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Effects of *Clostridium butyricum* and *Enterococcus faecalis* on growth performance, immune function, intestinal morphology, volatile fatty acids, and intestinal flora in a piglet model

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We investigated the effects of *Clostridium butyricum* and *Enterococcus faecalis* (probiotics) in a piglet model. Weaned piglets (180) were randomly divided into three treatment groups and fed basal diet and basal diet supplemented with 6×10^9 CFU *C. butyricum* per kg and 2×10^{10} CFU *E. faecalis* per kg, respectively. The probiotics improved the final body weight, average daily gain, and feed conversion rate, while they reduced the diarrhea rate. The serum aspartate aminotransferase and alanine aminotransferase activities in probiotic-supplemented piglets were decreased on days 14 and 28. Piglets supplemented with probiotics presented an increased serum immunoglobulin (Ig)M level on day 14 and IgA, IgG, and IgM levels on day 28 compared with control piglets, respectively. Moreover, the probiotics increased the jejunal villus length and jejunal villus height to crypt depth ratio, while they decreased the jejunal crypt depth compared with those of the control. Similarly, an increase in inflammation-related pathway factor expression was observed after probiotic administration. Piglets supplemented with probiotics had a higher concentration of volatile fatty acids in the colonic contents than that in the control. High-throughput sequencing indicated that the probiotics modulated the colon bacterial diversity. Species richness and the alpha diversity index of bacterial samples in probiotic-supplemented piglets were higher than those in the control. Piglets supplemented with *C. butyricum* presented a considerably high relative abundance of *C. butyricum* compared with that in the control. Overall, *C. butyricum* and *E. faecalis* can promote growth performance, protect the intestinal villi morphology, improve immunity, and optimize the intestinal flora in weaned piglets.

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Introduction

Weaning in piglets might increase diarrhea chances and reduce body metabolism and immune function, due to changes in food and environment.[1–4] Alleviating stress damage caused by weaning in piglets is an urgent problem to be solved. In the past decade, antibiotics have played an important role in controlling diseases in animals and enhancing animal growth and reproductive performance.[5,6] Antibiotics have been used as an additive to protect the health of weaned piglets. However, antibiotics can improve the tolerance of pathogenic bacteria[7,8] and kill beneficial bacteria.[9] Subsequently, the Food Agriculture Organization (FAO) and World Health Organization (WHO) in 2001 proposed that moderate amounts of probiotics can improve animal health.[10] Probiotics have the potential to replace antibiotics. Several studies have demonstrated that probiotics are beneficial for improving growth performance and immune function in animals,[11,12] maintaining the intestinal mucosal barrier integrity,[13] inhibiting pathogenic microorganisms,[14] and optimizing the intestinal flora structure.[15]

Clostridium butyricum (*C. butyricum*) and *Enterococcus faecalis* (*E. faecalis*) are important probiotics, and several studies have demonstrated their beneficial effects on animal health. *Clostridium butyricum* is a Gram-positive anaerobe that produces butyric acid, which exists in the intestine of healthy animals and humans.[16,17] Furthermore, *C. butyricum* has a stronger tolerance ability to lower pH, bile salt, a higher temperature environment, and several antibiotics than other probiotics. Therefore, *C. butyricum* has been regarded a good and safe food additive.[18] In a previous study, *C. butyricum* has been shown to improve growth performance, enhance the immune function, and adjust the microflora structure in broilers,[19]

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which is similar to the results of Chen in piglets.[20] Moreover, *C. butyricum* can repair the intestinal barrier in weaned piglets[21] and can improve the content of volatile fatty acid of caecal digesta in broilers.[22]

Studies have proven that enterococci can enhance growth performance,[23] maintain the balance of intestinal flora,[24] promote the absorption of nutrients,[25] and improve the immunity of host.[26] *Enterococcus faecalis* is a facultative anaerobic Gram-positive coccus, which colonizes the gastrointestinal tract and the oral cavity in humans as a normal commensal.[27] It improved product performance and affected the cecal microbial community structure in laying hens[28] and improved growth performance, reduced the diarrhea rate, and increased beneficial bacteria in weaned piglets.[29] In addition, *E. faecalis* increased the average daily gain, while it decreased the diarrhea rate and mortality of suckling piglets according to Liu's study.[30]

Although there are several studies on *C. butyricum* in broilers, there are only a few studies in piglets; moreover, studies on the effects of *E. faecalis* on the intestinal morphology and microbial community structure in weaned piglets are limited. Therefore, the present study was conducted to assess the effects of *C. butyricum* and *E. faecalis* on growth performance, serum parameters, immune function, intestinal morphology, volatile fatty acids, and intestinal microbial community in weaned piglets.

