



Probiotics-fermented Massa Medicata Fermentata ameliorates weaning stress in piglets related to improving intestinal homeostasis

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Abstract

Weaning stress has serious negative effects on piglets' health and the swine industry. Probiotics-fermented Chinese herbal medicines are potential feed additives to ameliorate weaning stress. In this study, the effects of probiotics-fermented Massa Medicata Fermentata (MMFP) on intestinal homeostasis were evaluated in weaning piglets. Dietary supplementation with MMFP promoted the development of the intestinal structure and elevated the concentrations of lactic acid and short-chain fatty acids (SCFAs) in the intestinal contents and antioxidant capacities in serum. MMFP reduced the levels of inflammatory factors in the intestinal mucosa. Microbial community analysis demonstrated that MMFP led to the selective and progressive enrichment of lactic acid- and SCFA-producing bacteria along the gastrointestinal tract, in particular, OTUs corresponding to *Lactobacillus*, *Streptococcus*, *Acetivomaculum*, *Roseburia*, and *Eubacterium xylanophilum* group, while MMFP reduced the relative abundance of pathogenic bacteria. On the contrary, antibiotics had negative effects on intestinal histology and increased the relative abundance of pro-inflammatory bacterium, such as *Marvinbryantia*, *Peptococcus*, *Turicibacter*, and *Blautia*. Correlation analysis reflected that the bacteria enriched in MMFP group were positively correlated with enhanced intestinal homeostasis, which suggested that dietary supplementation with MMFP enhanced host intestinal homeostasis by modulating the composition of gut microbiota and the levels of beneficial SCFAs, thus ameliorating weaning stress in piglets.

Keywords Weaning stress · Probiotics-fermented Massa Medicata Fermentata · Microbial community · Intestinal homeostasis · SCFAs

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Introduction

Weaning transition is a crucial period in the piglet's growth cycle. In the swine industry, compared with the natural environment, shorter suckling period as well as abrupt changes in dietary and social environment frequently lead to anorexia, growth retardation, severe enteric infections, or even death (Gresse et al. 2017; Lalles et al. 2007). In general, antibiotics are widely used to treat bacterial infections and promote animal growth. However, the overuse of antibiotics results in a growing number of drug-resistant pathogens and raises important concerns about animal and human health. Antibiotic-resistant strains of enterobacteria, such as *Clostridium difficile*, *Enterobacteriaceae*, *Enterococcus* spp., and *Staphylococcus aureus*, pose serious risks to human health (Gross 2013; Tanne 2012). Antibiotics are being gradually banned in various countries. Therefore, new and efficient feed additives to improve weaning stress in piglets are needed.

Intestinal dysfunction, such as stomachache, diarrhea, constipation, and anorexia, is the most common and primary symptom during the animal weaning period. Huge diversity of microorganisms inhabit in the gastrointestinal (GI) tract of mammals and provide many beneficial effects to the host (Buffie and Pamer 2013; Kamada et al. 2013). Commensal bacteria in the intestine can inhibit the expansion of pathogens through colonization resistance and defensive metabolites. Besides, organic acids, produced by the commensal bacteria, contribute to intestinal homeostasis and systemic immunity (Louis et al. 2014). Therefore, manipulation of intestinal microbiota is likely to be one of the keys to ameliorate weaning stress.

Massa Medicata Fermentata (MMF) is a widely clinically used Chinese herbal medicine, mainly for intestinal dysfunction (Zhang et al. 2012). In general, MMF is spontaneously fermented with red bean, *Semen Armeniacae Amarum*, *Artemisia carvifolia*, *Polygonum hydropiper* L., *Xanthium sibiricum*, wheat bran, and flour. Chinese herbal formulas containing MMF have attenuated ethanol-induced acute gastric injury in rats (Shin et al. 2013) and alleviated neomycin-induced colon injury and disorders in gut microbiota (Bose et al. 2013). However, pathogens and fungal toxins, such as aflatoxin, are usually detected in traditional MMF (Liu et al. 2017). This restricts its clinical application. According to the microbial community dynamics during MMF fermentation (Xu et al. 2013), we modified the fermentation strains with definite probiotics (*Bacillus* sp., *Lactobacillus* sp., *Streptococcus thermophilus*, *Enterococcus faecalis*, *Saccharomyces cerevisiae*, *Aspergillus oryzae*, and *Mucor racemosus*) instead of random and spontaneous strains (Wang et al., submitted for publication). These probiotics were widely used in food industry and have been proved beneficial to the health of the host (de Almada et al. 2015). Probiotics-fermented Massa Medicata Fermentata (MMFP) combines the advantages of Chinese herbal medicines, digestive enzymes, and probiotics, which has given it great potential value for development and application.

In this study, we aimed to evaluate the effects of MMFP as a novel feed additive on weaning stress in piglets. We systematically analyzed the effects of dietary supplementation with MMFP on intestinal histology, the intestinal immune system, the antioxidant capacities in serum, and the levels of organic acids in intestinal contents. The microbial composition along the GI tract and feces was analyzed through high-throughput sequencing. This is the first report about the ability of probiotics-fermented Chinese medicine to improve weaning stress in piglets. The current study may contribute to the development of new and efficient feed additives. As reviewed by Heinritz et al. (Heinritz et al. 2013), there are strong physiological similarities between humans and pigs, so this study could serve as a reference for the clinical application of probiotics-fermented Chinese medicines.

