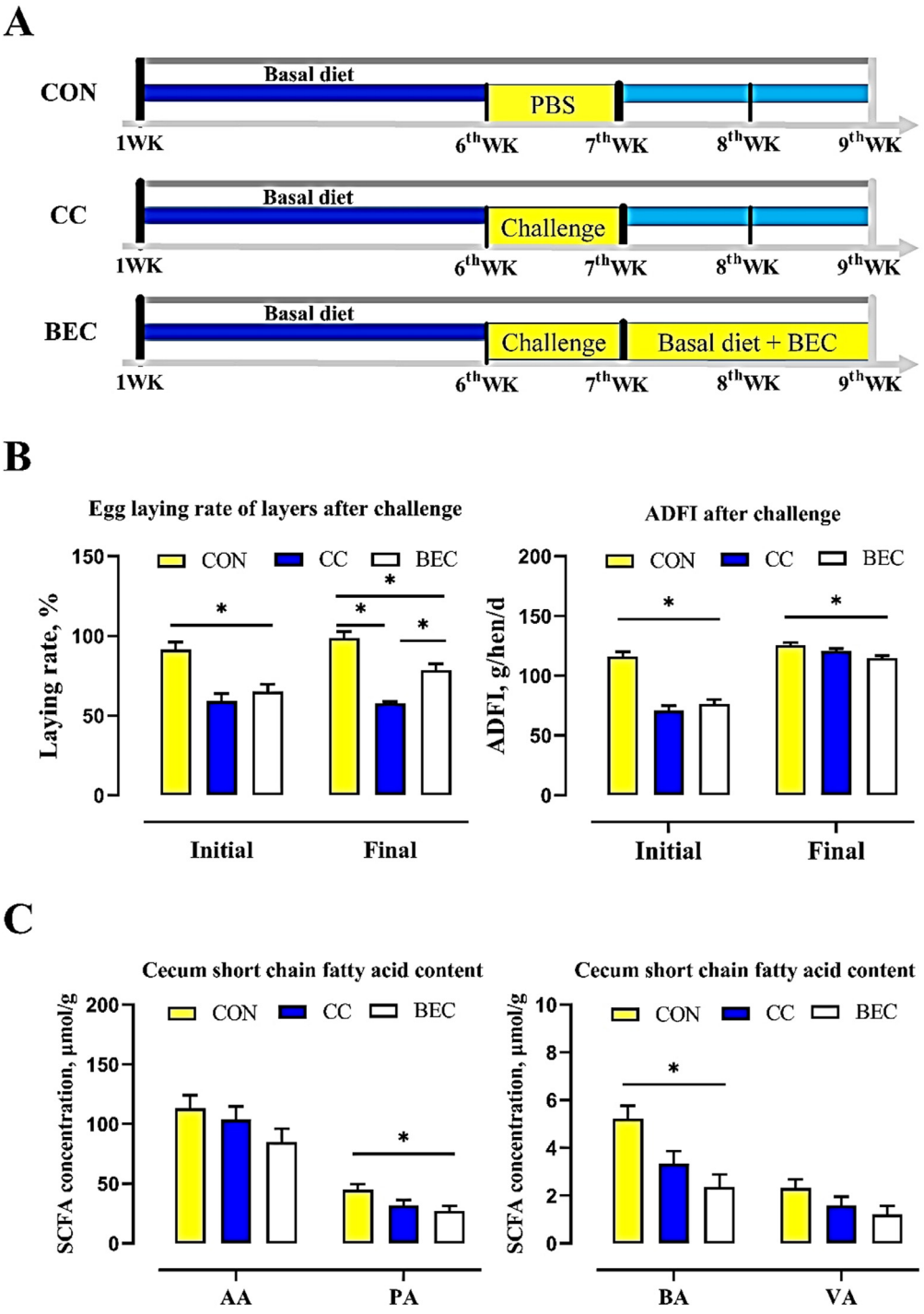

Figure 1. The effect of dietary BEC supplementation on the egg-laying rate, ADFI and SCFA. (A) Experiment design. (B) Egg-laying rate and average daily feed intake after challenge. (C) Short-chain fatty acid concentration in cecal digestion. Abbreviations: AA, acetate; ADFI, average daily feed intake; BA, butyrate; BEC, 2,000 mg/kg BEC (1,400 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 100 mg/kg essential oil complex) after challenge; CC, challenge group; CON, control group; PA, propionate; SCFA, short-chain fatty acid; VA, valerate. Data are means ± SEM, $^*P < 0.05$.

Mucin-2, ZO-1, ZO-2, Claudin-1, Claudin-2, and *occludin*) in jejunum of layers (Figure 3A–D; $P > 0.05$).