Experimental

Source of *C. butyricum* and *E. faecalis*

The probiotics used in this study were provided by Vegamax Biological Technology Co., Ltd (Huzhou, China). The *Clostridium butyricum* product was used at a concentration of 3×10^9 colony-forming unit (CFU) per gram *C. butyricum* powder. The *Enterococcus faecalis* product was used at a concentration of 1×10^{10} CFU per gram of *E. faecalis*. These two strains have been saved in the China General Microbiological Culture Collection Center (CGMCC). The collection number of *C. butyricum* is CGMCC 9386 and *E. faecalis* is CGMCC 9382.

Animals and feeding

The following procedures were approved by the ethics committee of the Zhejiang A&F University (Anji, China). One hundred and eighty Duroc × Landrace × Yorkshire weaned piglets (8.91 ± 0.1 kg, half male and half female) were selected according to the age, weight, and divided into three treatment groups. Six replicates per group and 10 piglets per replicate were used. Each group was fed a basal diet, the basal diet supplemented with 6×10^9 *C. butyricum* per kg, and the basal diet supplemented with 1×10^{10} *E. faecalis* per kg. The piglets were raised in Zhejiang Zhongtai Animal Husbandry Technology Co., Ltd (Shaoxing, China). After transferring to a cleaned and disinfected pig house, the piglets had a one-week feeding transition period. The piglets were fed at 8 : 00 a.m, 2 : 00 p.m, and 8 : 00 p.m everyday to ensure free access to drinking water and feed. The pig house disinfection and immunization procedures were carried out according to the routine method of the farm. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Zhejiang A&F University and approved by the Animal Ethics Committee of Zhejiang A&F University (Table 1).

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

Ingredients	Content (%)	Nutrient level/kg[b]	
Corn	52.00	DE, MJ	13.52
Wheat middling	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[a]	4.00	AP, %	0.42
Total	100		

[a] 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; and Se, 0.3 mg. [b] Values for net energy were calculated, and the contents of dry matter, crude protein, amino acid, Ca, and P were analyzed. DE, digestible energy; CP, crude protein; Lys, lysine; Met, methionine; Cys, cystine; Thr, threonine; Ile, isoleucine; Ca, calcium; TP, total phosphorus; AP, available phosphorus; and MJ, megajoule.

Growth performance

The body weight (BW) of piglets was measured at the beginning (day 1) and on day 28. The average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F : G) were calculated by recording the feed intake of piglets in each pen during the experiment period. The number of piglets with diarrhea in each pen was recorded during the experiment to calculate the diarrhea rate. Diarrhea rate = number of piglets with diarrhea per pen during the trial/(number of test pigs × number of test days) × 100.

Sample collection

On days 14 and 28, six healthy and similar weight pigs from each treatment group were selected and blood samples were collected from the vascular veins. On day 28, the piglets were sacrificed after blood collection. The blood samples were immediately centrifuged to obtain serum, which was stored at −20 °C. The middle segments of jejunum were stored in 4% paraformaldehyde at 4 °C, in which the jejunal mucosa and colonic contents were stored at −80 °C for further analysis.

Serum parameter analysis

Reagent kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), immunoglobulin A (IgA), immunoglobulin M (IgM), and immunoglobulin G (IgG) were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing,

China). Each parameter was strictly analyzed according to the instruction manual.

Jejunal morphology analysis

The jejunal intestine was dehydrated, embedded in paraffin, cut into 6 μm sections, dewaxed with xylene, hydrated, stained with hematoxylin and eosin, and sealed. Images were observed and captured under a microscope (NIKON Eclipse ci, NIKON digital sight DS-FI2; Japan). Ten fluffs and crypts were randomly selected in each picture to measure the villus height and crypt depth.

Immunohistochemical analysis

The sections of jejunal tissue were prepared for the analysis of toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), and nuclear factor-kappa B (NF-κB). An optical microscope was used to observe sections and capture images and calculate the cumulative optical density (IOD) and obtain the average. Cells colored brown–yellow when viewed under the microscope were identified as positive cells.

Volatile fatty acid (VFA) analysis

Colonic VFAs (acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids) were assayed by gas chromatography. Briefly, 2 g of colonic content samples was weighed into a 10 mL centrifuge tube and 2 mL of pre-cooled deionized water was added. The contents were vortex mixed for 30 s, allowed to stand at 4 °C for 30 min, and then centrifuged at 4 °C (10 000*g*) for 10 min. To 1 mL of the supernatant, 0.2 mL of 25% metaphosphoric acid (w/v) was added, mixed well, allowed to stand at 0 °C for more than 30 min, and centrifuged at 4 °C (10 000*g*) for 10 min; the temperature was set at 4 °C and the rotation speed was 15 000 rpm. One microliter of the supernatant was centrifuged at 10 000*g* for 4 min at 4 °C. Then, 500 μL of the supernatant was taken into a sample bottle for gas chromatography analysis.