Materials and methods

Animals, housing, and diets

The experiment was conducted by completely random design. A total of 24 healthy and well-developed piglets (Songliao black swine, initial body weight 15.61 ± 1.52 kg) were used in this study. These pigs were randomly assigned to three treatments (eight pigs per treatment) the day after weaning (day 0). The treatment groups were (a) Con group, in which pigs were fed a basal diet (Supplementary Table S1); (b) Ant group, in which pigs were fed the basal diet supplemented with colistin sulphate (0.3 g/kg feed) and bacitracin zinc (0.5 g/kg feed); and (c) MMFP group, in which pigs were fed the basal diet supplemented with MMFP (2 g/kg feed). MMFP was fermented with *Bacillus licheniformis* CICC24236, *Bacillus subtilis* CICC24434, *Lactobacillus reuteri* CICC6119, *Lactobacillus plantarum* CICC20261, *Streptococcus thermophilus* CICC6216, *Enterococcus faecalis* CGMCC1.10682, *Saccharomyces cerevisiae* CICC1005, *Aspergillus oryzae* CICC2013, and *Mucor racemosus* CICC40491 according to the National Drug Standards (1998) (Wang et al., submitted for publication). The composition of nutritional ingredients was according to the National Research Council (2012). Animals were housed in a controlled environment (26–28 °C, humidity 65–70%) with feed and water ad libitum during a 49-day trial period. The animal experiment was approved by the Institutional Animal Care and Use Committee of Jilin University (IACUC), and the animals were maintained according to the laboratory animal-guideline for ethical review of animal welfare.

Performance measurements and sample collection

Body weight and feed consumption were measured weekly, and average daily gain (ADG) and average daily feed intake (ADFI) were calculated. In this study, ADG was calculated as follows: $ADG = (\text{final body weight} - \text{initial body weight}) / 49$. Only survived pigs on day 49 were calculated. The ADFI was calculated as follows: $ADFI = (\text{feed consumption each week}) / (7 \text{ days} \times n)$, n = the number of survived pigs. Fecal consistency score (0 = normal feces; 1 = soft and moist feces; 2 = mild diarrhea; 3 = severe, watery diarrhea) was recorded daily (Casey et al. 2007). The diarrhea incidence was evaluated according to a previous study (Sun et al. 2008). Piglets' morbidity and mortality was recorded throughout the trial period.

Fresh fecal samples were collected at day 0 and day 49 as follows: nine sampling points were randomly selected in each pen, and then fecal samples from three points were mixed together as a specimen (three fecal samples per pen). At the end of the trial period, carotid artery blood samples from all of the survived pigs were collected by vacuum blood collection

tubes and centrifuged at 3000 rpm for 10 min at 4 °C. One animal from each pen was selected randomly and sacrificed. Intestinal tissues were collected from standardized areas of the ileum (100 cm from the ileocecal valve), cecum (central part of the cecum), and colon (central part of the colon). Contents from the cecum, proximal colon (colon 1), medial colon (colon 2), distal colon (colon 3), and rectum were collected with sterile tubes. Mucosa from the jejunum, ileum, cecum, proximal colon (colon 1), medial colon (colon 2), distal colon (colon 3), and rectum was gently scraped with sterile glass slides. All of the samples were stored at −80 °C before processing.

Histological analysis

Intestinal tissues were flushed with sterile PBS, fixed in 10% neutral-buffered formalin, processed, embedded in paraffin, cut at 5 μm thickness, and stained with hematoxylin and eosin (H&E). More than six complete crypt-villus units were randomly selected from each sample. Measurements of mucosal thickness, villus height, and crypt depth were performed using a Nikon biological microscope (E100) equipped with an ISH500 camera (Tucsen, Fuzhou, China) and ISCapture (version 3.5) morphological analysis software.

Analysis of antioxidant capacities in serum

The levels of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and malondialdehyde (MDA) in serum were determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Analysis of inflammatory factors in serum and intestinal mucosa

The levels of monocyte chemotactic protein 1 (MCP-1), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 10 (IL-10) in serum and the intestinal mucosa were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO®, Wuhan, China) according to the manufacturer's instructions.

Analysis of microbial metabolites in intestinal contents

Concentrations of lactic acid and SCFAs in intestinal contents were analyzed by high-performance liquid chromatography (HPLC). Sample pretreatment and derivatization were according to previous studies (Gao et al. 2017) with slight modifications. Briefly, 1 g of intestinal contents were suspended with 10 mL ddH₂O and thoroughly homogenized by vortexing for

1 min. Then, samples were centrifuged at 12,000×g for 5 min. 3-Nitrophenylhydrazine hydrochloride (3-NPH·HCl, Aladdin®, Shanghai, China) was dissolved in methanol at a concentration of 20 mM. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1-EDC·HCl, Aladdin®, Shanghai, China) was dissolved in 3% pyridine–methanol solution at a concentration of 0.25 M. Each sample (50 μL) was mixed with 200 μL of 3-NPH·HCl solution and 100 μL of 1-EDC·HCl solution, followed by incubation at 60 °C for 20 min. After cooling, the mixture was filtered through a 0.22 μm filter.

HPLC was performed using an ACCHROM S6000 high-performance liquid chromatograph (ACCHROM, Japan) equipped with a XAqua C18 4.6 mm × 250 mm column (ACCHROM, China). The mobile phase was composed of methanol–water (32:68), with the column temperature of 40 °C, flow rate of 1.2 mL/min, and wavelength of 230 nm.

