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Effects of *Clostridium butyricum* and *Enterococcus faecalis* on growth performance, intestinal structure, and inflammation in lipopolysaccharide-challenged weaned piglets

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Abstract

This study was conducted to investigate the effects of *Clostridium butyricum* and *Enterococcus faecalis* on growth performance, immune function, inflammation-related pathways, and microflora community in weaned piglets challenged with lipopolysaccharide (**LPS**). One hundred and eighty 28-d-old weaned piglets were randomly divided into 3 treatments groups: piglets fed with a basal diet (Con), piglets fed with a basal diet containing 6×10^9 CFU *C. butyricum*·kg^{-1} (CB), and piglets fed with a basal diet containing 2×10^{10} CFU *E. faecali*·kg^{-1} (EF). At the end of trial, 1 pig was randomly selected from for each pen (6 pigs per treatment group) and these 18 piglets were orally challenged with LPS 25 μg·kg^{-1} body weight. The result showed that piglets fed *C. butyricum* and *E. faecalis* had greater final BW compared with the control piglets ($P < 0.05$). The *C. butyricum* and *E. faecalis* fed piglets had lower levels of serum aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), IL-1β, tumor inflammatory factor-α (**TNF-α**), and had greater level of serum interferon-γ (**IFN-γ**) than control piglets at 1.5 and 3 h after injection with LPS ($P < 0.05$). Furthermore, piglets in the *C. butyricum* or *E. faecalis* treatment groups had a greater ratio of jejunal villus height to crypt depth (V/C) compared with control piglets after challenge with LPS for 3 h ($P < 0.05$). Compared with the control treatment, the CB and EF treatments significantly decreased the expression of inflammation-related pathway factors (TLR4, MyD88, and NF-κB) after challenge with LPS for 3 h ($P < 0.05$). High-throughput sequencing revealed that *C. butyricum* and *E. faecalis* modulated bacterial diversity in the colon. The species richness and alpha diversity (Shannon) of bacterial samples in CB or EF piglets challenged with LPS were higher than those in LPS-challenged control piglets. Furthermore, the relative abundance of *Bacteroidales-Rikenellanceae* in the CB group was higher than that in the control group ($P < 0.05$), whereas EF piglets had a higher relative abundance of *Lactobacillus amylovorus* and *Lactobacillus gasseri* ($P < 0.05$). In conclusion, dietary supplementation with *C. butyricum* or *E. faecalis* promoted growth performance, improved immunity, relieved intestinal villus damage and inflammation, and optimized the intestinal flora in LPS-challenged weaned piglets.

Key words: Clostridium butyricum, Enterococcus faecalis, growth performance, jejunal morphology, microflora community, weaned piglets

Introduction

Since the early 1950s, studies have shown that antibiotics can promote the growth performance (Dibner et al., 2005; Huyghebaert et al., 2011) and reduce the rate of diarrhea in animal (Walsh et al., 2007; Andreas et al., 2016). However, the development of resistance to many antibiotics has necessitated the search for alternatives to antibiotics. Recently, the provision of probiotics has achieved notable results in improving growth performance, maintaining intestinal health, and enhancing immunity in animals (Truusalu et al., 2004; Asai et al., 2011; Dheilly et al., 2011). *Clostridium butyricum*, a gram-positive anaerobic bacterium, is found in healthy animals and the human gut (Finegold et al., 1983; Zhang et al., 2017), and has the characteristics of showing resistance to acidic pH, high temperature, and bile salts (Kong et al., 2011; Zhang et al., 2016). Previous studies have shown that *C. butyricum* has effects related to the improvement of broiler growth performance and immune function, and optimization of intestinal microflora structure (Cao et al., 2012; Yang et al., 2012; Liao et al., 2015), whereas the intestinal inflammatory response in mice is effectively alleviated after feeding with *C. butyricum* (Hayashi et al., 2013). Similarly, Sumon et al. (2018) have reported that dietary *C. butyricum* can improve the growth performance and immune response of *Macrobrachium rosenbergii*. Studies have also shown that *C. butyricum* can improve growth performance in weaned piglets (Takahashi et al., 2018) and optimizes the microbial community (Zhang et al., 2018). Studies on *Enterococcus faecalis*, a lactic acid bacterium that occurs in the intestines of healthy animals (Toit et al., 2000; Gaggìa et al., 2010), have shown that it can improve growth performance and reduce diarrhea in weaned piglets (Hu et al., 2015), whereas Tsukahara et al. (2011) have shown that *E. faecalis* improves villous atrophy in early-weaned mice and pigs. Although some previous studies have investigated the use of *C. butyricum* and *E. faecalis* as probiotics, there have been few studies on the effects of such probiotics on intestinal inflammation and microbial community structure in weaned piglets.

Lipopolysaccharide (**LPS**) is a class of endotoxin present in the outer membrane of gram-negative bacteria, which is essential for the physical integrity and function of the bacterial outer membrane (Moran et al., 1995). Many animal studies have used LPS to generate acute inflammation models (Lei et al., 2015; Wyns et al., 2015). However, few studies have investigated the effects of *C. butyricum* and *E. faecalis* on weaned piglets in response to exposure to LPS.

Therefore, the present study mainly investigated the effects of *C. butyricum* and *E. faecalis* on growth performance, immune function, intestinal structure, inflammation-related pathways, and intestinal flora structure in weaned piglets challenged with LPS.

Materials and Methods

Animals, Housing, and Experimental Design

The following procedures were approved by the Ethics Committee of Zhejiang Agricultural and Forestry University. A total of 180 28-d-old weaned piglets (Duroc × Landrace × Yorkshire) were randomly assigned to 3 treatments (6 replicate pens per treatment with 10 pigs in each pen). The treatments were as follows: piglets fed a basal diet (Con), piglets fed a basal diet supplemented with 6×10^9 CFU of *C. butyricum*·kg^{-1} (CB), and piglets fed a basal diet supplemented with 2×10^{10} CFU of *E. faecalis*·kg^{-1} (EF). At the end of trial, one pig was randomly selected from for each pen (6 pigs per treatment group) and these 18 piglets were challenged with LPS 25 μg·kg^{-1} BW. The basal diet was formulated to meet the nutrient requirements suggested by the National Research Counci (1998) and contained no antibiotics (Table 1). Water and feed were provided ad libitum. Piglets were housed in a temperature-controlled room maintained at 25.0 to 28.0 °C.

Bacterial Preparations

The commercial preparations of *C. butyricum* and *E. faecalis* were provided by Zhejiang Vegamax Biological Technology Co., Ltd. China. The basal diet was formulated to contain either 6×10^9 CFU *C. butyricum*·kg^{-1} or 1×10^{10} CFU *E. faecalis*·kg^{-1}.

Growth Performance and Occurrence of Diarrhea

On the day of weaning and on day 28 postweaning, all piglets were weighed individually. Feed consumption per pen was measured throughout the experiment. Average daily feed intake (ADFI), ADG, and feed to gain (**F: G**) were calculated for the piglets in each pen. Instances of the diarrhea in each piglet were recorded daily in order to calculate the rate of diarrhea

Sample Collection

On day 28 postweaning, 1 pig was randomly selected from for each pen (6 pigs per treatment group) and these 18 piglets were injected with 25 μg·kg^{-1} LPS. Vascular blood (5 mL) was collected from each pig at 1.5 and 3 h after injection. Piglets were anesthetized and killed at 4 h after injection. Blood samples were allowed to clot 4 °C and then centrifuged at 5,000 × *g* for 10 min. The resulting serum was collected and stored at –20 °C for further analysis. Jejunum segments were preserved in 4% paraformaldehyde and stored at 4 °C, and samples of the empty field mucosa were collected in Eppendorf tubes and stored at –80 °C.

Table 1. Composition and nutrient levels of the basal diet (air-dry basis) %

Ingredients	Content	Nurtient level[2]	
Corn	52.00	DE	13.52
Wheat midding	8.90	CP	20.96
Soybean meal	10.00	Lys	0.98
Extruded soybean	8.00	Met+Cys	0.58
Imported fish meal	8.00	Thr	0.59
Whey	7.00	Ile	0.67
Choline chloride	0.10	Ca	0.82
Phospholipid	2.00	TP	0.60
Premix[1]	4.00	AP	0.42
Total	100.00		

[1]Supplied the following per kg of diet: vitamin A, 10,500 IU; vitamin D3, 450 IU;vitamin E, 10 mg; pantothenic acid, 20 mg; vitamin B6, 2 mg; biotin, 0.3 mg; folic acid, 5 mg; vitamin B12, 0.009 mg; ascorbic acid, 50 mg; Fe, 160 mg; Cu, 140 mg; Mn, 50 mg; Zn, 130 mg; I, 0.5 mg; Se, 0.3 mg.
[2]Values for net energy were calculated, the contents of ether extract, crude protein, acid-detergent fibre, neutral-detergent fibre, ash, Ca, and P were analyzed. DM, dry matter; T, Total; AA, amino acid.

Serum Parameter Analysis

The concentrations of serum factors, including aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), IgA, IgM and IgG, IL-1β, tumor inflammatory factor-α (**TNF-α**), and interferon-γ (**IFN-γ**), were measured using a multifunction microplate reader and kits purchased from the Jiancheng Biological Engineering Research Institute, Nanjing, China.