Colonic content flora structure

The colonic content samples from each piglet were used for the microbial community analysis. Microbial genomic DNA was extracted from fecal samples using the QIAamp DNA stool Mini Kit (Qiagen GmbH, Hilden, Germany). The V4 region of 16S rRNA was PCR-amplified using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The reaction conditions are as follows: 98 °C for 1 min; 30 cycles at 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s; and 72 °C for 5 min. The PCR products were purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA). Libraries were prepared using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, USA). Related sequencing and biological analysis on the Illumina HiSeq platform (Novogene Bioinformatics Technology Co., Ltd, Beijing, China) were performed. The alpha and beta diversity indices were calculated using SPSS 25 software (SPSS Inc., USA).

Statistical analysis

All statistical analyses were performed using the one-way ANOVA, with SPSS 25 software (SPSS Inc., USA). Data are presented as least squares means plus pooled SEM. The results with a P value of <0.05 were considered statistically significant. The histograms were developed using GraphPad Prism 6 software (GraphPad Prism Inc., USA).

Results

Growth performance

Clostridium butyricum-supplemented piglets had higher BW than the control group on day 28, which is consistent with the ADG results ($P < 0.05$) (Table 2). The piglets fed *E. faecalis* presented a significant increase in BW and ADG compared with the piglets fed the basal diet ($P < 0.05$). The ratio of feed to gain in the probiotic-supplemented groups was significantly lower than that in the non-supplemented group ($P < 0.05$). Diarrhea in piglets fed probiotics was also significantly reduced compared with that in the control group ($P < 0.05$). The results indicated that *C. butyricum* and *E. faecalis* significantly improved the growth performance of piglets.

Serum parameters

The piglets in the probiotic-supplemented groups had significantly decreased ($P < 0.05$) ALT and AST activities compared with piglets in the control group on day 14 and 28 (Fig. 1). This indicated that *C. butyricum* and *E. faecalis* have a protective effect on the liver. The serum IgM level of the probiotic-supplemented groups was significantly increased ($P < 0.05$) compared with that in the control group on day 14, whereas the levels of serum IgA, IgG, and IgM of the probiotic-supplemented groups were significantly increased ($P < 0.05$) compared with those of the control piglets on day 28 (Fig. 2). *Clostridium butyricum* and *E. faecalis* can protect the liver and improve the immune function in piglets.

Table 2 Effects of *C. butyricum* and *E. faecalis* on growth performance in weaned piglets

	Treatment[a]				
Item	Con	CB	EF	SEM[b]	*P* Value
Initial BW, kg	8.81	9.10	9.04	0.10	0.45
Final BW, kg	17.63^{c}	19.63^{a}	18.86^{b}	0.21	<0.01
ADG, g	314.80^{c}	382.14^{a}	351.02^{b}	6.60	<0.01
ADFI, g	640.72^{b}	656.19^{a}	622.34^{c}	3.27	<0.01
F : G	2.04^{a}	1.72^{b}	1.77^{b}	0.03	<0.01
Diarrhea rate, %	5.00^{a}	0.66^{c}	1.17^{b}	0.00	<0.01

In the same row, values with no letter or the same letter superscripts had no significant difference ($P > 0.05$), while values with different letter superscripts had a significant difference ($P < 0.05$). [a] Con, CB and EF represent the piglets supplemented with the basal diet on d 28, piglets supplemented with *C. butyricum* and piglets supplemented with *E. faecalis*, respectively. Piglets were regarded as the experimental units. [b] Pooled SEM; $n = 6$ per treatment.

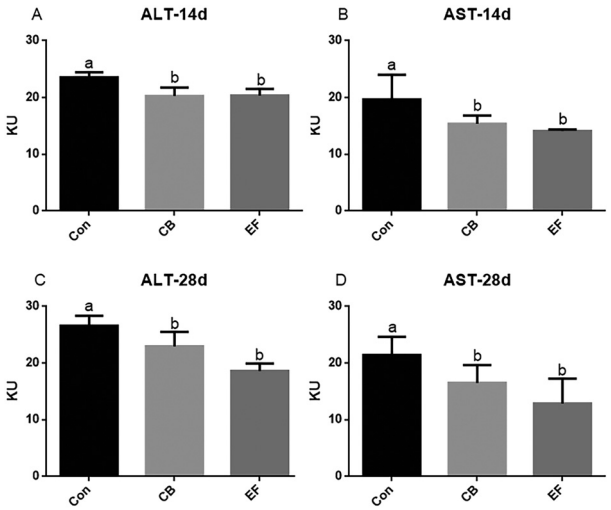

Fig. 1 Effect of *C. butyricum* and *E. faecalis* on the serum parameter indexes in weaned piglets. Con represents the control piglets on d 14 and 28, respectively; CB represents the piglets supplemented with *C. butyricum* on d 14 and 28, respectively; EF represents the piglets supplemented with *E. faecalis* on d 14 and 28, respectively. The letters indicate significant difference ($P < 0.05$). Mean values ($n = 6$) for the analysis of serum parameter indexes.