DNA extraction, sequencing, and data processing

Genomic DNA was extracted from intestinal contents and feces using a TIANamp Stool DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The final yield and quality of the extracted DNA were determined spectrophotometrically using a NANODROP 1000TM UV/VIS Spectrophotometer (Thermo, USA). The v3 and v4 regions of the 16S rRNA gene were amplified using forward primers of the sequence 5'-CCTACGRRBGCASCAGKVRVGAAT-3' and reverse primers of the sequence 5'-GGAC TACNVGGGTWTCTAATCC-3'. PCR was performed in a total of 20 μL volume, containing 1 × PCR Buffer (Mg²⁺ plus), 200 μM of dNTP, 0.1 μM each of the primer, 1 U of TaKaRa Taq Polymerase (Takara, Dalian, China), and 10 ng of template DNA. The v3–v4 regions were amplified at 94 °C for 3 min, followed by 24 cycles of 94 °C for 5 s, 57 °C for 90 s, and 72 °C for 10 s and a final extension at 72 °C for 5 min. The first round PCR products were used as templates for second round amplicon enrichment PCR. At the same time, indexed adapters were added to the ends of the 16S rDNA amplicons to generate indexed libraries ready for downstream NGS sequencing on Illumina Miseq.

Statistical analysis

The data are expressed as the mean values ± SEM. Statistical analyses were performed in SPSS Statistics 17.0 using the tables and data generated above. A value of $p < 0.05$ was considered statistically significant. Multiple comparisons were conducted using one-way analysis of variance (ANOVA). Figures were plotted with OriginPro 8 SR0 (v8.0724). The alpha-diversity of microbial communities was calculated by Mothur software (v.1.30). Heatmaps of bacterial abundance were made using R (V.3.2.3). Principal component analysis

and correlation analysis were performed using MetaboAnalyst 3.6 (<http://www.metaboanalyst.ca/faces/upload/StatUploadView.xhtml>).

Sequence data accession number

High-throughput sequencing data have been submitted to the NCBI Sequence Read Archive (SRA) under accession numbers SRP126208 and SRP126217.

Results

MMFP ameliorates weaning stress in piglets

Antibiotic treatment significantly improved the growth rate (Fig. 1, $p < 0.01$) and ADFI (Table 1) of piglets compared with Con group. Although MMFP lagged behind the Ant group, pigs in the MMFP group showed a higher growth rate (Fig. 1a, $p = 0.065$) and ADFI (Table 1) compared with the Con group from the 6th week on. At the 7th week, the body weight and ADG of pigs in the MMFP group were significantly higher than those in the Con group (Fig. 1, Table 1, $p < 0.05$). MMFP decreased diarrhea incidence compared with the Con group (Table 1). Two pigs in the Con group died from diarrhea, and one pig in the Ant group died from acute encephalitis. There was no death in the MMFP group. Thus, dietary supplementation with MMFP improved the growth performance and stress response caused by weaning effectively, and reduced mortality.

MMFP promotes the development of intestinal histology

Results from tissue slices of small intestine (Fig. 2a) showed that there were no obvious structural characters of Paneth cells or goblet cell in pigs from the Ant group and no obvious border of intestinal villi. The microvilli in pigs from the Con

Table 1 Effects of MMFP on the growth performance and state of health in piglets

	Con	Ant	MMFP
ADFI (kg)			
Day 0–7	0.85	0.88	0.88
Day 8–14	0.86	0.89	0.88
Day 15–21	0.88	1.08	0.95
Day 22–28	0.73	1.00	0.82
Day 28–35	0.98	1.32	1.00
Day 36–42	0.97	1.50	1.21
Day 43–49	0.87	1.38	1.19
Average daily gain (kg)	0.23 ± 0.08a	0.48 ± 0.08b	0.36 ± 0.13b
Diarrhea incidence	10.7%	1.5%	3.1%
Death rate	25%	12.5%	0

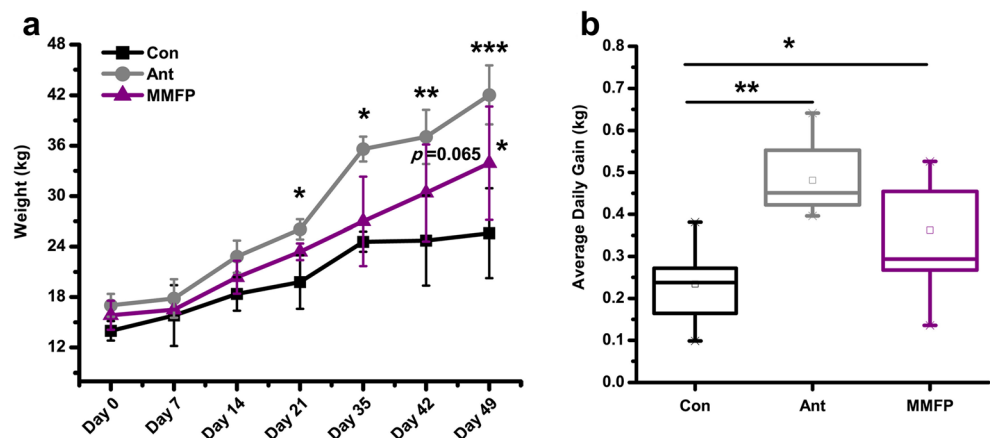
Data represent mean values ± SEM ($n = 6–8$ pigs for each group). Student's t test was used to calculate p values. a, b: Different letters mean significant difference ($p < 0.05$)

and MMFP groups were much more integrated than those from the Ant group. In the MMFP group, the mucosal thickness and villus height in small intestine were significantly higher than those in Con group. Villus height in MMFP group was significantly higher than that in the Ant group (Fig. 2d). However, the ratio of villus height to crypt depth in the Ant group was significantly lower than that in the Con and MMFP groups (Fig. 2e). Measurements of cecum and colon (Fig. 2f) showed that the thickness of the mucous layer in the Ant group was lower than that in the Con and MMFP groups. These results suggest that dietary supplementation with MMFP could promote the development and maturation of intestinal histology, while antibiotics might have negative effects.