Cecum Microbiota Composition

The shared OUT among 3 groups were presented in Figure 4A. Relative microbial abundances of the cecum at phylum level indicated that *Firmicutes* and *Bacteroidota* were the dominant phylum in all dietary treatments (CON, 77.56%; CC, 80.42%; BEC, 77.10%). The BEC group had higher abundance of *Desulfobacterota* and *Halobacterota* ratio value at the phylum level (Figure 4B; $P < 0.01$). At the genus level, we observed that enrichment of *Lactobacillus* and *Bacteroides* on 3 groups. The *Bacteroides* and *Megamonas* were increased in CC group, the *Faecalibacterium* was decreased compare with CON and BEC

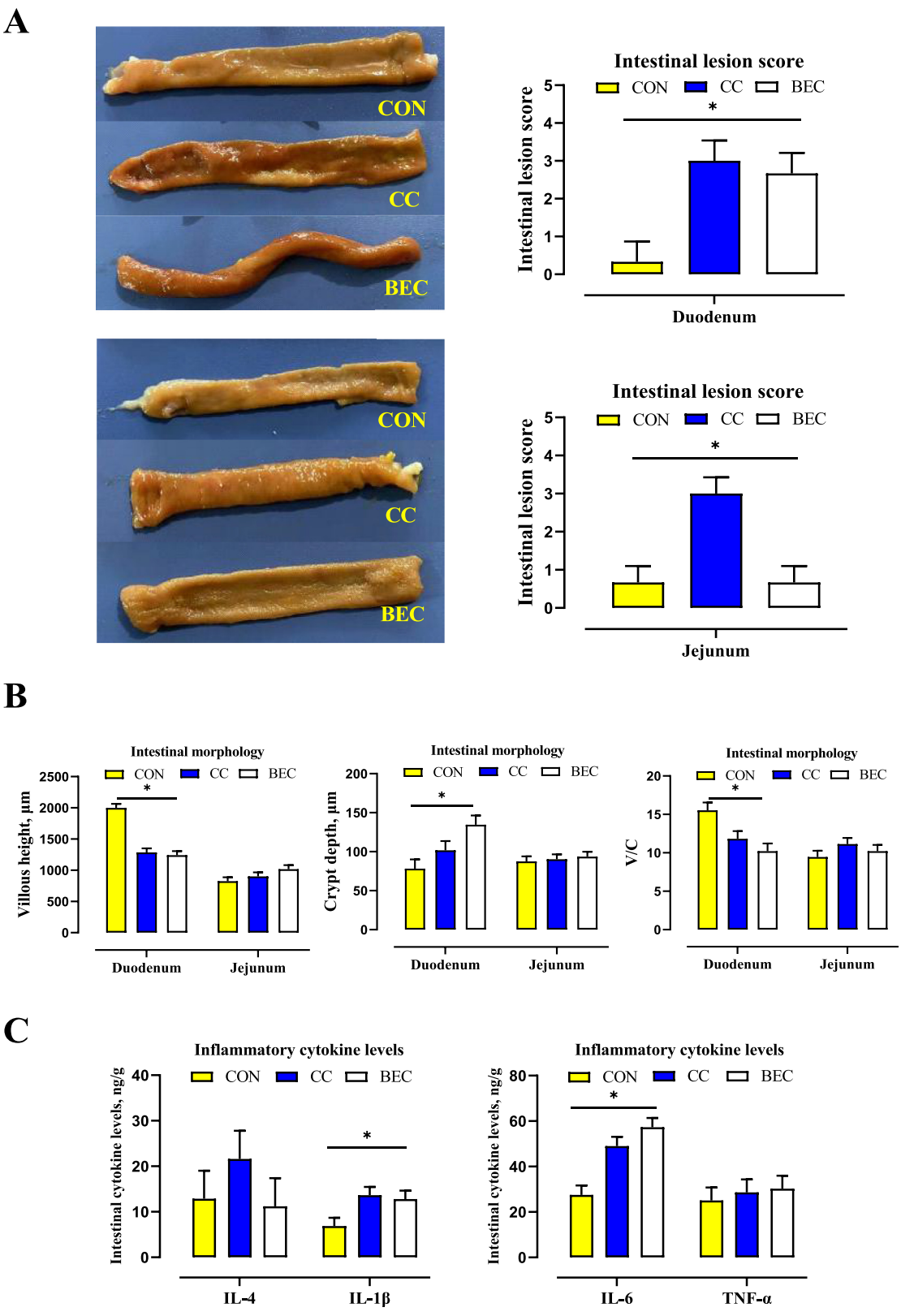

Figure 2. The effect of dietary BEC supplementation on the intestinal lesion score, morphology, and inflammatory cytokine levels of laying hens after challenged. (A) Intestinal lesion score. (B) Intestinal morphology. (C) Jejunum inflammatory cytokine levels. Abbreviations: BEC, 2,000 mg/kg BEC (1,400 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 100 mg/kg essential oil complex) after challenge; CC, challenge group; CON, control group; IL-4, interleukin-4; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; V/C, villus height/crypt depth. Data are means ± SEM, $^*P < 0.05$.

groups (Figure 4C; $P < 0.05$). These data showed that while the CC and BEC led to microbial variation but did not change the dominant species at phylum and genus level in the layer cecum.