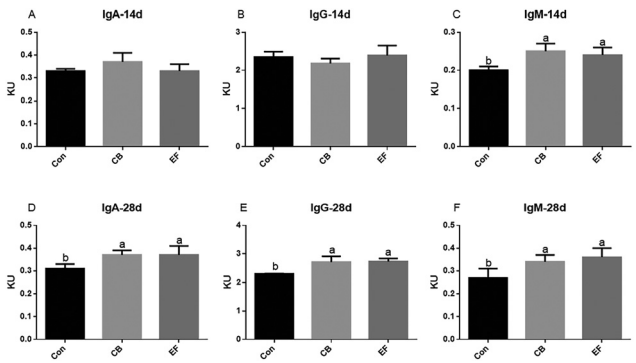

Fig. 2 Effect of *C. butyricum* and *E. faecalis* on the serum immunoglobulins in weaned piglets. Con represents the control piglets on d 14 and 28, respectively; CB represents the piglets supplemented with *C. butyricum* on d 14 and 28, respectively; EF represents the piglets supplemented with *E. faecalis* on d 14 and 28, respectively. The letters indicate significant difference ($P < 0.05$). Mean values ($n = 6$) for the analysis of serum immunoglobulins.

Jejunal morphology analysis

Piglets administered *C. butyricum* or *E. faecalis* had a higher ($P < 0.05$) jejunal villus height and ratio of the jejunal villus height to jejunal crypt depth (V/C) than the piglets fed the basal diet, while the jejunal crypt depth decreased ($P < 0.05$) (Fig. 3). Thus, probiotics can protect the integrity of intestinal villi.

Expression of proteins related to the TLR4 signaling pathway in the jejunal mucosa

As shown in Fig. 4, we evaluated the expression of proteins related to the TLR4 signaling pathway in piglets. A considerable increase in the protein expression of jejunal TLR4 ($P < 0.05$), MyD88 ($P < 0.05$), and NF-κB ($P < 0.05$) was observed in the probiotic-supplemented piglets compared with that in the control. *Clostridium butyricum* and *E. faecalis* can improve the protein level of the TLR4 signaling pathway.

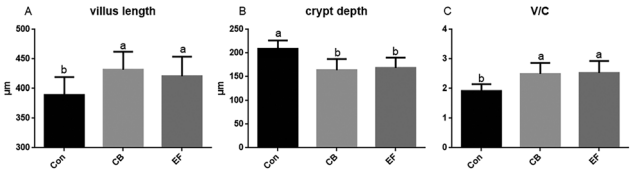

Fig. 3 Effects of *C. butyricum* and *E. faecalis* on the jejunum form of weaned piglets. A, The villus length in the jejunum of weaned piglets. B, The crypt depth in the jejunum of weaned piglets. C, The ratio of the villus length and crypt depth. Con represents the control piglets on d 28; CB represents the piglets supplemented with *C. butyricum* on d 28; and EF represents the piglets supplemented with *E. faecalis* on d 28. The letters indicate significant difference ($P < 0.05$). Mean values ($n = 10$) for the analysis of the jejunum form.

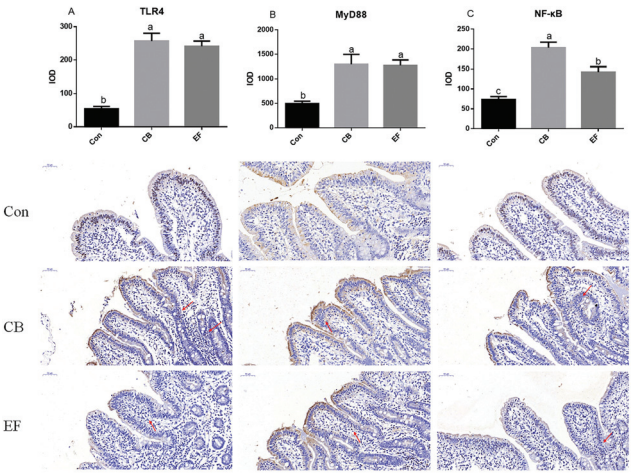

Fig. 4 Effect of *C. butyricum* and *E. faecalis* on the protein expression about TLR4 signaling pathways in the jejunum of weaned piglets. A, Immunohistochemical results of TLR4. B, Immunohistochemical results of MyD88. C, Immunohistochemical results of NF-κB. Con represents the control piglets on d 28; CB represents the piglets supplemented with *C. butyricum* on d 28; and EF represents the piglets supplemented with *E. faecalis* on d 28. Shooting multiples: 400×. The letters indicate significant difference ($P < 0.01$). Mean values ($n = 6$) for the analysis of jejunal mucosa.