Jejunal Morphological Analysis

Jejunal samples were washed with normal saline, fixed with 4% paraformaldehyde phosphate buffer for 48 h. The tissues were then cut into 4 μm sections, which were routinely dehydrated, dipped in wax, embedded in paraffin, and sectioned. The processed samples were subsequently dewaxed with xylene, stained with hematoxylin and eosin, dehydrated with ethanol, and covered with coverslips. The villus height and crypt depth were observed under an optical microscope (NIKON Eclipse ci, Japan) and photographed (NIKON digital sight DS-FI2, Japan). Ten fields of view were selected for the measurement of villus height and crypt depth.

Immunohistochemical Analysis

Immunohistochemistry for the detection of Toll-like receptors 4 (**TLR4**), Myeloid differentiation factor 88 (**MyD88**), and Nuclear factor-kappa B (**NF-κB**) was performed using 4-μm thick paraffin sections of jejunal tissues prepared as described previously. Sections were incubated with primary antibodies against TLR4, MyD88, and NF-κB, and then incubated with secondary antibody and diaminobenzidine. The sections were observed under an optical microscope and photographed, and the cumulative optical density (IOD) in the photograph was calculated and averaged. Cells colored brown-yellow when viewed under the microscope were identified as positive cells.

16S rRNA Sequencing of Colonic Microflora

Following the procedures described in our previous study (Yang et al., 2018), the colonic microbiota was analyzed by high-throughput sequencing using the colonic contents of the different treatment groups. The main methods included DNA extraction, PCR amplification of 16S rRNA, amplicon sequencing, and sequence data processing. Total genomic DNA was extracted using a QIAamp DNA stool Mini Kit, followed by the measurement of DNA concentration and purity. Genomic DNA integrity was determined by electrophoresis on a 1% agarose gel. 16S rRNA gene sequencing and related biological analyses was performed by Novogene (Beijing, China) using on the Illumina HiSeq platform. The composition and abundance of the microflora were determined by further alpha and beta diversity analyses.

Statistical Analysis

SPSS Statistics 21.0 (SPSS Inc., USA) and GraphPad Prism 7 (GraphPad Software Inc., USA) were used for statistical analyses. One-way ANOVA was performed and differences among the means of different treatment were compared using least significant difference tests. An alpha value of 0.05 was used to assess the significance among means.

RESULTS

Growth Performance

The results showed that inclusion of *C. butyricum* and *E. faecalis* in the diet increased the ADG ($P < 0.05$) and G:F ratio ($P < 0.05$) of weaned piglets. Furthermore, we found that the rate of diarrhea among piglets in the probiotic groups was lower than that in control group animals ($P < 0.05$) (Table 2).

Liver Function

Compared with piglets fed the control diet, those fed diets supplemented with *C. butyricum* or *E. faecalis* showed reduced levels of serum AST and ALT at 1.5 and 3 h postchallenge with LPS ($P < 0.05$) (Fig. 1). These results indicated that *C. butyricum* and *E. faecalis* can alleviate the liver damage caused by stress.

Serum Inflammatory Factors

Piglets fed *C. butyricum* or *E. faecalis* had higher concentrations of serum IgM than those fed the basal diet at 1.5 h postchallenge with LPS ($P < 0.05$) (Fig. 2). Compared with the control group piglets, those in the probiotic groups had lower serum concentrations of IL-1β and TNF-α at 1.5 and 3 h postchallenge with LPS ($P < 0.05$), whereas the concentration of IFN-γ was increased (Fig. 3).

Jejunal Morphology Analysis

Although piglets fed *C. butyricum* or *E. faecalis* had higher jejunum villus lengths than the control piglets when challenged with LPS ($P < 0.05$), they showed no significant increase in crypt depth. Moreover, compared with control group piglets, those fed *C. butyricum* or *E. faecalis* had an increased the villus length to crypt depth ratio (V/C) following LPS challenge ($P < 0.05$) (Fig. 4).

Table 2. Effects of *C. butyricum* and *E. faecalis* on growth performance of the weaned piglets[1]

	Treatment				
Item	Control	CB	EF	SEM2	*P* Value
Initial BW, kg	8.86	9.10	9.04	0.10	0.47
Final BW, kg	17.61[b]	19.60[a]	18.44[a]	0.24	0.02
ADG, g	312.76[b]	375.00[a]	347.96[a]	6.85	<0.01
ADFI, g	641.88[b]	654.17[a]	626.29[c]	2.93	<0.01
F:G	2.05[a]	1.75[c]	1.81[b]	0.04	<0.01
Diarrhea rate, %	6.67[a]	0.50[c]	1.30[b]	0.00	<0.01

[1]Control, CB and EF represents the piglets supplemented with basal diet, piglets supplemented with the *C. butyricum* and piglets supplemented with *E. faecalis*, respectively. Piglets were regarded as the experimental units.
[2]Pooled SEM; $n = 6$ per treatment.
[a,b]means within the same raw with different superscripts differ significantly ($P < 0.05$).

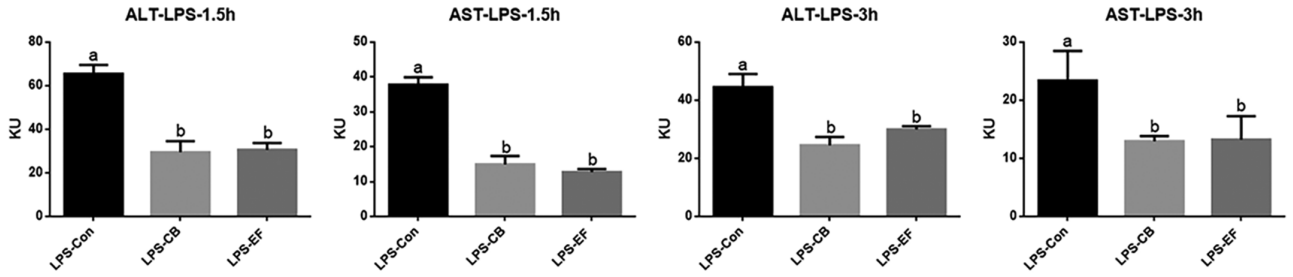

Figure 1. Effects of *C. butyricum* and *E. faecalis* on AST and ALT in weaned piglets challenged with LPS involved 1.5 h and 3 h. LPS-Con represents the control piglets challenged with LPS on day 30; LPS-CB represents the piglets supplemented with *C. butyricum* and challenged with lipopolysaccharide on day 30; LPS-EF represents the piglets supplemented with *E. faecalis* and challenged with LPS on day 30; Small superscript letter indicates differences ($P < 0.05$). Values means $n = 6$ for the analysis of liver function indexes.

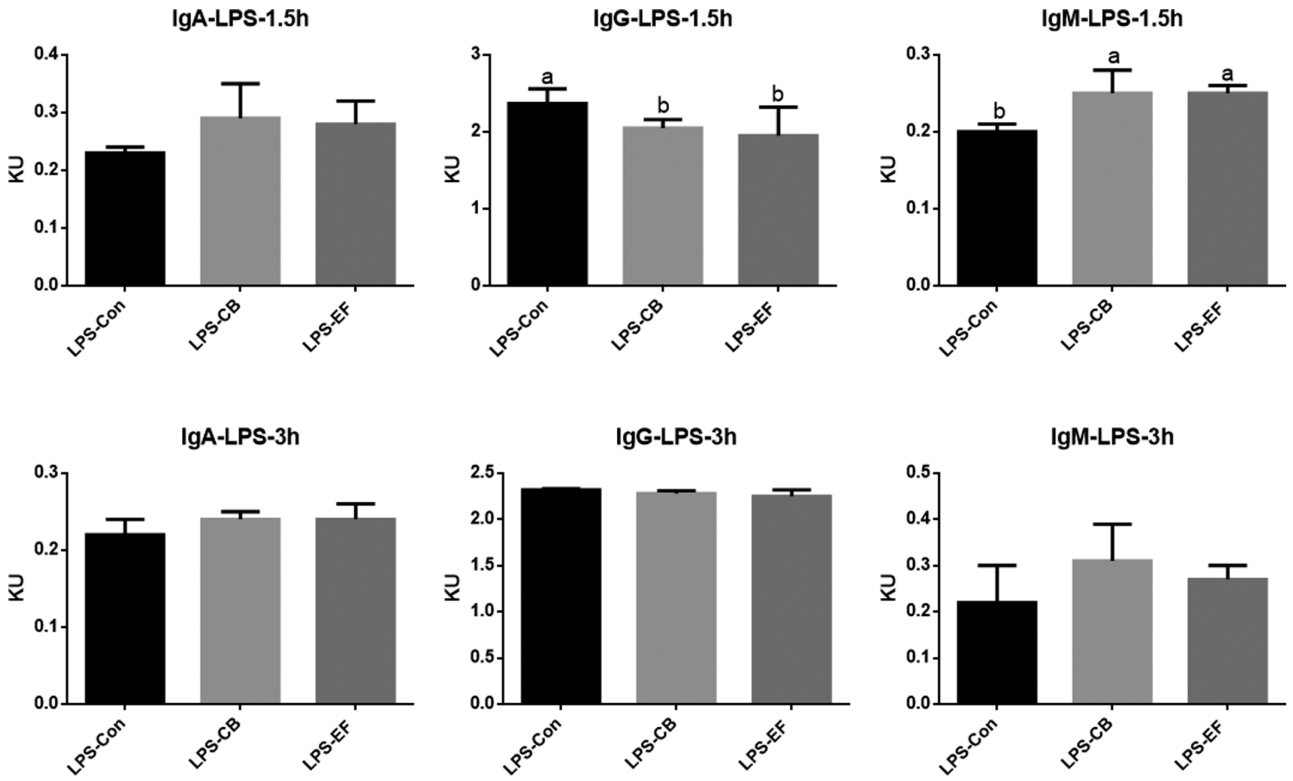

Figure 2. Effects of *C. butyricum* and *E. faecalis* on IgA, IgG, IgM in weaned piglets challenged with LPS involved 1.5 h and 3 h. LPS-Con represents the control piglets challenged with LPS on day 30; LPS-CB represents the piglets supplemented with *C. butyricum* and challenged with LPS on day 30; LPS-EF represents the piglets supplemented with *E. faecalis* and challenged with LPS on day 30; Small superscript letter indicates differences ($P < 0.05$). Values means $n = 6$ for the analysis of serum inflammatory factor indexes.