MMFP improves antioxidant capacities in serum

The levels of T-AOC, SOD, GSH-Px, CAT, and MDA in serum were analyzed to evaluate the effects of MMFP and

Fig. 1 The growth performance of piglets. **a** Growth rate. **b** Average daily gain. Data represent mean values ± SEM ($n = 6–8$ pigs for each group). Student's t test was used to calculate p values. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



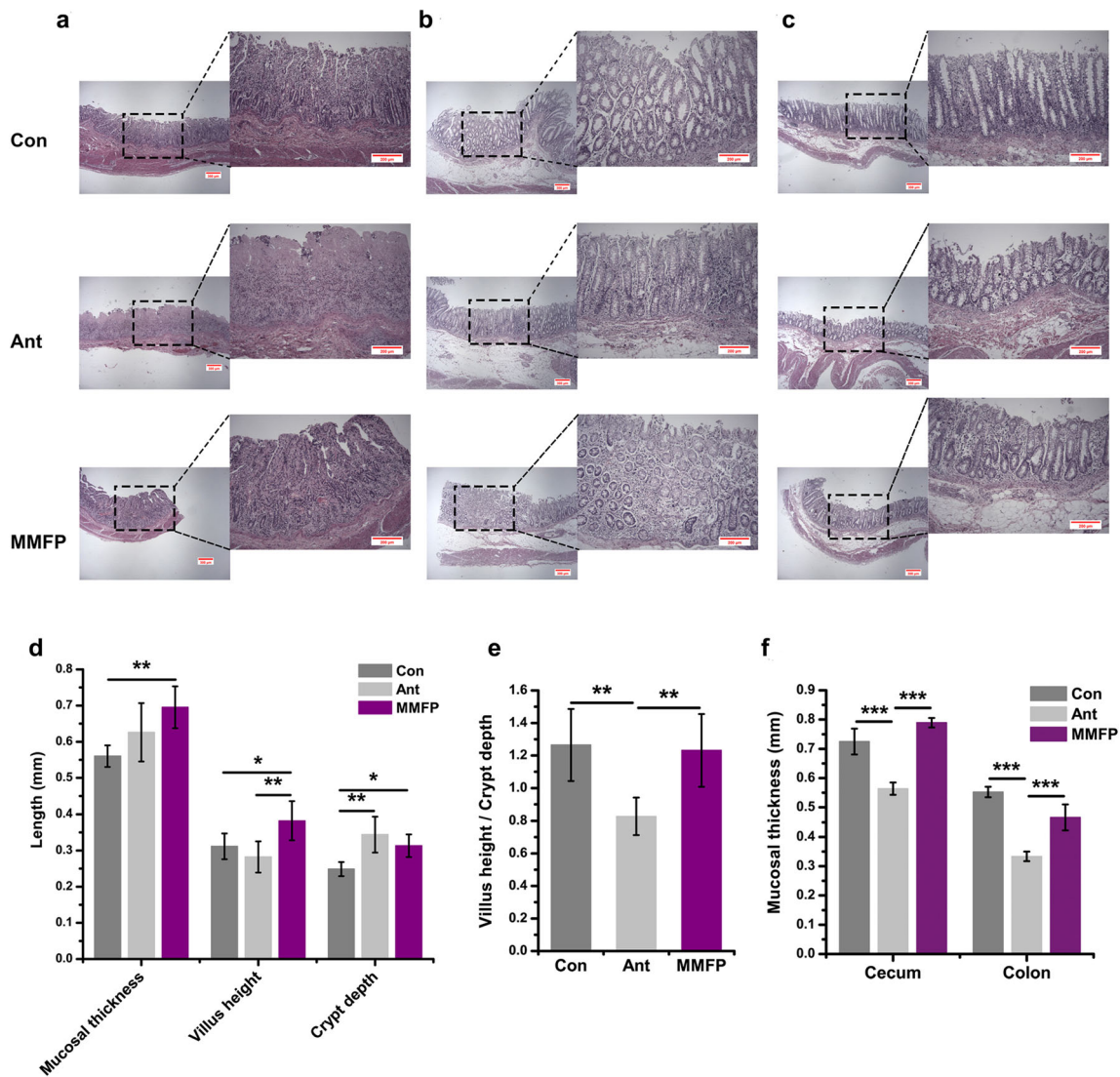


Fig. 2 Effects of MMFP on intestinal histology. On day 49, one pig from each pen was selected randomly and slaughtered. Photomicrographs of small intestine (a), photomicrographs of cecum (b), and

photomicrographs of colon (c) were analyzed. Statistical analysis of intestinal histology (d–f). Data represent mean values \pm SEM. Student's *t* test was used to calculate *p* values. **p* < 0.05; ***p* < 0.01; ****p* < 0.001

antibiotic on antioxidant capacities. The levels of T-AOC, SOD, GSH-Px, and CAT in the MMFP group were the highest among the three groups (Fig. 3a–d). The level of MDA in the Ant group was significantly higher than that in the Con and MMFP groups (Fig. 3e). These results indicate that dietary supplementation with MMFP could enhance the antioxidant capacities and reduce lipid peroxidation in serum.

MMFP reduces inflammatory factors in intestinal mucosa

Levels of inflammatory factors in serum and the mucosa along the gastrointestinal tract were analyzed by ELISA to evaluate the effects of MMFP and antibiotic on immunologic homeostasis. Except for the cecum, MMFP reduced the expression levels of MCP-1, IL-1 β , IL-6, and IL-10 in the whole of GI

tract compared with the Con and Ant groups (Fig. 3f–i). Levels of inflammatory factors in serum were not significantly different between the Con, Ant, and MMFP groups (Fig. S1).