Concentration of VFAs

The concentrations of butyric, propionic, butyric, isobutyric, and isovaleric acids in the colonic contents of the probiotic-supplemented piglets were significantly increased ($P < 0.05$) compared with those in the control piglets, while the concentration of valeric acid presented no significant change ($P > 0.05$) between the control and probiotic-supplemented groups (Fig. 5). Probiotics might increase the concentration of VFAs in the intestine.

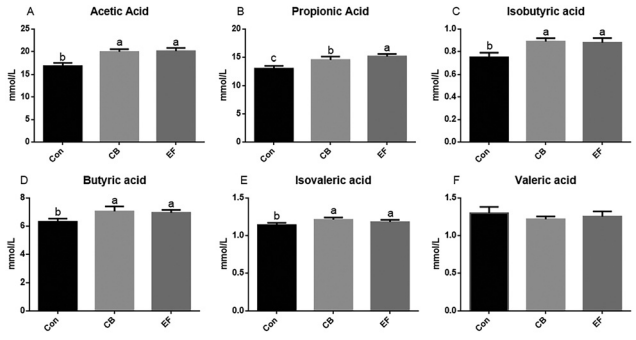

Fig. 5 Effect of *C. butyricum* and *E. faecalis* on the VFAs in the colonic contents of weaned piglets. Con represents the control piglets on d 28; CB represents the piglets supplemented with *C. butyricum* on d 28; EF represents the piglets supplemented with *E. faecalis* on d 28. The letters indicate significant difference ($P < 0.05$). Mean values ($n = 6$) for the analysis of VFAs.

Microflora structure in the colonic contents

The microbiota in the colonic content is shown in the Venn diagram (Fig. 6A). A total of 1115 observed taxonomic units (OTUs) were shared among the three treatment groups. The control group piglets had 54 unique OUTs, CB group piglets had 58 OTUs, and EF group piglets had 40 unique OTUs (Fig. 6A). There was a difference in microflora between the CB and control groups as indicated by the principal component analysis (PCA), whereas the EF and control groups had similar microflora (Fig. 6B). The alpha diversity (Shannon) of piglets supplemented with *C. butyricum* or *E. faecalis* was higher than that of the control piglets (Fig. 6C). The microbial composition of the colonic contents in piglets comprised Firmicutes, Bacteroidetes, Proteobacteria, and Tenericutes at the phylum level (Fig. 6G). The relative abundance of Actinobacteria in the CB group was significantly higher than that in the control group (Fig. 6D). At the genus level, we observed that *Clostridium*, *Subdoligranulum*, *Terrisporobacter*, *Streptococcus*, and *Ruminococcus* were the dominant genera in all the groups (Fig. 6H). At the species level, *Selenomonas bovis*, *Megasphaera elsdenii*, *Ruminococcus bromii*, *Clostridium butyricum*, and *Roseburia inulinivorans* were the dominant species in all samples (Fig. 6F). The abundance of *C. butyricum* in the CB group piglets was higher ($P < 0.01$) than that in the control piglets (Fig. 6E).