Beta Diversity of Cecum Microbiota

PCA of related bacterial communities indicate that the separation in the CON, CC and BEC groups could

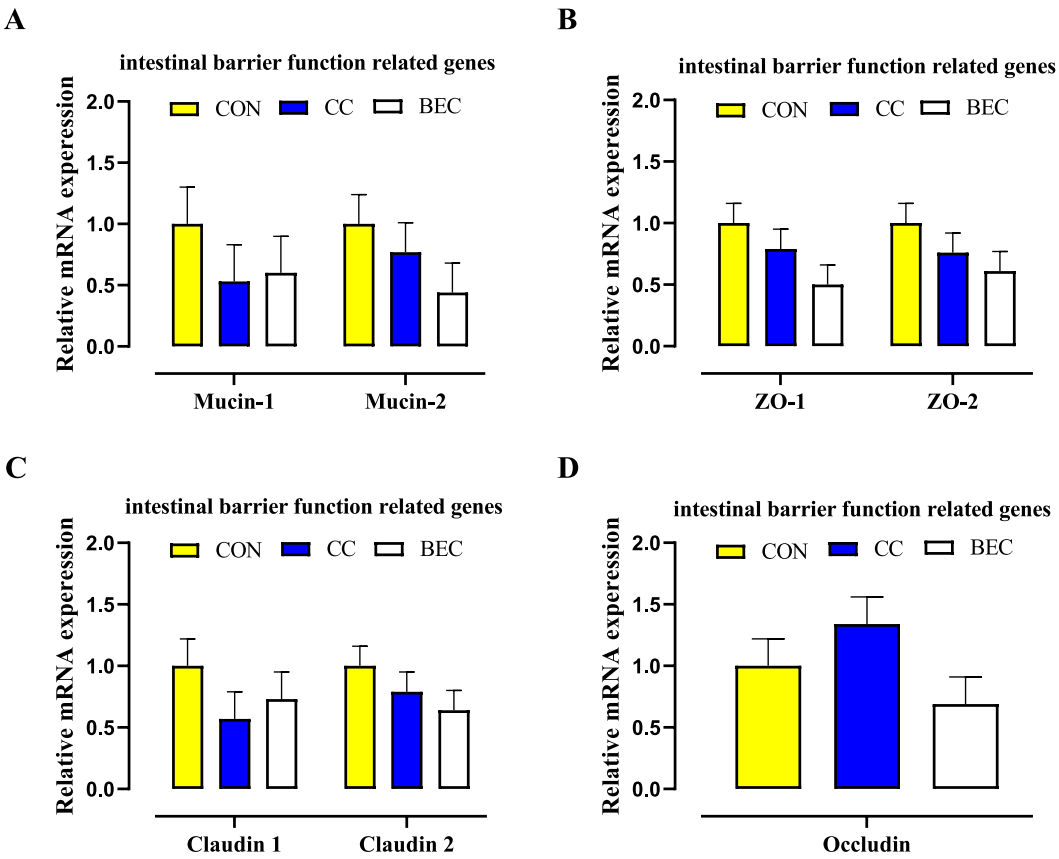

Figure 3. The effect of dietary BEC supplementation on the expression of genes related to intestinal barrier function of laying hens after challenged. (A–D) Intestinal barrier function-related genes. Abbreviations: BEC, 2,000 mg/kg BEC (1,400 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 100 mg/kg essential oil complex) after challenge; CC, challenge group; CON, control group; *ZO-1, zonula occluden-1; ZO-2, zonula occluden-2*. Data are means ± SEM, $^*P < 0.05$.

be hardly detected (Figure 4B; PC1 vs. PC2). As shown in Figure 5A and B (LEfSe), an increase in the abundance of *Bacteroidoes* (genus), *Bacteroidaceae* (family), *Bacteroidoes* sp. *Marseille P3166* (species), *Bacteroidoes caecicola* (species) was observed in the CC group, whereas the BEC group had higher abundance of *Bacteroides caecigallinarum* (species).

Correlations Between Cecum Microbiota and Laying Rate or Inflammatory Cytokines of Laying Hens

A Spearman correlation analysis was performed to evaluate the potential link between alterations in gut microbiota composition and the ELR or inflammatory cytokines in laying hens (Figure 6A and B). The ELR positively correlated with genus *Faecalibacterium, Asteroleplasma, Rikenellaceae RC9 gut, Olsenella*, and *Alloprevotella* ($r = 0.57, 0.60, 0.48, 0.48, 0.42$; $P < 0.05$), but negatively correlated with *Bacteroides, Romboutsia*, and *Megamonas* ($r = -0.58, -0.73, -0.48$; $P < 0.05$). The genera *Bacteroides, Lactobacillus, Romboutasi*, and *Megamonas* were positively correlated with IL-1β ($r = 0.61, 0.60, 0.59, 0.53$; $P < 0.05$), but genera *Rikenellaceae RC9 gut, Faecalibacterium, Olsenella, Prevotellaceae UGG. 001*, and *Escherichia. Shigella* ($r = -0.56, -0.57, -0.61, -0.47$; $P < 0.05$) were negatively correlated with IL-1β. The genera *UCG. 005, Bacteroides, Lactobacillus*, and *UCG.008* positively correlated with IL-6 ($r = 0.58, 0.48, 0.47, 0.47$; $P < 0.05$), but genera *Rikenellaceae RC9 gut, Faecalibacterium, Asteroleplasma*, and *Olsenella* ($r = -0.62, -0.46, -0.47, -0.46$; $P < 0.05$) were negatively correlated with IL-6.

DISCUSSION

NE generally present as acute clinical and subclinical, the acute clinical NE was characterized by livestock mortality, while the subclinical NE caused intestinal mucosa damage, disrupted the villous crypt microarchitecture, and prevented digestion and absorption of nutrients, thereby impaired the growth performance of the chicken (Immerseel et al., 2004). In our study, we used a cochallenge of *C. perfringens* (CVCC2030) with coccidia, similar to our previous study using the same challenge model to create subclinical NE (Zhang et al., 2022). Different from the previous study, the BEC were supplemented after rather than before challenge, and the objective of current study is to evaluate whether the effect of BEC could help the laying recover from coccidia and *C. perfringens* challenge.