Expression of Jejunal TLR4, MyD88, and NF-κB

Compared with the control piglets, CB and EF supplementation decreased the protein expression of TLR4, MyD88, and NF-κB following challenge with LPS ($P < 0.05$) (Fig. 5). *Clostridium butyricum* or *E. faecalis* therefore appeared to have a significant effect on suppressing inflammation.

Analysis of Colon Microbial Community Structure

The abundance and diversity of microorganisms in colonic contents were determined based on high-throughput 16S-rRNA sequencing. A Venn diagram showed that a total of 1,404 operational taxonomic units (**OTUs**) were shared among the 3 treatment groups. The control group piglets had 79 unique OTUs, whereas CB and EF group piglets had 68 and 35 unique OTUs, respectively (Fig. 6A). Beta diversity (UPGMA cluster tree, Principal component analysis) analysis indicated that the microbial communities derived from CB or EF group animals showed varying degrees of difference from the control group piglets (Fig. 6B and C). The alpha diversity (Shannon) of bacteria in *C. butyricum* and *E. faecalis* piglets was found to be higher than that in control piglets (Fig. 6D). At the phylum level, the microbial composition of colonic contents in piglets was Firmicutes, Bacteroidetes, Proteobacteria, and Tenericutes (Fig. 6E), whereas at the family level, *Clostridiaceae*, *Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were predominant (Fig. 6F). We found that the relative abundance of *Bacteroidales-Rikenellanceae* in the CB group was significantly higher than that in the control group

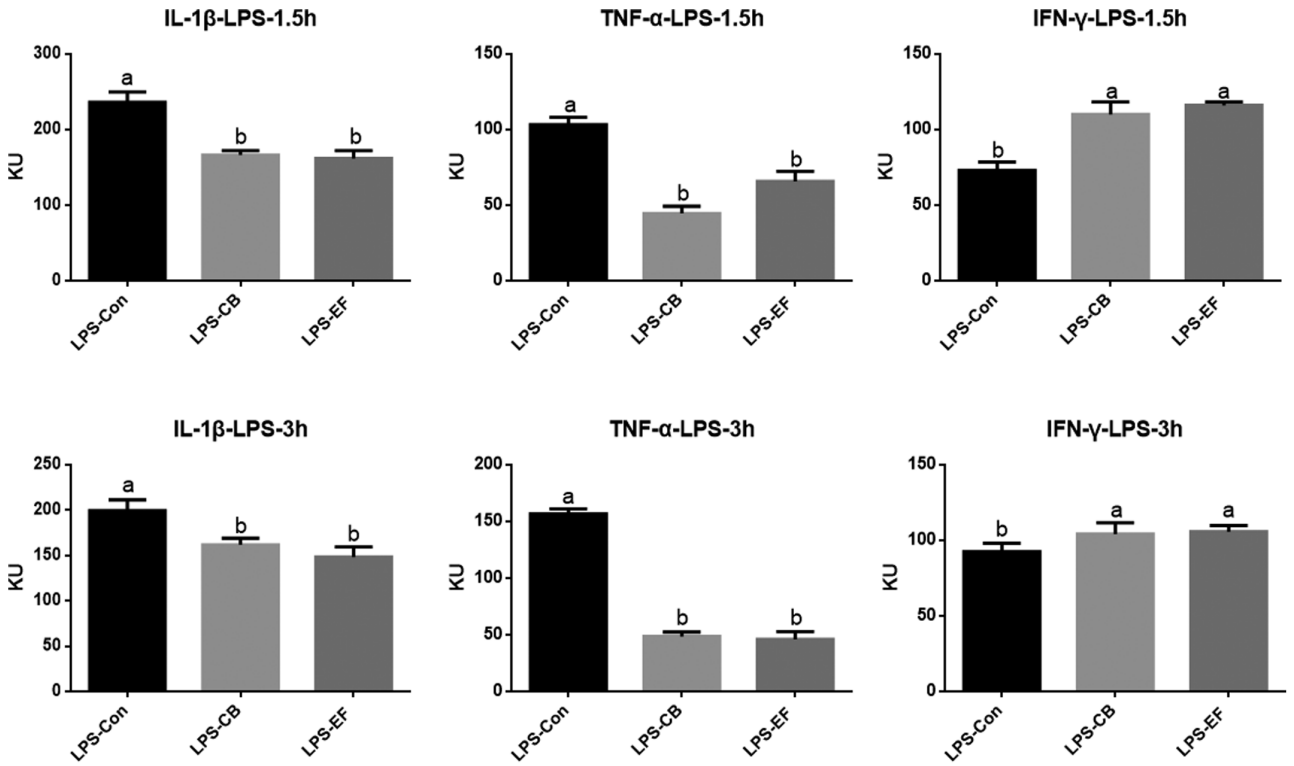

Figure 3. Effects of *C. butyricum* and *E. faecalis* on serum IL-1β, TNF-α, IFN-γ in weaned piglets challenged with LPS involved 1.5 h and 3 h. LPS-Con represents the control piglets challenged with LPS on day 30; LPS-CB represents the piglets supplemented with *C. butyricum* and challenged with LPS on day 30; LPS-EF represents the piglets supplemented with *E. faecalis* and challenged with LPS on day 30; Small superscript letter indicates differences ($P < 0.05$). Values means $n = 6$ for the analysis of serum inflammatory factor indexes.

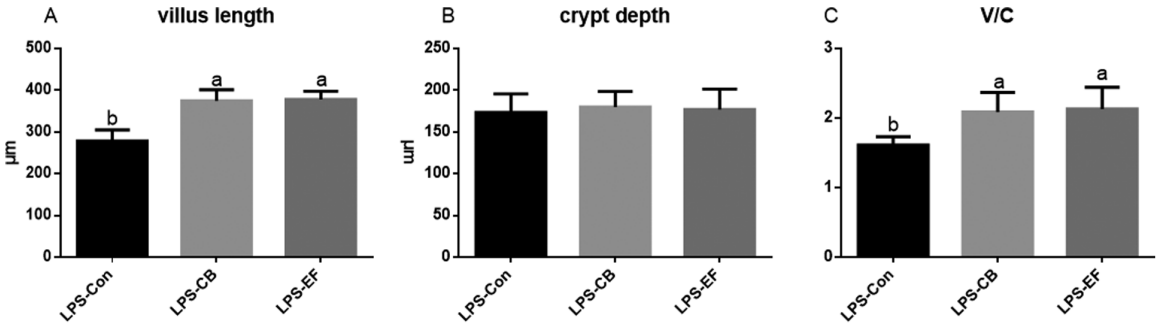

Figure 4. Effects of *C. butyricum* and *E. faecalis* on the jejunal villus height and the depth of jejunal crypt and the jejunal V/C in weaned piglets challenged with LPS. (A) The villus length in the jejunum of weaned piglets; (B) the crypt depth in the jejunum of weaned piglets; (C) the ratio of the villus length and crypt depth. LPS-Con represents the control piglets challenged with LPS on day 30; LPS-CB represents the piglets supplemented with *C. butyricum* and challenged with LPS on day 30; LPS-EF represents the piglets supplemented with *E. faecalis* and challenged with LPS on day 30; small superscript letter indicates differences ($P < 0.05$). Values means $n = 10$ for the analysis of the jejunum form.

($P < 0.05$) (Fig. 6J). At the genus level, *Clostridium*, *Lactobacillus*, *Prevotellaceae*, *Terrisporobacter*, and *Roseburia* were the dominant genera in all samples, with the abundance of *Lactobacillus* in EF group being higher than that in the control group (Fig. 6G). Furthermore, the relative abundance of *unidentified Prevotellaceae* in the CB group was higher than that in control group ($P < 0.05$) (Fig. 6L). At the species level, we found that *Ruminococcus* spp, *Lactobacillus amylovorus*, *Selenomonas bovis*, *Alloprevotella* spp, *Lactobacillus gasseri*, and *Megasphaera elsdenii* were the dominant species (Fig. 6H), and the abundance of *L. amylovorus* and *L. gasseri* in the EF group being were higher than that in the control group (Fig. 6L).