MMFP elevates organic acids in intestinal contents

Concentrations of organic acids in the intestinal contents were analyzed by HPLC to evaluate the effects of MMFP and antibiotics on microbial metabolism. The concentrations of lactate, acetate, propionate, butyrate, isobutyrate, and total SCFAs were significantly increased in all segments of the large intestine in MMFP group compared with the Con and Ant groups (Fig. 4, *p* < 0.001). These data indicate that dietary supplementation with MMFP enhanced microbial metabolic activities in the intestinal tract.

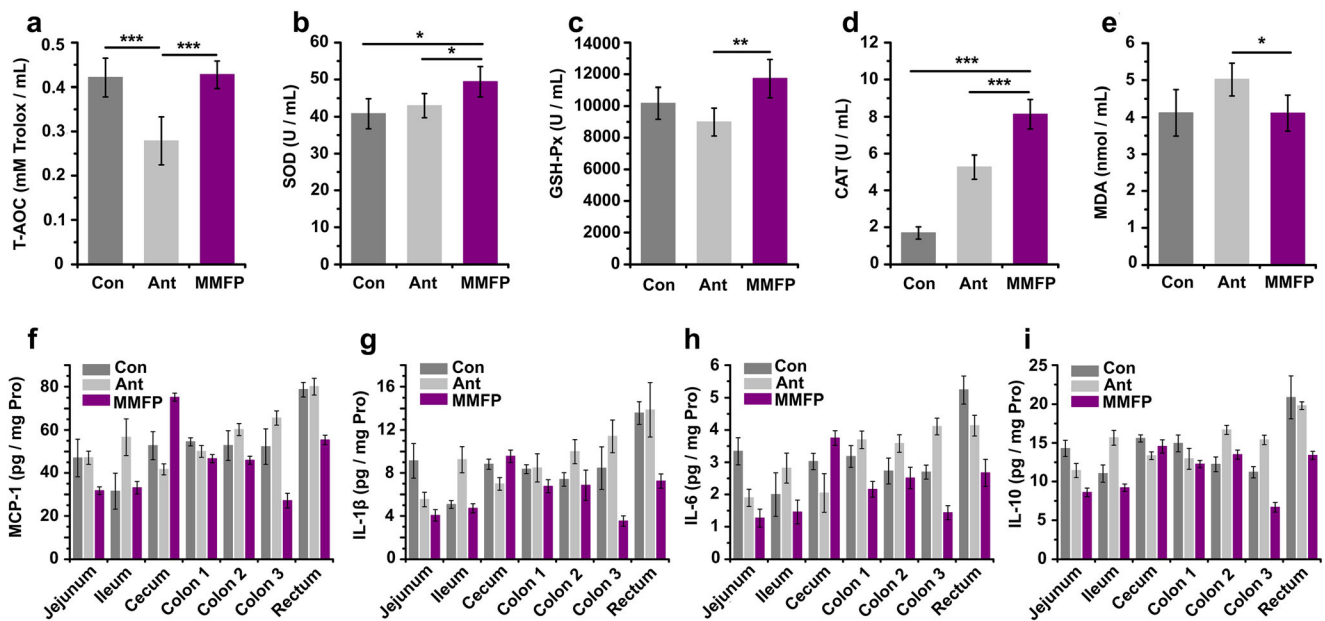


Fig. 3 Effects of MMFP on antioxidant capacities in serum and intestinal immune system. On day 49, one pig from each pen was selected randomly and slaughtered. Antioxidant capacities in serum (a–e) and the levels of inflammatory factors in mucosa from different segments of

GI tract (f–i) were analyzed. Data represent mean values \pm SEM. Student's *t* test was used to calculate *p* values. **p* < 0.05; ***p* < 0.01; ****p* < 0.001

MMFP affects microbial community structure of intestinal contents

A total of 1,124,498 paired-end reads were obtained from 15 intestinal content samples. After jointing, filtering, and removing chimeras, a total of 909,431 effective sequences were reserved for later analysis (Table S2). Rarefaction curves and

Shannon index curves showed that the sequencing depth was enough (Fig. S2a, b). Based on 97% sequence similarity, sequences were assigned to 692 unique OTUs. Phylum-level analysis of the gut microbiome revealed that all regions of the intestinal tract were dominated by *Firmicutes* (72.81–99.14%), followed by *Bacteroidetes* (0.45–13.97%). At the genus level, *Clostridium sensu stricto* 1, *Peptoclostridium*,