Usually, NE could lead to the decrease in feed intake, body weight gain and feed conversion of broilers (Du et al., 2015; Awawdeh, 2021). Similar to our

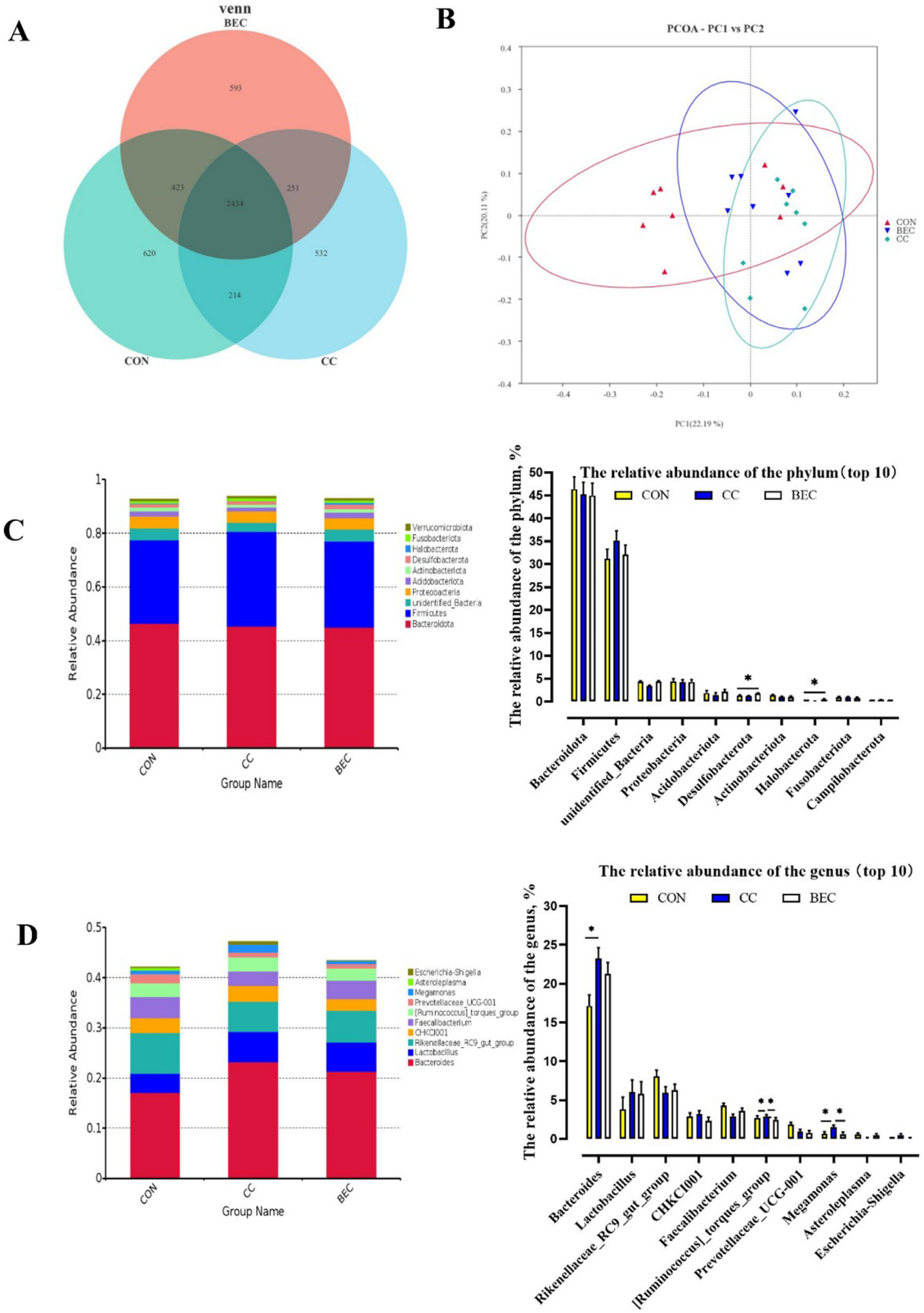

Figure 4. The effect of dietary BEC supplementation in microbiota diversity. (A) Venn diagram. (B) The principal coordinate analysis (PCoA) of the cecum microbiota based on unweighted UniFrac metric. (C, D) The relative abundance of the top 10 phylum (C) and genus (D) from groups. Abbreviations: BEC, 2,000 mg/kg BEC (1,400 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 100 mg/kg essential oil complex) after challenge; CC, challenge group; CON, control group. Data are means ± SEM, * $P < 0.05$.

findings, both egg production and ADFI decreased significantly after challenge. And the dietary supplementation BEC after challenge had an increase trendy in laying rate. Soltan (2008) showed that organic acids had no significant effect on egg production. But there was a study had shown that adding 150 mg/kg of organic acid and essential oil mixture could increase egg production and reduce ADFI of laying hens (Wang et al., 2019a).