Discussion

In the past few decades, probiotics have been demonstrated to have positive effects in animals. However, previous studies have tended to report inconsistent observations regarding the effect of probiotics on growth performance. For example, whereas *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* have been shown to be beneficial in enhancing the growth performance in broilers (Mountzouris et al., 2007). Biernasiak et al. (2009) reported that probiotics have no significant effect on broiler growth performance. Similarly, although Yang et al. (2012) and Liao et al. (2015) have shown that *C. butyricum* promotes

growth performance in broilers. Zhang et al. (2011) showed that supplementation with 1 × 10^9 CFU *C. butyricum*·kg^{-1} had no effect on the growth performance of these birds. In piglets, Lu et al. (2018) found that *Enterococcus faecium*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Lactobacillus paracasei* improved piglet growth performance and relieved diarrhea caused by weaning stress, whereas Liu et al. (2018) showed that *Lactobacillus plantarum* increased daily weight gain and feed conversion ratio. Zong et al. (2019) showed that feeding piglets with *C. butyricum* effectively improved growth performance and reduced the rate of diarrhea. Similarly, Chen et al. (2018a) showed that dietary supplementation with *C. butyrium* had positive effects on the growth of weaned piglets, although it had no significant effect on elevation of the G:F ratio. Furthermore, Hu et al. (2015) indicated that *E. faecalis* can promote the growth performance and reduce diarrhea in weaned piglets. In the present study, we found that the dietary supplementation with *C. butyricum* or *E. faecalis* led to a higher final BW and ADG. In addition, G:F ratio of animals in the groups supplemented with probiotics was reduced compared with that in control piglets. Moreover, the ratio of diarrhea piglets fed *C. butyricum* or *E. faecalis* was markedly reduced compared with of control group piglets.

Amino acid metabolic enzymes, such as AST and ALT, are highly active in the liver and are important indicators of liver function (Hijmans et al., 2014). Previous studies have indicated that probiotics have no significant effects on serum AST and ALT concentrations in broilers under either normal conditions or heat stress (Sohail et al., 2011). However, probiotics have been shown reduce serum AST and ALT concentrations in patients with alcoholic liver disease (Kirpich et al., 2008). Cao et al. (2019) showed that the *Clostridium butyricum*-based probiotics reduced the concentration of AST and ALT in serum, improved liver function. In the present study, we found that LPS-challenged piglets fed *C. butyricum* or *E. faecalis* had lower AST and ALT levels than LPS-challenged control piglets, which indicates that probiotic *C. butyricum* or *E. faecalis* can alleviate liver damage induced by LPS.

Lipopolysaccharide treatment causes an inflammatory reaction in hosts, via changes in various inflammatory factors in blood. Dietary supplementation with probiotics has been shown to enhance serum IgA, IgM, and IL-6 concentrations in broilers (Zhang et al., 2015), and compared with control broilers, birds fed *C. butyricum* have been found to have greater serum IgM at day 21(Han et al., 2018). In the present study, we showed that dietary supplementation with *C. butyricum* or *E. faecalis* increased the serum concentrations IgA and IgM and improved immune function. Previously, it has been shown that dietary supplementation with *C. butyricum* can prevent experimental acute colitis in mice through induction of IL-10 (Kanai et al., 2015), and can significantly reduce concentrations of TNF-α in broiler (Chen et al., 2018b). Furthermore, *E. faecalis* has been shown to induce the secretion of IL-6 and IFN-γ in the K mouse intestinal epithelial cell line (Hoffmann et al., 2011). Studies have also found that piglets infected with only enterotoxigenic *Escherichia coli* K88 (ETEC K88) have elevated mRNA levels of the proinflammatory cytokine IL-1β compared with untreated pigs at 6 and 24 h post-ETEC K88 infection (Li et al., 2018). In the present study, we found that concentrations of the proinflammatory factors IL-1β and TNF-α in the serum of CB or EF group piglets challenged with LPS were lower than those in control group piglets, whereas serum concentrations of the serum anti-inflammatory factor IFN-γ were significantly increased compared with the control group following LPS challenge. Collectively, these findings indicate that dietary

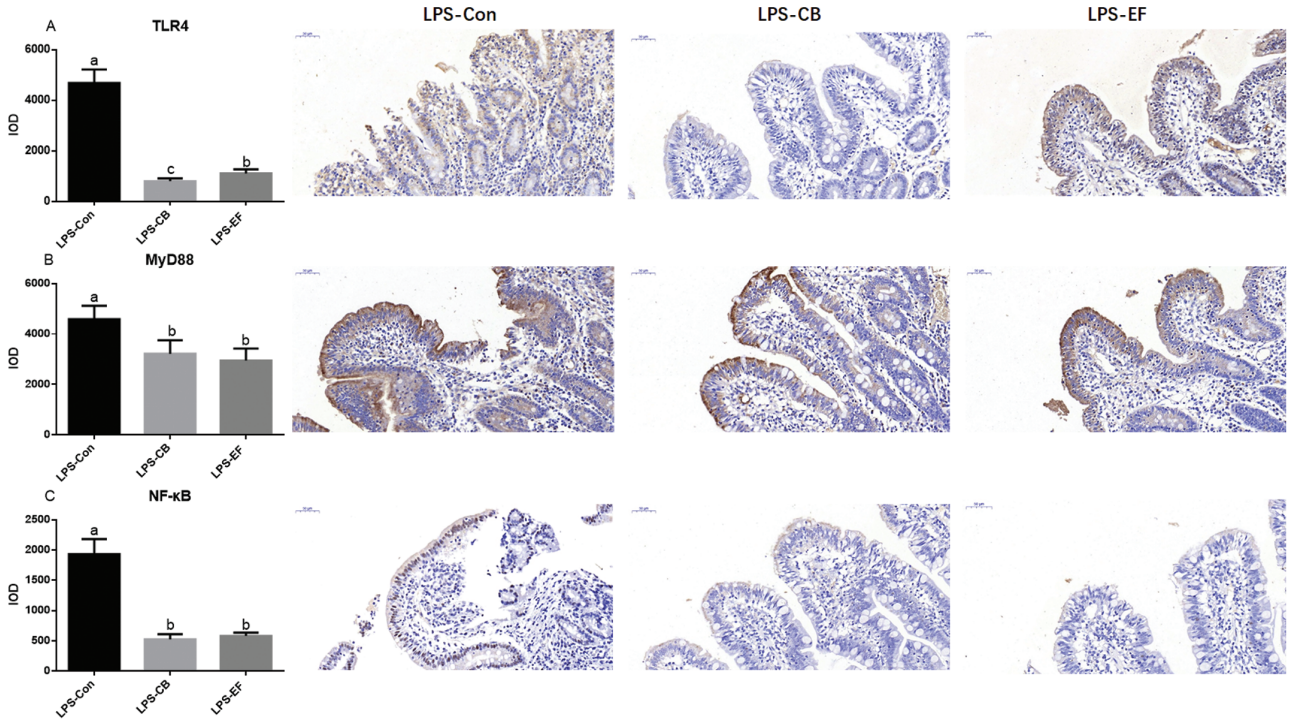

Figure 5. Effects of *C. butyricum* and *E. faecalis* on jejunal about TLR4, MyD88, and NF-κB in weaned piglets challenged with LPS. This is the result of immunohistochemistry in jejunal mucosa. (A) Immunohistochemical results of TLR4; (B) immunohistochemical results of MyD88; (C) immunohistochemical results of NF-κB. LPS-Con represents the control piglets challenged with LPS on day 30; LPS-CB represents the piglets supplemented with *C. butyricum* and challenged with LPS on day 30; LPS-EF represents the piglets supplemented with *E. faecalis* and challenged with LPS on day 30; Small superscript letter indicates differences ($P < 0.05$). Values means $n = 6$ for the analysis of jejunal mucosa.