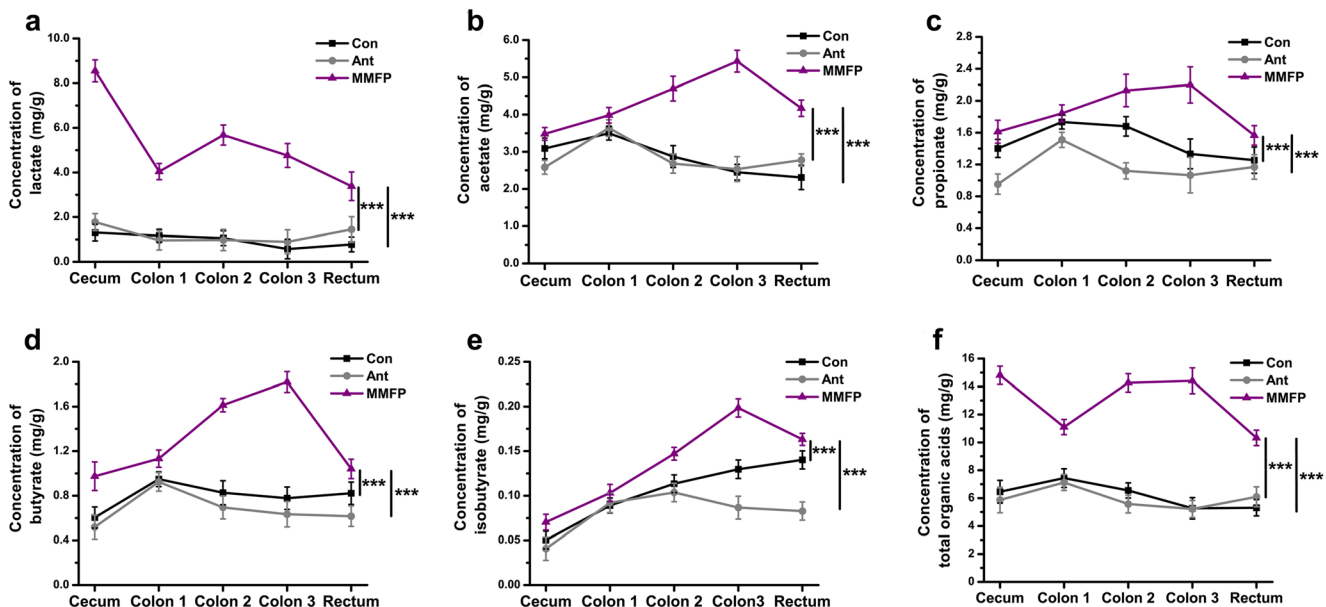


Fig. 4 The concentrations of organic acids in the intestinal contents. On day 49, one pig from each pen was selected randomly and slaughtered. The concentrations of lactate (a), acetate (b), propionate (c), butyrate (d), isobutyrate (e), and total organic acids (f) in intestinal contents from

different segments of GI tract were analyzed. Data represent mean values \pm SEM. Student's *t* test was used to calculate *p* values. ****p* < 0.001

Streptococcus, *Turicibacter*, *Parabacteroides*, and *Intestinibacter* were predominant in the intestinal contents (Fig. S2d, e). Principal component analysis (PCA) showed that the microbial compositions of the colonic and rectal contents were more similar to each other than to the cecal contents (Fig. S2c). Alpha diversity analysis (Table S3) revealed that microbial abundance (ACE and Chao1 index) and diversity (Simpson and Shannon index) in cecal contents were lower than those in the colonic and rectal contents.

The heatmap (Fig. 5) revealed that the composition of the microbial community in intestinal contents varied with treatment. In the MMFP group, the relative abundances of *Eubacterium xylanophilum* group, *Clostridium sensu stricto* 6, *Streptococcus*, *Acetivomaculum*, *Roseburia*, *Coprococcus* 1, and *Lactobacillus* were higher than those in the Con and Ant groups along the GI tract (Fig. 6a). The relative abundance of *Ruminococcus* was higher in the cecum and proximal colon of the MMFP group compared with the Con and Ant groups (Fig. 6a). The relative abundances of *Marvinbryantia*, *Peptococcus*, *Turicibacter*, and *Blautia* were increased in the colorectum of the Ant group compared with the Con and MMFP groups (Fig. 6b). However, *Candidatus Saccharimonas*, *Mogibacterium*, *Solobacterium*, *Lachnoclostridium*, *Escherichia-Shigella*, and *Methanobacteriaceae* were more abundant in the colorectum of the Con group than those in the Ant and MMFP groups (Fig. 6c).

Effects of MMFP on microbial community structure of feces

The microbial composition of feces on day 0 and day 49 was analyzed in this study. A total of 1,494,890 paired-end reads were obtained from 18 stool samples. After jointing, filtering, and removing chimeras, a total of 456,963 effective sequences were reserved for later analysis (Table S4). Rarefaction curves and Shannon index curves showed that the sequencing depth was enough (Fig. S3a, b). Based on 97% sequence similarity, sequences were assigned to 1195 unique OTUs. Phylum-level analysis of the feces microbiome revealed that *Bacteroidetes* (34.09–70.22%) and *Firmicutes* (27.80–61.52%) were the dominant phyla (Fig. S3d). Alpha-diversity analysis revealed that species richness and diversity increased over time. At the end of the experiment, the number of unique OTUs in the MMFP and Ant group was significantly higher than that in the Con group, and the richness and diversity index in the Ant and MMFP groups showed higher trends than the Con group (Table 2).

PCA analysis (Fig. S3c), histogram of species richness at the genus level (Fig. S3e), and the heatmap (Fig. S4) showed pronounced changes in microbial composition between the start (day 0) and end (day 49) of the experiment. Compared with the start, the relative abundance of *Lactobacillus* decreased significantly at the end of the experiment. However, *Lactobacillus* was much more abundant in the MMFP group

compared with the Con and Ant groups on day 49 (Fig. 6d). In addition, at the end of the experiment, the relative abundance of *Campylobacter* in the Con group was significantly higher than that in the Ant and MMFP groups. These results indicate that MMFP could not only ameliorate the reduction of *Lactobacillus* caused by weaning but also inhibited the proliferation of pathogenic bacteria.