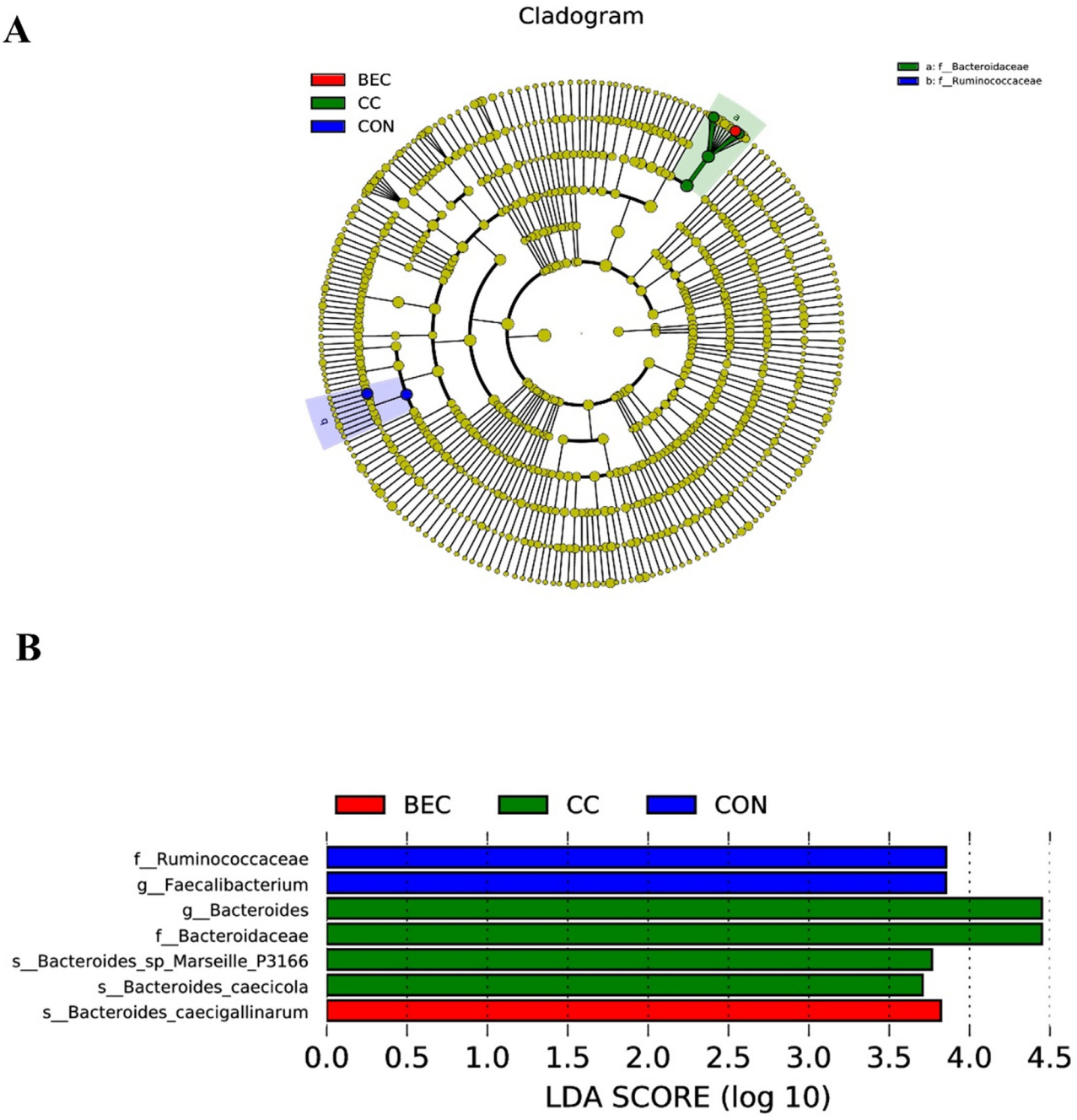

Figure 5. The effect of dietary BEC supplementation in microbiota diversity. (A) Taxonomic cladogram obtained from LEfSe analysis of 16S rRNA sequencing. Biomarker taxa are heighted by colored circles and shaded areas. Each circle's diameter is relative to abundance of taxa in the community. (B) Only taxa meeting an LDA significant threshold >3.5 are shown. (Red) BEC enriched taxa; (Green) CC enriched taxa; (Blue) CON enriched taxa. Abbreviations: CC, challenge group; CON, control group; BEC, 2,000 mg/kg BEC (1,400 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 100 mg/kg essential oil complex) after challenge.

Therefore, the effects of organic acids and EOs on production performance may be influenced by different type and concentration, different dietary compositions, feeding, and management conditions (Gheisar et al., 2014; Habibi et al., 2014; Zeng et al., 2015).