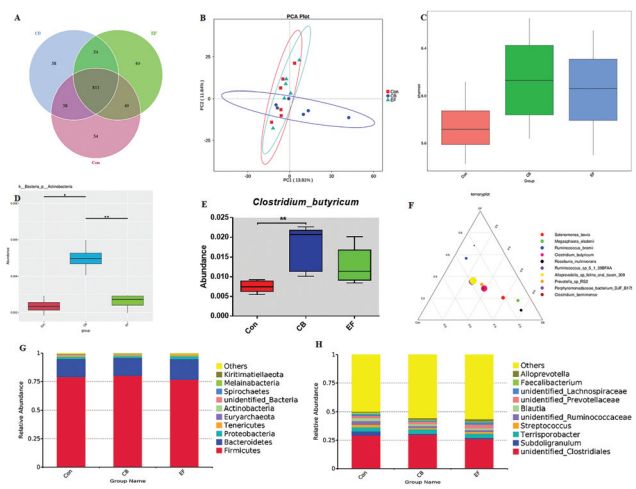

Fig. 6 Summary of the microbial community in the colonic contents of weaned piglets. A, The Venn diagram summarizing the numbers of common and unique observed taxonomic units (OTUs) in the microflora community in the colonic contents of weaning piglets on d 28. B, The principal component analysis (PCA) plot about the colonic microflora in the colonic contents of weaning piglets on d 28. C, Shannon index reflecting species diversity within and between groups on d 28. D, E, A species with significant differences between groups on d 28 (level phylum, species). F, Differences of dominant species between groups on d 28 (level species). G, H, The top 10 relative abundances of the microflora community between groups on d 28 (level phylum, genus).

Discussion

In recent years, several studies have confirmed that probiotics can improve animal growth performance, such as *Lactobacillus* spp. and *Bacillus* spp.[13] and *Saccharomyces* spp.[31] Li *et al.* showed that 10^{11} and 10^{12} cfu kg^{-1} *C. butyricum* supplemented into feed significantly improved the growth performance in *Litopenaeus vannamei*.[32] Abdel-Latif *et al.* proved that the dietary supplementation of a mixture of *C. butyricum* and *Saccharomyces cerevisiae* at an equal ratio was effective in improving growth performance in broilers.[33] Similarly, Chen reported that *C. butyricum* promoted broiler's growth performance.[20] However, a study revealed that supplementation with 9.6×10^4 cfu *C. butyricum* per g feed did not have any effect on growth performance in pigs.[34] In the present study, there was a significant improvement in growth performance in piglets fed *C. butyricum*-supplemented diet compared with that in piglets fed the basal diet. Takahashi *et al.* recently reported that the use of *C. butyricum* might improve the growth performance of weaned piglets,[35] which is consistent with the findings of our study. Zhao *et al.* demonstrated that the use of *Enterococcus faecium* improved the growth performance in weaned pigs.[36] Sato *et al.* showed that diet supplemented with live or heat-killed *E. faecium* improved the BW compared with that of the control group in post-weaned pigs.[34] However, Aluko *et al.* have shown that *E. faecalis* CG1.0007 had no significant effect on improving growth performance or reducing diarrhea in enterotoxigenic *Escherichia coli* K88+-challenged piglets.[37] Meanwhile, Hu *et al.* found that supplementation with *E. faecalis* improved growth performance in weaned piglets.[29] In this study, the growth performance of piglets that were fed *E. faecalis* was higher than that of the control piglets, which is similar to the findings of Hu. However, AST and ALT play an important role in the liver function as main indicators. Recently, *Lactobacillus rhamnosus* MTCC-5897 has been confirmed to reduce the activity of serum ALT and lactate dehydrogenase (LDH) and maintain normal functions of the liver and kidney.[38] Ozcan *et al.* have demonstrated that *E. faecium* decreased the concentration of serum AST and ALT in broi-

lers.[39] Gancarčíková *et al.* demonstrated that *Lactobacillus reuteri* relieved liver damage caused by *Salmonella typhimurium* infection, by decreasing the activities of serum AST and ALT.[40] In the mice model of carbon tetrachloride-induced acute liver damage, *C. butyricum* reduced the serum activities of ALT and AST.[41] Our results indicated that *C. butyricum* or *E. faecalis* resulted in a considerable reduction in the serum ALT and AST activities in piglets.

Immunoglobulin mainly exists in the serum, which is one of the components of the immune system of an animal. Li *et al.* have shown that the oral administration of *Lactobacillus delbrueckii* can improve the concentration of secretory IgA in the intestinal mucosa of piglets.[42] In Wang's study, the oral administration of *B. subtilis* significantly improved the levels of serum IgG and ileum IgA antibodies of piglets infected by porcine epidemic diarrhea virus.[43] Tsukahara *et al.* have confirmed that diet supplemented with compound probiotics (*Bacillus mesentericus*, *C. butyricum*, and *E. faecalis*) increased the concentration of milk IgA compared with that in the control lactating sows.[44] According to Zong *et al.*, diet supplemented with *C. butyricum* alone or combined with *Bacillus licheniformis* enhanced the concentrations of serum IgG, IgA, and complement protein C3 compared with those of the control piglets.[21] Similarly, the results of Han *et al.* indicated that the diet supplementation of *C. butyricum* increased serum IgM and IgA secretion in broilers.[22] Similarly, the present study results indicated that *C. butyricum* or *E. faecalis* increased the level of serum IgM in piglets on day 14, whereas there was a considerable increase in the serum IgG, IgA, and IgM levels in piglets on day 28.