Correlation analysis between metabolic profiles and microorganisms

Correlation analysis (Fig. 7) showed significant correlations between piglet metabolic profile and the relative abundances of bacteria in GI tract. The relative abundances of *Clostridium sensu stricto* 6, *Acetivomaculum*, *Ruminococcaceae* UCG-013, *Coprococcus* 1, *Peptoclostridium*, *Streptococcus*, *Roseburia*, *Eubacterium xylanophilum* group, and *Lactobacillus* had positive correlations with the levels of T-AOC, GSH-Px, and CAT in serum and the concentrations of SCFAs in the intestinal contents, while these bacteria were negatively correlated with levels of MDA in serum and inflammatory factors in intestinal mucosa. In contrast, the relative abundances of *Turicibacter*, *Blautia*, *Marvinbryantia*, *Peptococcus*, *Methanobacteriaceae*, Family XIII, and *Parabacteroides* had negative correlations with the levels of T-AOC, GSH-Px, and CAT in serum and the concentrations of SCFAs in the intestinal contents, and they had positive correlations with the levels of MDA in serum and inflammatory factors in the intestinal mucosa. There was no obvious correlation between the growth performance of piglets and changes in the relative abundances of fecal microbiota (data not shown).

Discussion

Traditional MMF is spontaneously fermented by bacteria, mold, and yeast (Xu et al. 2013). However, the quality of traditional MMF is unstable, and pathogens have been detected in some MMFs (Liu et al. 2017). This restricts its clinical application. Guided by the Chinese Pharmacopeia, we improved the fermentation of MMF with probiotics, i.e., *Bacillus* sp., *Lactobacillus* sp., *S. thermophilus*, *E. faecalis*, *Saccharomyces cerevisiae*, *Aspergillus oryzae*, and *Mucor ramosus*. Bacterial strains used in MMFP fermentation are safer and more controllable compared with traditional MMF. Therefore, MMFP combines the advantages of Chinese herbal medicine, digestive enzymes, and probiotics. The current study confirmed that dietary supplementation with MMFP effectively alleviated weaning stress in piglets, reduced the levels of inflammatory factors in the intestinal mucosa, and elevated the antioxidant capacities in serum and the concentrations of organic acids in the intestinal contents.

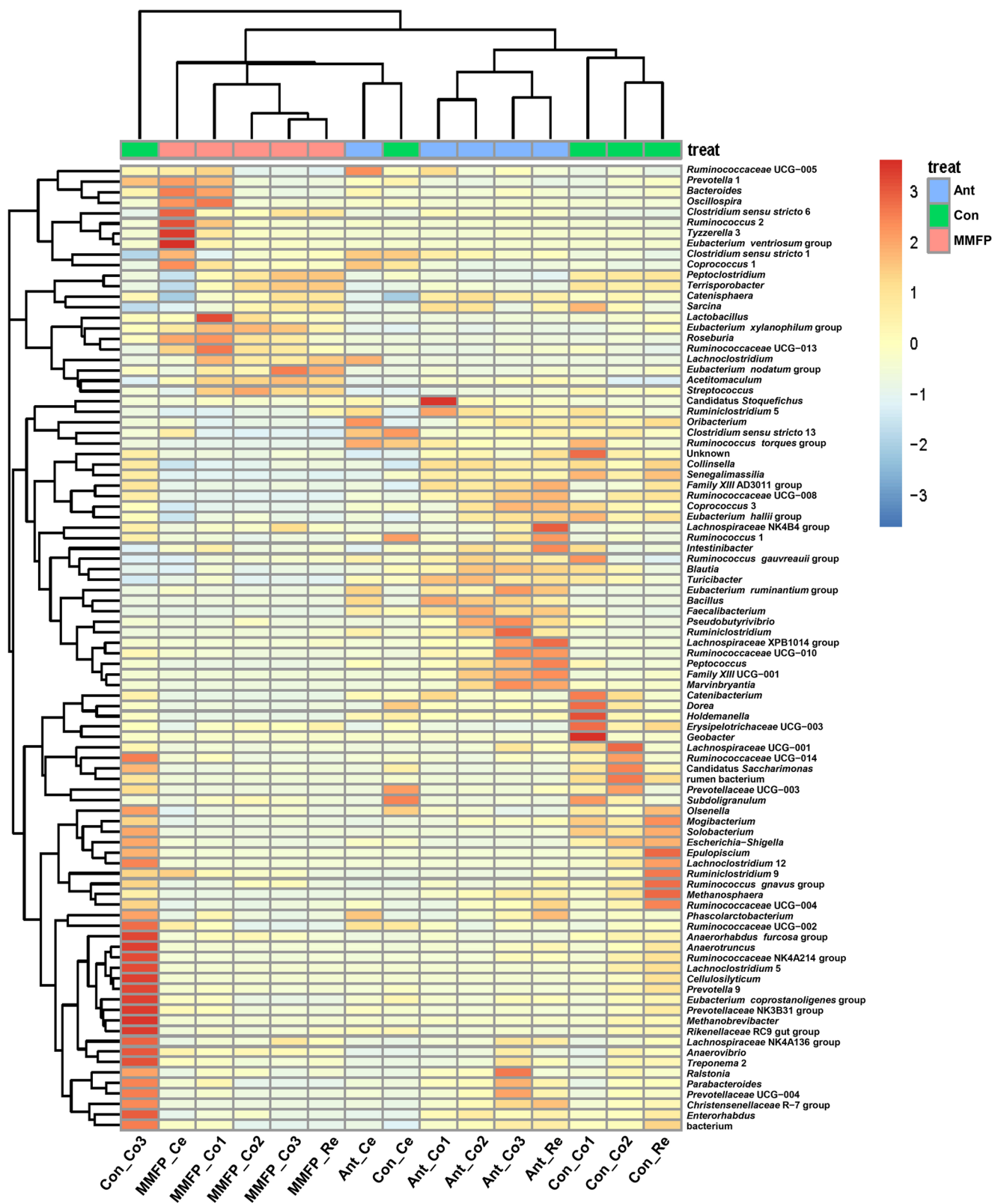


Fig. 5 Heatmap of microbial community along the gastrointestinal tract. On day 49, one pig from each pen was selected randomly and slaughtered. The microbial community of intestinal contents from different segments of GI tract was analyzed through high-throughput sequencing. Sample ID: Con_Ce, Ant_Ce, and MMFP_Ce: Cecal

contents; Con_Co1, Ant_Co1, and MMFP_Co1: contents of proximal colon; Con_Co2, Ant_Con2, and MMFP_Co2: contents of medial colon; Con_Co3, Ant_Con3, and MMFP_Co3: contents of distal colon; Con_Re, Ant_Re, and MMFP_Re: rectal contents