The indigestible dietary fiber produced SCFA in the cecum of poultry under the action of anaerobic microbial fermentation, which could promote intestinal mucus secretion and protect the intestinal barrier by regulating the tight junction protein (Walugembe et al., 2015; Molnár et al., 2020). In this experiment, the concentration of cecal butyrate and propionate decreased when the laying hens were challenged with coccidia and *C. perfringens*, which indicated that challenge-induced stress lead to a depression in nutrients fermentation of cecum. Our observation was in agreement with that of Lin et al. (2022), who found that the content of acetate, butyrate and total SCFA in the cecum of broiler been lowered after challenged with the Eimeria. The short-chain fatty acids played a critical role in maintaining the physical health of animals. Among them, butyrate was an important energy source for promoting intestinal development and intestinal epithelial cells growth, while propionate was closely related to liver metabolism

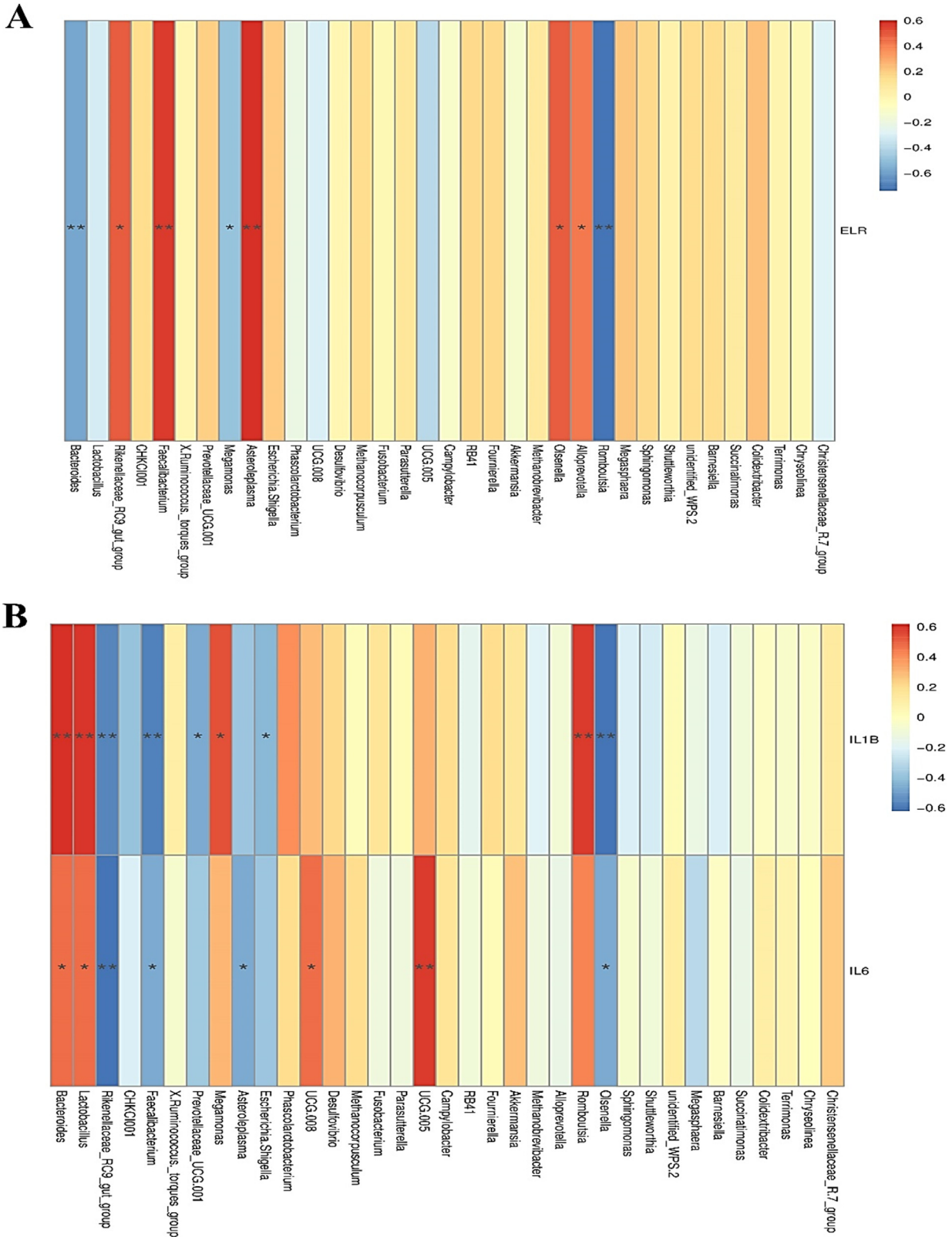

Figure 6. Heatmap of spearman r correlations between the gut microbiota significantly modified by different (A) egg-laying rate (B) inflammatory factors at genus level (Top 35). Red indicates positive correlation, and blue indicates negative correlation; while the color is darker, the correlation is higher. $^{*}P < 0.05$ and $^{**}P < 0.01$. Abbreviations: BEC, 2,000 mg/kg BEC (1,400 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 100 mg/kg essential oil complex) after challenge; CC, challenge group; CON, control group; ELR, egg-laying rate; IL1B, IL-1β (interleukin-1 β); IL-6, interleukin-1.

(Liao et al., 2020). There were some studies have shown that adding plant essential oil, organic acids or beneficial bacteria to the diet increased the concentration of SFCA (Yang et al., 2019; Xu et al., 2022). However, we suggested that the dietary supplementation with high level of BEC could not increase the content of cecal butyrate and propionate of laying hens which had been challenged with it was speculated that the NE caused by

challenging might be more severe, or the individual beneficial substance in the BEC was lower coccidia and *C. perfringens*.