The intestine is the main site of nutrient absorption, and the health of villi is a key factor influencing nutrient absorption. *Lactobacillus plantarum* favorably recovered the cyclophosphamide-induced abnormal intestinal morphology in mice by improving the villus height and crypt depth.[45] Li *et al.* reported that *Bacillus coagulans* TBC169 reduced the V/C ratio in the jejunum of birds compared with that in the control group.[46] However, no difference was observed among the mean crypt depth, mean villus height, and mean V/C ratio of the duodenum, jejunum, and ileum of broilers fed a basal diet and diets supplemented with probiotics (*B. subtilis*, *E. faecium*, *Bifidobacterium* spp., *Pediococcus* spp., and *Lactobacillus* spp.) according to Dela Cruz *et al.*[47] *E. faecium* improved the length of the intestinal villi in weaned piglets.[48] Moreover, Long *et al.* reported that the combined use of *C. butyricum* and *Lactobacillus salivarius* could further improve the length of the intestinal villi in mice.[49] According to Chen *et al.*, *C. butyricum* increased the duodenal, jejunal, and ileal villus height and jejunal V/C ratio in lipopolysaccharide-challenged weaned piglets.[20] Meanwhile, *E. faecalis* increased the villus length in the jejunum of suckling piglets.[30] Our results indicated that dietary *C. butyricum* or *E. faecalis* enhanced the intestinal health development and nutrient absorption in weaned piglets by decreasing the crypt depth and increasing the villus height and V/C ratio in weaning piglets.

Weaning stress can result in histological and biochemical changes in the intestine, which are necessary for piglets to grow.[50] Toll-like receptors are phylogenetically conserved mediators of innate immunity which can discriminate the intestinal microbiota and respond to pathogenic microbes.[51,52] TLR4 is one of the members of TLRs, inducing the secretion of inflammatory factors *via* the activation of the MyD88 pathway.[53] MyD88 is an important adaptor protein in the TLR4 signal transduction process, which promotes the release of inflammatory factors and induces an immune response in the intestinal mucosa.[54] NF-κB is closely related to the transcriptional processes of genes, such as those encoding inflammatory transmitters and cytokines, and primarily initiates the inflammatory reaction by binding to the κB site, disrupting immune balance in the intestine, leading to the occurrence of ulcerative colitis.[55] In addition, Chen's study also found that the inhibition of the TLR4/NF-κB signaling pathway and the activation of the Nrf2/HO-1 signaling pathway ameliorated intestinal barrier disruption in weaned piglets.[56] Mountzouris *et al.* have indicated that viable compound probiotics (*L. reuteri*, *E. faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, and *Lactobacillus salivarius*) reduced the expression of cecal TLR4 in broilers compared with that in the control group.[57] Similarly, the findings of Kanmani *et al.* showed that the expression of NF-κB was reduced in lipopolysaccharide-challenged Caco2 cells (intestinal epithelial cells), which is related to *L. plantarum*.[58] *Enterococcus faecium* can downregulate the mRNA level of TLR4, but it did not significantly affect the mRNA or protein level of MyD88 in human enterocyte-like HT-29 cells.[59] Interestingly, our study proved that *C. butyricum* or *E. faecalis* decreased the protein expression of TLR4, MyD88, and NF-κB in the jejunum of piglets compared with that in the control. Both *C. butyricum* and *E. faecalis* were found to adjust the inflammatory responses by modulating the TLR4 inflammatory signaling pathway.

The colon is the main site of microbial colonization, which plays a key role in animal health.[60] Previous studies showed that *Lactobacillus plantarum* increased *Bifidobacterium* and *Lactobacillus*, decreased *Escherichia* in the cecal content of cyclophosphamide-treated mice,[45] and also increased *Prevotellaceae* and *Bifidobacteriaceae*,[61] resulting in flora balance. Sato *et al.* demonstrated that *E. faecium* optimized the structure of the gut microbiota compared with that in piglets fed the basal diet, which had discrete microbiota with beta diversity (weighted UniFrac distances),[34] similar to the findings of Chae *et al.*[62] Our findings indicated that the PCA of the CB group was different from the PCA of the control group, while the alpha diversity (Shannon) in *C. butyricum*- or *E. faecalis*-supplemented piglets was higher than that in the control piglets. The results of Baños's study showed the ability of *E. faecalis* UGRA10 to inhibit pathogenic bacteria *in vitro* in co-culture and *in vivo* in infected-rainbow trout.[63] Furthermore, *E. faecalis* also affected the cecal microbial community structure in laying hens according to Zhang *et al.*[28] Administration of live *E. faecium* and *C. butyricum* decreased the relative abundance of *Treponema*, and *Treponema* causes colon inflammation and swine blood dysentery, suggesting that these probiotics inhibited the growth of harmful bacteria,