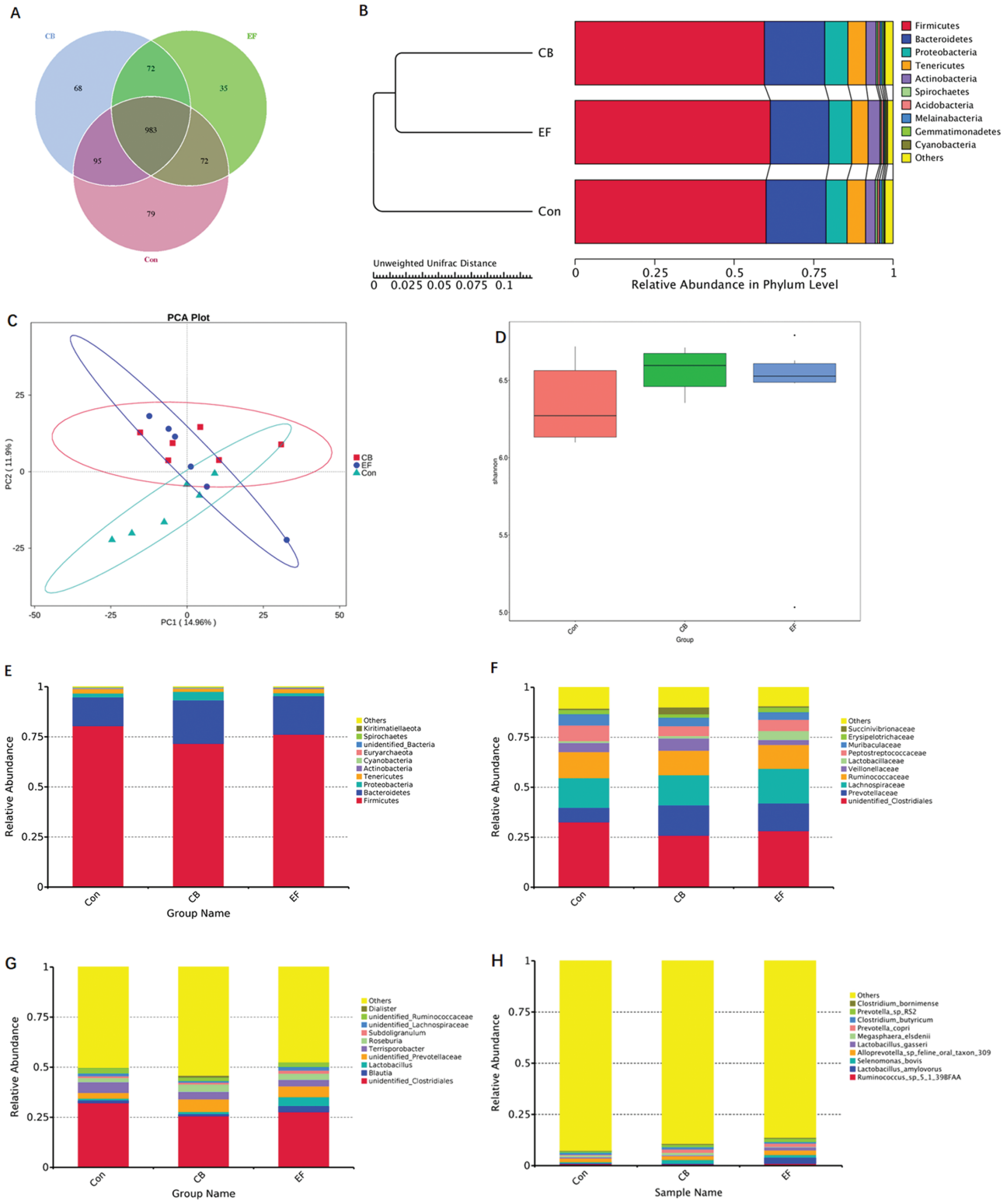

Figure 6. Summary of microbial community in colon contents of weaned piglets. (A) The Venn diagram summarizing the numbers of common and unique OTUs in the microflora community in colonic contents of weaning piglets. (B) UPGMA Cluster Tree displaying the relative abundances of predominant bacteria at the species level in each group (unweighted UniFrac distance). (C) Principle component analysis plot about the colonic microflora. (D) Shannon index reflecting species diversity within and between groups. (E–H) The top 10 relative abundance of microflora community (level phylum, family, genus, species). (I) Differences of dominant species between groups (level species). (J–L) Species with significant differences between groups (level family, genus, species). Con represents the control piglets challenged with LPS; CB represents the piglets supplemented with the *C. butyricum* challenged with LPS; EF represents the piglets supplemented with *E. faecalis* challenged with LPS. OTU,

operational taxonomic unit. Piglets were regarded as the experimental units, each treatment with $n = 6$. *Means different ($P < 0.05$), ** means significant difference ($P < 0.01$)

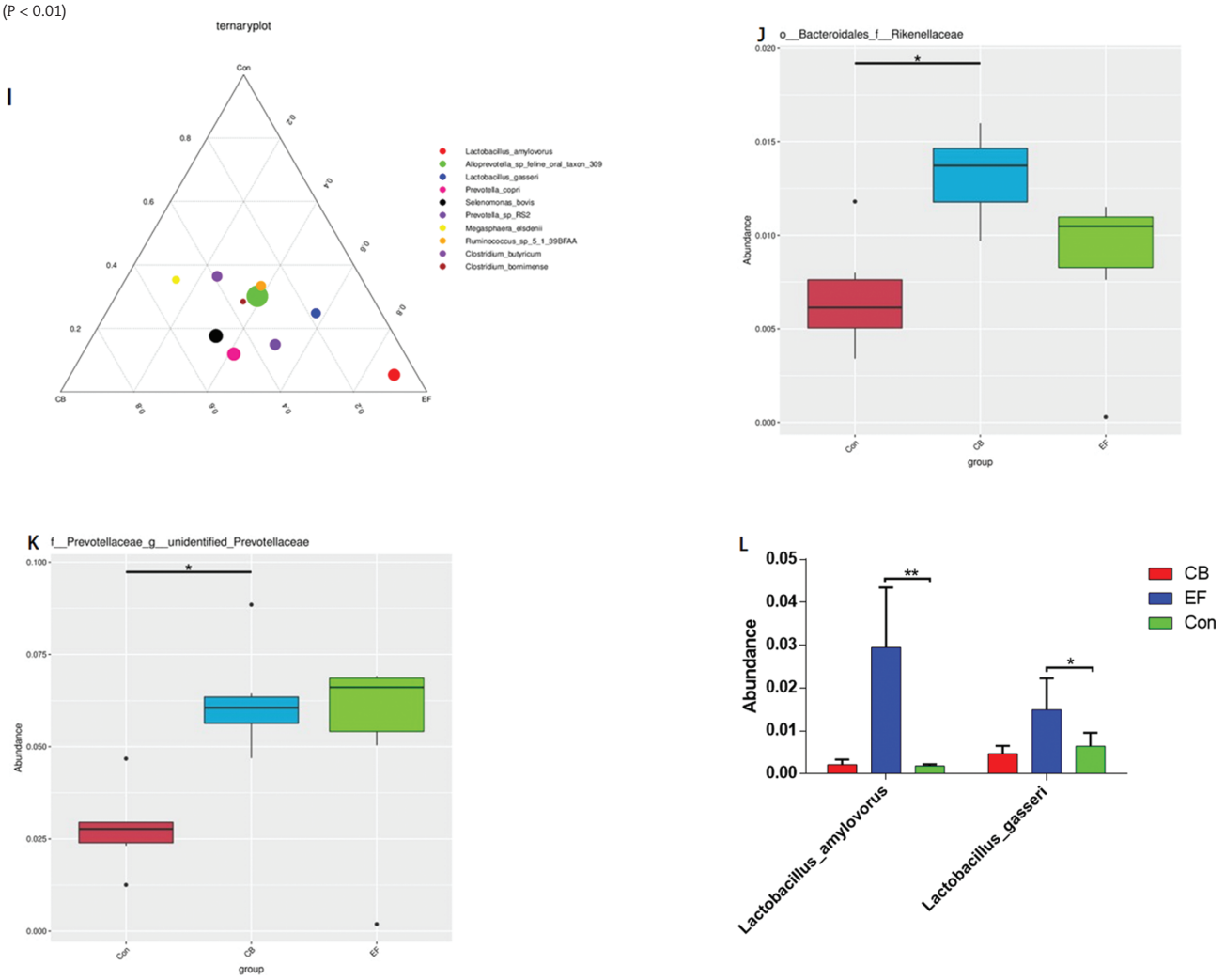

Figure 6. Continued

supplementation with *C. butyricum* or *E. faecalis* effectively alleviates the LPS-induced inflammatory response.

The length of villi and depth of crypts in the intestine are considered primary indicators for detecting any perturbation of intestinal function. Previous studies in broilers have shown that probiotic supplementation can increased villus height and the ratio of villus height to crypt depth, whereas the crypt depth was reduced (Awad et al., 2009; Kim et al., 2012). Research has also shown that daily oral administration of *E. faecalis* can alleviate the intestinal damage in piglets or young rats due to weaning, and significantly increase the height of the jejunal villi (Tsukahara et al., 2011). Furthermore, broilers fed *C. butyricum* have been found to have a significantly increased ratio of villus height to crypt depth (Cao et al., 2012), and Zhang et al. (2016) have shown that the supplementation of basal diet with *C. butyricum* increased jejunal villus height and decreased crypt depth in broiler chickens challenged with *E. coli* K88. In the present study, we demonstrated that inclusion of *C. butyricum* or *E. faecalis* in the diet increased the ratio of jejunum villus height to crypt depth, thereby indicating that *C. butyricum* or *E. faecalis* can protect the intestinal development and facilitate the maintenance of normal intestinal function following LPS stimulation.

Toll-like receptors 4 (TLR4), one of the Toll-like receptors (**TLRs**), regulates the inflammatory response by recognizing LPS and mediating its signal transduction, thereby playing an important role in the immune system in response to against the invasion of gram-negative bacteria (Gordon, 2002; Akira et al., 2004). TLR4, the first member of TLR family to be discovered, belongs to the type I transmembrane receptors of the TLR interleukin super-family, and functions in proinflammatory and immune responses (Lin et al., 2017). Nonspecific recognition, combined with pathogen-associated molecular patterns, activates NF-κB-related protein pathways, and promotes inflammatory reactions (Rashidian et al., 2016). TLR-mediated myocardial inflammation causes severe damages to the myocardium, and the TLR4/MyD88/NF-κB signaling pathway plays an important role in coronary microembolism (CME)-induced myocardial injury (Heusch et al., 2016). It has been demonstrated that *Lactobacillus* can inhibit the NF-κB activation and TLR4 expression induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) in mice (Lee et al., 2009), and Lim et al. (2017) have shown that *Bifidobacterium* can ameliorate high-fat diet induced colitis in mice by inhibiting the activation of NF-κB and production of LPS. Furthermore, it has been reported that *C. butyricum* inhibits the phosphorylation of NF-κB, nuclear

factor kappa-B RelA (NF-κB p65), and extracellular regulated protein kinases in the gastric tissues (Liu et al., 2016). Zhao et al. (2017) revealed that the inflammation of chickens were alleviated by *C. butyricum* via down-regulating TLR4, MyD88, and NF-κB-dependent pathways. Ushida et al. (2010) showed that *E. faecalis* promotes the production of inflammatory factor IL-12 by mediating MyD88 in mice. To date, however, there have been relatively few studies on the effects of *C. butyricum* and *E. faecalis* onTLR4 inflammation-related pathways in weaned piglets. In the present study, we found that among the piglets challenged with LPS, the protein expression levels of TLR4, MyD88, NF-κB in the jejunum of piglets fed *C. butyricum* or *E. faecalis* were significantly decreased compared with those in control piglets fed the basal diet. Both *C. butyricum* and *E. faecalis* were found to alleviate the inflammatory responses induced by LPS through modulating the TLR4 inflammatory signaling pathway.