The intestine is an important digestive organ of the body, and the morphology of intestine is one of the most critical indicators that reflect the intestine health. The intestinal surface area including villus height, crypt depth, and V/C, which could determine the rate of intestinal epithelial turnover and the nutrient digestion and absorption capacity (Yang et al., 2016). If the crypt depth deepened, it means that the body may rebuild the villi by increasing the rate of cell turnover to fight the damage caused by pathogenic bacteria or toxin infection (Paiva et al., 2014). In our study, the CC and BEC groups significantly increased crypt depth and decreased V/C of duodenum compared with the control group. In our previous study, *C. perfringens* and coccidia challenged could make the crypt depth was higher and V/C was lower in duodenum, jejunum, and ileum (Zhang et al., 2022). It is proved that the challenge had a certain damaged to the integrity of intestinal morphology. Gong et al. (2021) shown that dietary supplemented with 2,000 mg/kg benzoic acid had increased crypt depth in duodenum. In addition, Placha et al. (2010) shown that 0.4% EO may had effects on intestine integrity, and the probiotic strain *E. faecium* was able to eliminate negative effects and can strengthen nonspecific immunity, which is similar to our findings but not identical. Coccidia and *C. perfringens* challenge lesions in different parts of the poultry gut that depend entirely on the extent of the infection, thus leading to differences in pathological conditions (Tyzzer et al., 1932). Keyburn et al. (2006) defined the intestinal lesions scores include thin or friable walls, focal necrosis or ulceration and diffuse necrosis typical of field cases. Our results shown that the lesions scores of duodenum and jejunum in challenge group were higher than, the BEC group had higher lesions scores of duodenum, and the BEC group had lower pathological scores of jejunum than control group. Our findings were similar to many studies, which also determined that coccidia and *C. perfringens* challenged significantly increased duodenum and jejunum lesions scores (Attia et al., 2012; Rodrigues et al., 2017; Gharib-Naseri et al., 2019). Mustafa et al. (2021) indicated that the dietary supplementation with organic acids in coccidial challenged group could improve intestinal health, and reduce intestinal lesions scores of broiler chickens. Another study showed that adding 120 mg/kg of essential oils (thymol and carvacrol) after *C. perfringes* challenged significantly reduced lesions scores in chickens (Yin et al., 2017). Recently, there were many studies about the effects of organic acids, essential oils or probiotics on the intestinal of challenged poultry, the results were inconsistent and further research was needed.

NE is understood to be a stepwise process that begins with depletion of the mucosal layer of the intestine, it appears that under conditions where birds are already subject to infection, the activity of mucolytic bacteria becomes more than the host mucogenic activity, with the result that pathogenesis of NE proceeds in the presence of increased host mucogenic activity (Collier et al., 2008). Forder et al. (2012) considered that challenged with Eimeria, and *C. perfringens* could affect the intestinal mucin synthesis genes, and they reported that the expression of *Mucin2* and *Mucin13* were depressed by challenge. However, we observed that the CC and BEC groups had no significant effects on the expression of genes-related intestinal barrier. Although our result was different from previous studies, the experiment had shown that the intestinal barrier-related genes was only expressed when it yield an acute challenge response (Kitessa et al., 2014). Further study is required to elucidate the pattern of change in mucin synthesis genes as NE progresses clinically or subclinically.

The upregulation of intestinal inflammatory factors can induce inflammatory responses in the intestinal tract of broilers. The occurrence of inflammatory diseases in animals is often accompanied by an increase in the secretion of inflammatory factors such as IL-Iβ and IL-6. In our study, compared with the control group, the CC and BEC groups upregulated the levels of IL-1β and IL-6. Studies have shown that the challenge activates the intracellular signaling cascade, and finally activates the nuclear transcription factor NF-κB, which promotes the synthesis and secretion of proinflammatory cytokines such as IL-1β and IL-6, and activates the inflammatory response (Paul et al., 2011). Al-Sadi et al. (2008) found that the inflammatory factors increased the permeability of the mucosal barrier of intestinal epithelial cells, allowing foreign antibodies to enter, leading to the occurrence of intestinal diseases.

To further elucidate the effect of BEC on the gut health of laying hens challenged with coccidia and *C. perfringens*, we focused on the analysis of gut microbial community was closely related to NE (Alizadeh et al., 2021). The present study showed that coccidia and *C. perfringens* challenged and the dietary was supplemented with BEC all had no significantly effect on richness and diversity of cecum microbial community. There was a previous study demonstrated that *C. perfringens* was used to challenge broiler did not affect α-diversity index of cecal microbial community (Latorre et al., 2018), while another study had reported that *C. perfringens* infection of broiler reduced the diversity of ileum microbial community (Bortoluzzi et al., 2019). This phenomenon correlated with the time of infection of coccidia and *C. perfringens*, the site of sample collection and the growth stage of the animal. According to the PCA analysis of β diversity, we found that the bacterial community structure of each treatment group was relatively similar, and Bacteroidota were the dominant bacteria at the phylum level. It was reported that Firmicutes and Bacteroidetes constituted vast majority of intestinal microbiota in laying hens, and dynamic changing occurred at different physiological stages (Dai et al.,2022). The Firmicutes to Bacteroidetes ratio