which are associated with colitis in pigs.[64] The present study showed that Firmicutes, Bacteroidetes, Proteobacteria, and Tenericutes were the dominant phylum of the colonic contents in piglets. Chae *et al.* and Tran *et al.* have proved that Firmicutes and Bacteroidetes are the most dominant phyla in pig feces,[62,65] which is consistent with our results. In the present study, *C. butyricum* in the colonic contents of the CB group was more abundant than that in the control group, and the relative abundance of *Actinobacteria* in the CB group was higher than that in the control group. Hence, *C. butyricum* and *E. faecalis* effectively increased the intestinal microbial diversity and abundance, maintaining the intestinal flora balance.

Volatile fatty acids, also called short-chain fatty acids (SCFAs), especially butyric acid, produced by *C. butyricum* can provide nutrients for the regeneration and repair of intestinal epithelial cells,[66] decrease the intestinal pH, and inhibit the growth of certain harmful microorganisms.[67] *Lactobacillus reuteri* increased the SCFAs in the cecal contents, mainly increasing the concentration of butyric, acetic, and propionic acids in the cecal contents, consistent with the findings of Gancarčíková *et al.*[40] Similarly, there was an increase in the VFA concentrations in the cecal digesta of broilers, especially acetic acid.[22] In the study of Mishra *et al.*, *E. faecalis* AG5 has been shown to promote the production of SCFAs, especially propionic acid.[68] In the present study, piglets supplemented with *C. butyricum* and *E. faecalis* presented a significant increase in the concentration of acetic, isobutyric, butyric, propionic, and isovaleric acids in the colonic contents.

Many researchers have shown that there is a close relationship among intestinal microbes, volatile fatty acids, and immune function. Members of the phylum Firmicutes can favor digester fiber and produce SCFAs from dietary compounds.[69] Bacteroidetes produce acetate and propionate, while Firmicutes members (*Lachnospiraceae* and *Ruminococcaceae*) mainly produce butyrate as the primary metabolic end product.[70,71] This is consistent with our finding that the abundance of Firmicutes in the CB group was higher than that in the control group, and the abundance of Bacteroidetes in the EF group was higher, explaining the increase in VFAs. Similarly, Chen *et al.* also showed that the piglets fed chlorogenic acids increased the abundance of phyla Firmicutes and Bacteroidetes in the cecum digesta, as well as the concentrations of acetate, propionate and butyrate.[72] Interestingly, SCFAs can improve the immune function of mice by promoting B cell antibody production.[73] Short-chain fatty acids inhibited human monocyte inflammatory responses by inducing the release of prostaglandin E2 and expression of the anti-inflammatory cytokine IL-10, thereby protecting the body's normal immune function.[74] In our study, the levels of IgA, IgG, and IgM in serum of piglets in the probiotics group were increased compared with those in the control group, which is consistent with the result of VAFs. Moreover, in the study of Cremon, *Lactobacillus paracasei* CNCM I-1572 resulted in a considerable reduction in the genus *Ruminococcus*, a significant improvement in the SCFA acetate and butyrate, and an obvious reduction in the pro-inflammatory cytokine interleukin-15 (IL-15), thereby alleviating human intestinal damage induced by irritable bowel syndrome.[75] Similarly, in mice treated with 3% dextran sulfate sodium, *L. fermentum* supplementation to the basal diet increased the colonic expression of miR-155 and miR-223, which are the markers of immune response, restored SCFA- and lactic acid-producing bacterial populations, and improved Chao richness and Shannon diversity.[76] This is consistent with our finding that the administration of probiotics resulted in an increase in the richness and diversity of colonic microbial communities, promotion of VAF secretion, and expression of serum immunoglobulin, which indicated a correlation among the three parts. Therefore, we suspect that *C. butyricum* and *E. faecalis* promote the production of VAFs by regulating the structure of the intestinal flora, thereby improving the immune function of weaned piglets.

Conclusions

In summary, the dietary supplementation of *C. butyricum* and *E. faecalis* can improve growth performance, protect the liver, protect intestinal villi, might regulate intestinal morphology by activating the TLR4-mediated MyD88-dependent signaling pathway, and might promote the immune function by enhancing the concentration of VFAs in the colon contents which were increased by enriching the colonic microbial population and diversity in weaned piglets. *Clostridium butyricum* and *E. faecalis* are conducive for the healthy development of weaned piglets. Our finding described the relationship among the immune function, volatile fatty acids, and intestinal flora and laid a foundation for further study of the mechanism of them.

Conflicts of interest

This work has no conflict of interest.

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