The structure of the intestinal microbial community is extremely complex and plays a key role in feed utilization, digestive tract integrity, and animal health. In piglets, the colon is the main site of microbial colonization (Mazmanian et al., 2008; Kong et al., 2011; Luo et al., 2013). A study by Duan et al. (2018) confirmed that *C. butyricum* optimized the intestinal microbial structure of Penaeus vannamei, as determined by alpha diversity analysis (Shannon, abundance-based coverage estimator), which is consistent with the results of the present study. Also consistent with the findings of the present study, Hagihara et al. (2018) found that *C. butyricum* increased the abundance of the bacterial genera *Bifidobacterium*, *Coprococcus*, and *Bacteroides*. We detected a higher abundance of *Bacteroidetes* and *Proteobacteria* in the CB group piglets compared with those in the control group, as indicated by a species relative abundance map and UPGMA cluster tree. Miao et al. (2018) found that *C. butyricum* administered to breast-feeding maternal mice can regulate the balance of intestinal flora in their offspring. In our previous study, we demonstrated that *C. butyricum* reduces the number of *E. coli* in the intestines of broiler chickens and increases the number of *Bifidobacterium*, thereby regulating the intestinal flora (Cao et al., 2012). Zhang et al. (2014) showed that birds fed *C. butyricum* had decreased and increased populations of *E. coli* and *Lactobacillus*, respectively, compared with control birds challenged with *E. coli* K88, whereas Chen et al. (2018a) found that *C. butyricum* increased the levels of *Bacillus* and *Ruminococcaceae* in the intestines of LPS-challenged piglets, although there were no differences between CB group and control piglets with regards to the levels of *Bacillus* and *Ruminococcaceae*. In the present study, we found that among the piglets challenged with LPS, those receiving diets supplemented with *C. butyricum* showed enhanced growth performance compared with control group.

In addition, Samli et al. (2007) found that broilers fed with probiotic *E. faecium* were characterized by an increase in lactic acid bacteria colonization in the ileal content of chicks. *Bacillus* is one of the members of microbial that can be directly fed to animals (Gerritsen et al., 2011), and in this study, an increase in *L. amylovorus* and *L. gasseri* in LPS-challenged piglets fed an *E. faecalis*-supplemented diet indicates the beneficial effect of this probiotic bacterium. Oh et al. (2018) reported that *L. gasseri* has a strong ability to colonize the intestines, which has beneficial antioxidative, anti-inflammatory effects and also inhibits α-glucosidase activity and nitric oxide production in livestock. Moreover, *L. gasseri* has also been shown to inhibit the antiviral activity of respiratory syncytial virus, to upregulate the genes stimulated by interferon, and to increase the level of interferon (Eguchi et al., 2019), and effectively inhibits *Staphylococcus aureus* (Cifuentes et al., 2019). Moreover, Slavica et al. (2015) have reported that *L. amylovorus* converts glucose into lactate and acetate for absorption and utilization by host animals. *Lactobacillus amylovorus* has also been shown to protect the intestinal cells from ETEC K88 infection through inhibiting the ETEC-induced increase in the levels of TLR4 and MyD88, the phosphorylation of inhibitor of nuclear factor kappa-B kinase (IKK α), inhibitor of nuclear factor kappa-B kinase (IKK β), inhibitor of NF-κB (IκB α), and NF-κB p65, and over-production of the inflammatory cytokines IL-8 and IL-1β (Finamore et al., 2014). In the present study, we found that dietary supplementation with *C. butyricum* or *E. faecalis* increased the diversity of the intestinal microflora in LPS-challenged piglets, optimized intestinal flora structure, and effectively improved piglet health.

Conclusion

In this study, we demonstrate that dietary supplementation with either *C. butyricum* or *E. faecalis* enhances the growth performance of weaned piglets. We also observed a tendency of *C. butyricum* and *E. faecalis* to enhance immune function by alleviating liver damage. Moreover, *C. butyricum* and *E. faecalis* improved intestinal morphology of LPS-challenged piglets, decreased the expression of inflammation-related pathway proteins, and increased the abundance and diversity of the colonic microbial population in LPS-challenged piglets.

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Conflict of interest statement.

None declared.

Literature Cited

Akira, S., and K. Takeda. 2004. Toll-like receptor signaling. *Nat. Rev. Immunol.* **4**:499–511. doi:10.1074/jbc.R300028200

Andreas, P., C. Farfán-López, Y. Rondón, F. Mora, M. Rossini, and H. Araque. 2016. Effect of using mannoproteins and antibiotics as growth promoters in diets for weaned piglets on performance. *Rev. Cient-Fac. Cien. V.* **26**:26–32. http://www.redalyc.org/articulo.oa?id=95944832006

Asai, T., K. Masani, C. Sato, M. Hiki, M. Usui, K. Baba, M. Ozawa, K. Harada, H. Aoki, and T. Sawada. 2011. Phylogenetic groups and cephalosporin resistance genes of *Escherichia coli* from diseased food-producing animals in japan. *Acta Vet. Scand.* **53**:52. doi:10.1186/1751-0147-53-52.

Awad, W. A., K. Ghareeb, S. Abdel-Raheem, and J. Böhm. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. *Poult. Sci.* **88**:49–56. doi:10.3382/ps.2008-00244.

Biernasiak, J., and K. Slizewska. 2009. The effect of a new probiotic preparation on the performance and faecal microflora of broiler chickens. *Veter. Med-Czech.* **54**:525–531.

Cao, G. T., F. Tao, Y. H. Hu, Z. M. Li, Y. Zhang, B. Deng, and X. A. Zhan. 2019. Positive effects of a Clostridium butyricum-based compound probiotic on growth performance,

immune responses, intestinal morphology, hypothalamic neurotransmitters, and colonic microbiota in weaned piglets. *Food. Funct*. **10**: 2926–2934. doi:10.1039/c8fo02370k.

Cao, G. T., Y. P. Xiao, C. M. Yang, A. G. Chen, T. T. Liu, L. Zhou, L. Zhang, and P. R. Ferket. 2012. Effects of *Clostridium butyricum* on growth performance, nitrogen metabolism intestinal morphology and cecal microflora in broiler chickens. *J. Anim. Vet. Adv*. **11**: 2665–2671. doi:10.3923/javaa.2012.2665.2671

Chen, L., S. Li, J. Zheng, W. Li, X. M. Jiang, X. L. Zhao, J. Li, L. Q. Che, Y. Lin, S. Y. Xu, *et al*. 2018a. Effects of dietary *Clostridium butyricum* supplementation on growth performance, intestinal development, and immune response of weaned piglets challenged with lipopolysaccharide. *J. Anim. Sci. Biotechno*. **9**:62. doi:10.1186/s40104-018-0275-8

Chen, Y. P., H. Zhang, Y. F. Cheng, Y. Li, C. Wen, and Y. M. Zhou. 2018b. Dietary L-threonine supplementation attenuates lipopolysaccharide-induced inflammatory responses and intestinal barrier damage of broiler chickens at an early age. *Brit. J. Nutr*. **119**: 1254–1262. doi:10.1017/s0007114518000740

Cifuentes, G. C., M. Rodriguez-Palmero, J. Jimenez, and J. A. Moreno. 2019. Antimicrobial activity against human pathogenic strain of *Staphylococcus aureus* CECT4013 by *Lactobacillus gasseri* strains. *Ann. Nutr. Metab*. **74** (Supplement.1):27. doi:10.1159/000496759

Dheilly, A., A. Bouder, L. Le Devendec, G. Hellard, and I. Kempf. 2011. Clinical and microbial efficacy of antimicrobial treatments of exprimental avian colibacillosis. *Vet. Microbiol*. **149**:422–429. doi:10.1016/j.vetmic.2010.11.033

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: History and mode of action. *Poult. Sci*. **84**:634–643. doi:10.1093/ps/84.4.634

Duan, Y. F., Y. Wang, H. B. Dong, X. Ding, Q. S. Liu, H. Li, J. S. Zhang, and D. L. Xiong. 2018. Changes in the intestine microbial, digestive, and immune-related genes of *Litpoenaeus vannamei* in response to dietary probiotic *Clostridium butyricum* supplementation. *Front. Microbiol*. **9**. doi:10.3389/fmicb.2018.02191.