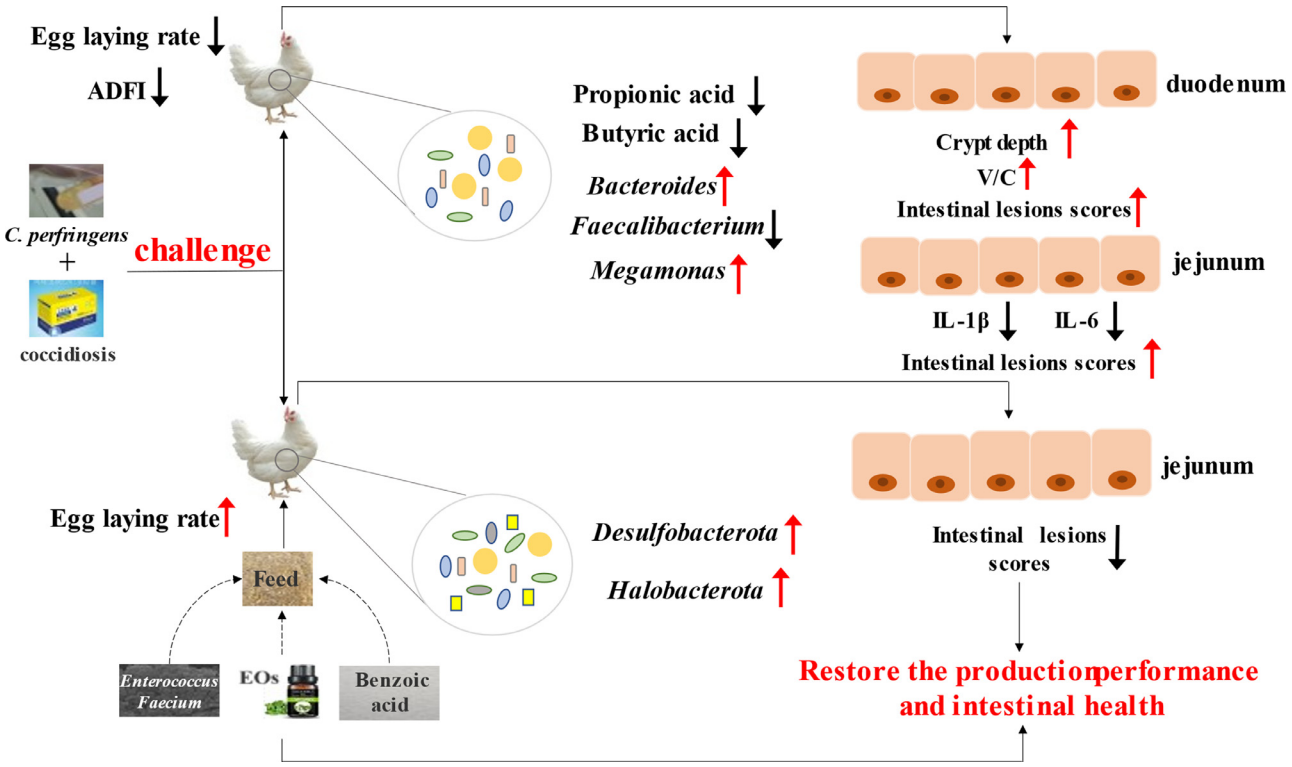

Figure 7. Graphical summary of the effect of benzoic acid, *Enterococcus faecium* and essential oil complex on intestinal microbiota of laying hens under coccidia and *Clostridium perfringens* challenge.

was considered a critical biomarker of healthy intestinal function and could be indicative of microecological balance conditions in the gastrointestinal tract (Pereira et al., 2016). Similar to previous reports (Wang et al., 2019b; Gong et al., 2021), the current study revealed that the coccidia and *C. perfringens* challenge and the dietary supplementation with BEC induced a conversion in gut microenvironment, increased the abundance of *Bacreroides* and *Lactobacillus* at the genus level. Simultaneously, we observed that the genus *Romboutsia, Lactobacillus* and *Bacreroides* were positively correlated with proinflammatory factor IL-1β. There was a study have suggested that the presence of *Bacreroides* and *Lactobacillus* linked to intestinal inflammation and barrier dysfunction (Las-Heras et al., 2019), which might be involved in regulating intestinal anti-inflammatory response. In addition, we also found that the genus *Asteroleplasma* and *Faecalibactenium* were positively correlated with ELR. According to experimental researches (Min et al., 2015; Maioli et al., 2021), an increase in the abundance of *Asteroleplasma* resulted in intestinal inflammation and other intestinal disease and *Faecalibactenium* was a highly abundant butyrate-producing bacterium that could product anti-inflammatory metabolites. We thought that the alternation in microbiota associated with inflammation such as *Bacreroides, Lactobacillus, Asteroleplasma, Faecalibactenium*, and so on might affect intestinal health, metabolic function, and production performance of laying hens. Therefore, understanding how NE and BEC induced variations on the intestinal microbiota could help reduce the negative impact of the disease in animal health.

CONCLUSIONS

In conclusion, the *C. perfringens* and coccidia challenge resulted in reduced performance, sustained damage to the intestine and changed cecal microbiomes. Dietary supplementation of BEC complex right after challenge (1,400 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 100 mg/kg essential oil) could help the layer recover from challenge by alleviating production performance, enriching microbial compositions, and promoting intestinal health of laying hens (Figure 7).

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Author contributions: H. Z. and J. W. conceived and designed the experiments; J. W., and H. Z. performed the experiments; H. Z. and J. W. analyzed the data; H. Z. wrote the paper; X. D., S. B., Z. Q., K. Z., X. M., C. L., and J. W. helped revise this manuscript. All authors read and approved the final manuscript.

DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2023.102490.

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