Eguchi, K., N. Fujitani, H. Nakagawa, and T. Miyazaki. 2019. Prevention of respiratory syncytial virus infection with probiotic lactic acid bacterium *Lactobacillus gasseri* SBT2055. *Sci. Rep*. **9**:4812. doi:10.1038/s41598-019-39602-7.

Finamore, A., M. Roselli, A. Imbinto, J. Seeboth, I. P. Oswald, and E. Mengheri. 2014. *Lactobacillus amylovorus* inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic *Escherichia coli* via modulation of the negative regulators and involvement of TLR2 in intestinal caco-2 cells and pig explants. *Plos One* **9**:e94891. doi:10.1371/journal.pone.0094891.

Finegold, S. M., V. L. Sutter, and G. E. Mathisen. 1983. *Normal indigenous intestinal flora. Human Intestinal Microflora in Health and Disease*. New York: Academic Press. 3–31. doi:10.1016/b978-0-12-341280-5.50007-0.

Gaggìa, F., B. Mattarelli, and B. Biavati. 2010. Probiotics and prebiotics in animal feeding for safe food production. *Int. J. Food Microbiol*. **141**:S15–S28. doi:10.1016/j.ijfoodmicro.2010.02.031

Gerritsen, J., H. Smidt, G. T. Rijkers, and W. M. de Vos. 2011. Intestinal microbiota in human health and disease: The impact of probiotics. *Genes Nutr*. **6**:209–240. doi:10.1007/s12263-011-0229-7.

Gordon, S. 2002. Pattern recognition receptors: Doubling up for the innate immune response. *Cell* **111**:927–930. doi:10.1016/s0092-8674(02)01201-1.

Hagihara, M., R. Yamashita, A. Matsumoto, T. Mori, Y. Kuroki, H. Kudo, K. Oka, M. Takahashi, T. Nonogaki, Y. Yamagishi, *et al*. 2018. The impact of clostridium butyricum MIYAIRI 588 on the murine gut microbiome and colonic tissue. *Anaerobe* **54**:8–18. doi:10.1016/j.anaerobe.2018.07.012.

Han, J. F., Y. W. Wang, D. Song, Z. X. Lu, Z. L. Dong, H. J. Miao, W. W. Wang, J. H. He, and A. K. Li. 2018. Effects of *Clostridium butyricum* and *Lactobacillus plantarum* on growth performance, immune function and volatile fatty acid level of caecal digesta in broilers. *Food. Agr. Immunol* **29**:797–807. doi:10.1080/09540105.2018.1457013

Hayashi, A., T. Sato, N. Kamada, Y. Mikami, K. Matsuoka, T. Hisamatsu, T. Hibi, A. Roers, H. Yagita, T. Ohteki, *et al*. 2013. A single strain of *Clostridium butyricum* induces intestinal IL-10-producing macrophages to suppress acute experimental colitis in mice. *Cell Host Microbe* **13**:711–722. doi:10.1016/j.chom.2013.05.013.

Heusch, G., and B. J. Gersh. 2016. The pathophysiology of acute myocardial infarction and of strategies of protection beyond reperfusion: A continual challenge. *Eur. Heart J*. **38**:774–784. doi:10.1093/eurheartj/ehw224

Hijmans, B. S., A. Grefhorst, M. H. Oosterveer, and A. K. Groen. 2014. Zonation of glucose and fatty acid metabolism in the liver: Mechanism and metabolic consequences. *Biochimie* **96**:121–129. doi:10.1016/j.biochi.2013.06.007.

Hoffmann, M., A. Messlik, S. C. Kim, R. B. Sartor, and D. Haller. 2011. Impact of a probiotic *Enterococcus faecalis* in a gnotobiotic mouse model of experimental colitis. *Mol. Nutr. Food Res*. **55**:703–713. doi:10.1002/mnfr.201000361.

Hu, Y., Y. Dun, S. Li, D. Zhang, N. Peng, S. Zhao, and Y. Liang. 2015. Dietary *Enterococcus faecalis* LAB31 improves growth performance, reduces diarrhea, and increases fecal *Lactobacillus* number of weaned piglets. *Plos One* **10**:e0116635. doi:10.1371/journal.pone.0116635.

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. *Vet. J*. **187**:182–188. doi:10.1016/j.tvjl.2010.03.003.

Kanai, T., Y. Mikami, and A. Hayashi. 2015. A breakthrough in probiotics: *Clostridium butyricum* regulates gut homeostasis and anti-inflammatory response in inflammatory bowel disease. *J. Gastroenterol*. **50**:928–939. doi:10.1007/s00535-015-1084-x.

Kim, J. S., S. L. Ingale, Y. W. Kim, K. H. Kim, S. Sen, M. H. Ryu, J. D. Lohakare, I. K. Kwon, and B. J. Chae. 2012. Effect of supplementation of multi-microbe probiotic product on growth performance, apparent digestibility, cecal microbiota and small intestinal morphology of broilers. *J. Anim. Physiol. Anim. Nutr. (Berl)*. **96**:618–626. doi:10.1111/j.1439-0396.2011.01187.x.

Kirpich, I. A., N. V. Solovieva, S. N. Leikhter, N. A. Shidakova, O. V. Lebedeva, P. I. Sidorov, T. A. Bazhukova, A. G. Soloviev, S. S. Barve, C. J. McClain, *et al*. 2008. Probiotics restore bowel flora and improve liver enzymes in human alcohol-induced liver injury: A pilot study. *Alcohol* **42**:675–682. doi:10.1016/j.alcohol.2008.08.006.

Kong, Q., G. Q. He, J. L. Jia, Q. L. Zhu, and H. Ruan. 2011. Oral administration of *Clostridium butyricum* for modulating gastrointestinal microflora in mice. *Curr. Microbiol*. **62**:512–517. doi:10.1007/s00284-010-9737-8.

Lee, J. H., B. Lee, H. S. Lee, E. A. Bae, H. Y. Lee, Y. T. Ahn, K. S. Lim, C. S. Huh, and D. H. Kim. 2009. *Lactobacillus suntoryeus* inhibits pro-inflammatory cytokine expression and TLR-4-linked NF- κB activation in experimental colitis. *Int. J. Colorectal Dis*. **24**:231–237. doi:10.1007/s00384-008-0618-6

Lei, Y. C., L. L. Yang, W. Li, and P. Luo. 2015. Sphingosine kinase 1 dependent protein kinase C-δ activation plays an important role in acute liver failure in mice. *World J. Gastroenterol*. **21**:13438–13446. doi:10.3748/wjg.v21.i48.13438.

Li, H. H., Y. P. Li, Q. Zhou, J. Y. Qiao, and W. J. Wang. 2018. Dietary supplementation with *Clostridium butyricum* helps to improve the intestinal barrier function of weaned piglets challenged with enterotoxigenic *Escherichia coli* K88. *J. Appl. Microbiol*. **125**:964–975. doi:10.1111/jam.13936

Liao, X. D., G. Ma, J. Cai, Y. Fu, X. Y. Yan, X. B. Wei, and R. J. Zhang. 2015. Effects of *Clostridium butyricum* on growth performance, antioxidation, and immune function of broilers. *Poult. Sci*. **94**:662–667. doi:10.3382/ps/pev038.

Lim, S. M., and D. H. Kim. 2017. *Bifidobacterium adolescentis* IM38 ameliorates high-fat diet-induced colitis in mice by inhibiting

NF-κb activation and lipopolysaccharide production by gut microbiota. *Nutr. Res.* **41**:86–96. doi:10.1016/j.nutres.2017.04.003.

Lin, S., Y. Li, L. Shen, R. Zhang, L. Yang, M. Li, K. Li, and J. Fichna. 2017. The anti-inflammatory effect and intestinal barrier protection of HU210 differentially depend on TLR4 signaling in dextran sulfate sodium-induced murine colitis. *Dig. Dis. Sci.* **62**:372–386. doi:10.1007/s10620-016-4404-y.

Liu, W. C., J. S. Kang, and I. H. Kim. 2018. Dietary *Lactobacillus plantarum* GB805 supplementation improves growth performance and nutrient digestibility in weaning pigs. *Indian. J. Anim. Res.* **52**:1313–1316. doi:10.18805/ijar.B-852

Liu, J. M., F. Y. Wang, H. H. Luo, A. H. Liu, K. X. Li, C. Li, and Y. Jiang. 2016. Protective effect of butyrate against ethanol-induced gastric ulcers in mice by promoting the anti-inflammatory, anti-oxidant and mucosal defense mechanisms. *Int. Immunopharmacol.* **30**:179–187. doi:10.1016/j.intimp.2015.11.018

Lu, X., M. Zhang, L. Zhao, K. Ge, Z. Wang, L. Jun, and F. Ren. 2018. Growth performance and post-weaning diarrhea in piglets fed a diet supplemented with probiotic complexes. *J. Microbiol. Biotechnol.* **28**:1791–1799. doi:10.4014/jmb.1807.07026.

Luo, J., A. Zheng, K. Meng, W. Chang, Y. Bai, K. Li, H. Cai, G. Liu, and B. Yao. 2013. Proteome changes in the intestinal mucosa of broiler (*Gallus gallus*) activated by probiotic *Enterococcus faecium*. *J. Proteomics* **91**:226–241. doi:10.1016/j.jprot.2013.07.017.

Mazmanian, S. K., J. L. Round, and D. L. Kasper. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* **453**:620–625. doi:10.1038/nature07008.

Miao, R. X., X. X. Zhu, C. M. Wan, Z. L. Wang, Y. Wen, and Y. Y. Li. 2018. Effect of *Clostridium butyricum* supplementation on the development of intestinal flora and the immune system of neonatal mice. *Exp. Ther. Med.* **15**:1081–1086. doi:10.3892/etm.2017.5461.

Moran, A. P. 1995. Structure-bioactivity relationships of bacterial endotoxins. *J. Toxicol-Toxin Rev.* **14**: 47–83. doi:10.3109/15569549509089968

Mountzouris, K. C., P. Tsirtsikos, E. Kalamara, S. Nitsch, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult. Sci.* **86**:309–317. doi:10.1093/ps/86.2.309.

Oh, N. S., J. Y. Joung, J. Y. Lee, and Y. Kim. 2018. Probiotic and anti-inflammatory potential of *Lactobacillus rhamnosus* 4B15 and *Lactobacillus gasseri* 4M13 isolated from infant feces. *Plos One* **13**:e0192021. doi:10.1371/journal.pone.0192021.

Rashidian, A., A. Muhammadnejad, A. R. Dehpour, S. E. Mehr, M. M. Akhavan, R. Shirkoohi, M. Chamanara, S. E. Mousavi, and S. M. Rezayat. 2016. Atorvastatin attenuates TNBS-induced rat colitis: The involvement of the TLR4/NF-kb signaling pathway. *Inflammopharmacology* **24**:109–118. doi:10.1007/s10787-016-0263-6.

Samli, H. E., N. Senkoylu, F. Koc, M. Kanter, and A. Agma. 2007. Effects of *Enterococcus faecium* and dried whey on broiler performance, gut histomorphology and intestinal microbiota. *Arch. Anim. Nutr.* **61**:42–49. doi:10.1080/17450390601106655.

Slavica, A., A. Trontel, N. Jelovac, Ž. Kosovec, B. Šantek, and S. Novak. 2015. Production of lactate and acetate by lactobacillus coryniformis subsp. Torquens DSM 20004(T) in comparison with *Lactobacillus amylovorus* DSM 20531(T). *J. Biotechnol.* **202**:50–59. doi:10.1016/j.jbiotec.2015.01.014.

Sohail, M. U., Z. U. Rahman, A. Ijaz, M. S. Yousaf, K. Ashraf, T. Yaqub, H. Zaneb, H. Anwar, and H. Rehman. 2011. Single or combined effects of mannan-oligosaccharides and probiotic supplements on the total oxidants, total antioxidants, enzymatic antioxidants, liver enzymes, and serum trace minerals in cyclic heat-stressed broilers. *Poult. Sci.* **90**:2573–2577. doi:10.3382/ps.2011-01502.

Sumon, M. S., F. Ahmmed, S. S. Khushi, M. K. Ahmmed, M. A. Rouf, M. A. H. Chisty, *et al.* 2018. Growth performance, digestive enzyme activity and immune response of *Macrobrachium rosenbergii* fed with probiotic *Clostridium butyricum* incorporated diets. *JKSUS.* **30**: 21–28. doi:10.1016/j.jksus.2016.11.003

Takahashi, M., E. McCartney, A. Knox, M. Francesch, K. Oka, K. Wada, M. Ideno, K. Uno, K. Kozłowski, J. Jankowski, *et al.* 2018. Effects of the butyric acid-producing strain *Clostridium butyricum* MIYAIRI 588 on broiler and piglet zootechnical performance and prevention of necrotic enteritis. *Anim. Sci. J.* **89**:895–905. doi:10.1111/asj.13006.

Toit, M. D., C. M. A. P. Franz, L. M. T. Dicks, and W. H. Holzapfel. 2000. Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig faeces. *J. Appl. Microbiol.* **88**:482–494. doi:10.1046/j.1365-2672.2000.00986.x

Truusalu, K., P. Naaber, T. Kullisaar, H. Tamm, and M. Mikelsaar. 2004. The influence of antibacterial and antioxidative probiotic Lactobacilli on gut mucosa in a mouse model of Salmonella infection. *Microb. Ecol. Health Dis.* **16**:180–187. doi:10.1080/08910600410021783

Tsukahara, T., Y. Yoshida, T. Tsushima, T. Watanabe, N. Matsubara, R. Inoue, and K. Ushida. 2011. Evaluation of the heat-killed and dried cell preparation of *Enterococcus faecalis* against villous atrophy in early-weaned mice and pigs. *Anim. Sci. J.* **82**:302–306. doi:10.1111/j.1740-0929.2010.00829.x.

Ushida, K., Y. Yoshida, T. Tsukahara, T. Watanabe, and R. Inoue. 2010. Oral administration of Enterococcus faecalis EC-12 cell preparation improves villous atrophy after weaning through enhancement of growth factor expression in mice. Biomedical. *Res-Tokyo.* **31**:191–198. doi:10.2220/biomedres.31.191.

Walsh, M. C., D. M. Sholly, R. B. Hinson, K. L. Saddoris, A. L. Sutton, J. S. Radcliffe, R. Odgaard, J. Murphy, and B. T. Richert. 2007. Effects of water and diet acidification with and without antibiotics on weanling pig growth and microbial shedding. *J. Anim. Sci.* **85**:1799–1808. doi:10.2527/jas.2006–049.

Wyns, H., E. Meyer, E. Plessers, A. Watteyn, T. van Bergen, S. Schauvliege, S. De Baere, M. Devreese, P. De Backer, and S. Croubels. 2015. Modulation by gamithromycin and ketoprofen of in vitro and in vivo porcine lipopolysaccharide-induced inflammation. *Vet. Immunol. Immunopathol.* **168**:211–222. doi:10.1016/j.vetimm.2015.09.014.

Yang, C. M., G. T. Cao, P. R. Ferket, T. T. Liu, L. Zhou, L. Zhang, Y. P. Xiao, and A. G. Chen. 2012. Effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecal microflora in broiler chickens. *Poult. Sci.* **91**:2121–2129. doi:10.3382/ps.2011–02131.

Yang, C. M., L. L. Zhang, G. T. Cao, J. Feng, M. Yue, Y. L. Xu, B. Dai, Q. J. Han, and X. Q. Guo. 2018. Effects of dietary supplementation with essential oils and organic acids on the growth performance, immune system, faecal volatile fatty acids and microflora community in weaned piglets. *J. Anim. Sci.* **97**:133–143. doi:10.1093/jas/sky426

Zhang, L., G. T. Cao, X. F. Zeng, L. Zhou, P. R. Ferket, Y. P. Xiao, A. G. Chen, and C. M. Yang. 2014. Effects of *Clostridium butyricum* on growth performance, immune function, and cecal microflora in broiler chickens challenged with *Escherichia coli* K88. *Poult. Sci.* **93**:46–53. doi:10.3382/ps.2013–03412.

Zhang, L., J. Li, T. T. Yun, W. T. Qi, X. X. Liang, Y. W. Wang, and A. K. Li. 2015. Effects of pre-encapsulated and pro-encapsulated *Enterococcus faecalis* on growth performance, blood characteristics, and cecal microflora in broiler chickens. *Poultry. Sci.* **94**:2821–2830. doi:10.3382/ps/pev262

Zhang, J., Z. L. Shi, M. H. Zeng, C. Wei, H. X. Wu, M. Y. Liu, J. Q. Huang, Y. J. Zheng, and X. Sun. 2017. Oral administration of *Clostridium butyricum* CGMCC0313-1 reduces

ovalbumin-induced allergic airway inflammation in mice. *Respirology*. **22**: 898–904. doi:10.1111/resp.12985

Zhang, J., J. Sun, X. Y. Chen, C. X. Nie, J. B. Zhao, W. Y. Guan, L. H. Lei, T. He, Y. Q. Chen, L. J. Johnston, *et al*. 2018. Combination of *Clostridium butyricum* and Corn Bran Optimized Intestinal Microbial Fermentation Using a Weaned Pig Model. *Front. Microbiol*. **9**. doi:10.3389/fmicb.2018.03091

Zhang, B., X. Yang, Y. Guo, and F. Long. 2011. Effects of dietary lipids and *Clostridium butyricum* on the performance and the digestive tract of broiler chickens. *Arch. Anim. Nutr*. **65**:329–339.

Zhang, L., L. L. Zhang, X. A. Zhan, X. F. Zeng, L. Zhou, G. T. Cao, A. G. Chen, and C. M. Yang. 2016. Effects of dietary supplementation of probiotic, *Clostridium butyricum*, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with *Escherichia coli K88*. *J. Anim. Sci. Biotechno*. **7**. doi:10.1186/s40104-016-0061-4.

Zhao, X. N., J. Yang, L. L. Wang, H. Lin, and S. H. Sun. 2017. Protection mechanism of Clostridium butyricum against Salmonella Enteritidis infection in broilers. *Front. Microbio*. **8**. doi:10.3389/fmicb.2017.01523

Zong, X., T. H. Wang, Z. Q. Lu, D. G. Song, J. Zhao, and Y. Z. Wang. 2019. Effects of Clostridium butyricum or in combination with Bacillus licheniformis on the growth performance, blood indexes, and intestinal barrier function of weanling piglets. *Livest. Sci*. **220**:137–142. dio:10.1016/j.livsci.2018.12.024