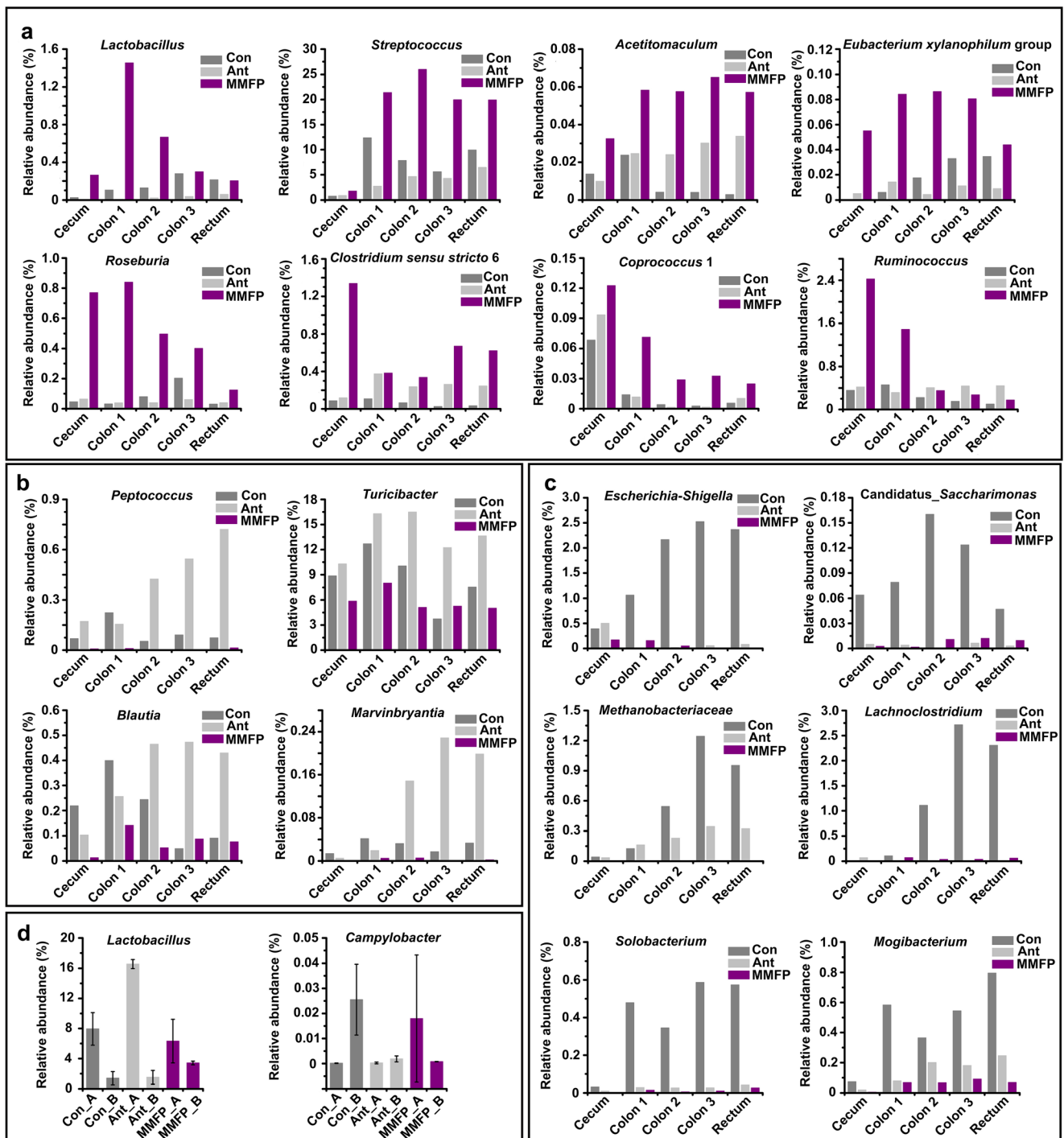


Fig. 6 Effects of MMFP on relative abundance of bacteria in different intestinal segments and feces. **a** Bacteria enriched in intestinal contents of MMFP group. **b** Bacteria enriched in intestinal contents of Ant group. **c** Bacteria enriched in intestinal contents of Con group. **d** Bacteria in fecal samples. Three fecal samples from each pen on day 0 and day 49 respectively were analyzed. On day 49, one pig from each pen was

selected randomly and slaughtered. The microbial community of intestinal contents from different segments of GI tract was analyzed. Sample ID: Con_A, Ant_A, and MMFP_A: fecal samples from Con, Ant, and MMFP group respectively on day 0; Con_B, Ant_B, and MMFP_B: fecal samples from Con, Ant, and MMFP group respectively on day 49

During the weaning period, weaning stress can cause anorexia, growth retardation, severe enteric infection, or even death, which inflicts significant losses on the swine industry. Many kinds of feed additives, such as a live oral vaccine

against ETEC F4, ZnO, essential oils, organic acids, prebiotics, and probiotics, have been used to alleviate weaning stress and pathogenic infections in piglets, as reviewed by Gresse et al. (2017). However, there was no systematic report

Table 2 The α -diversity of microbiota in the feces

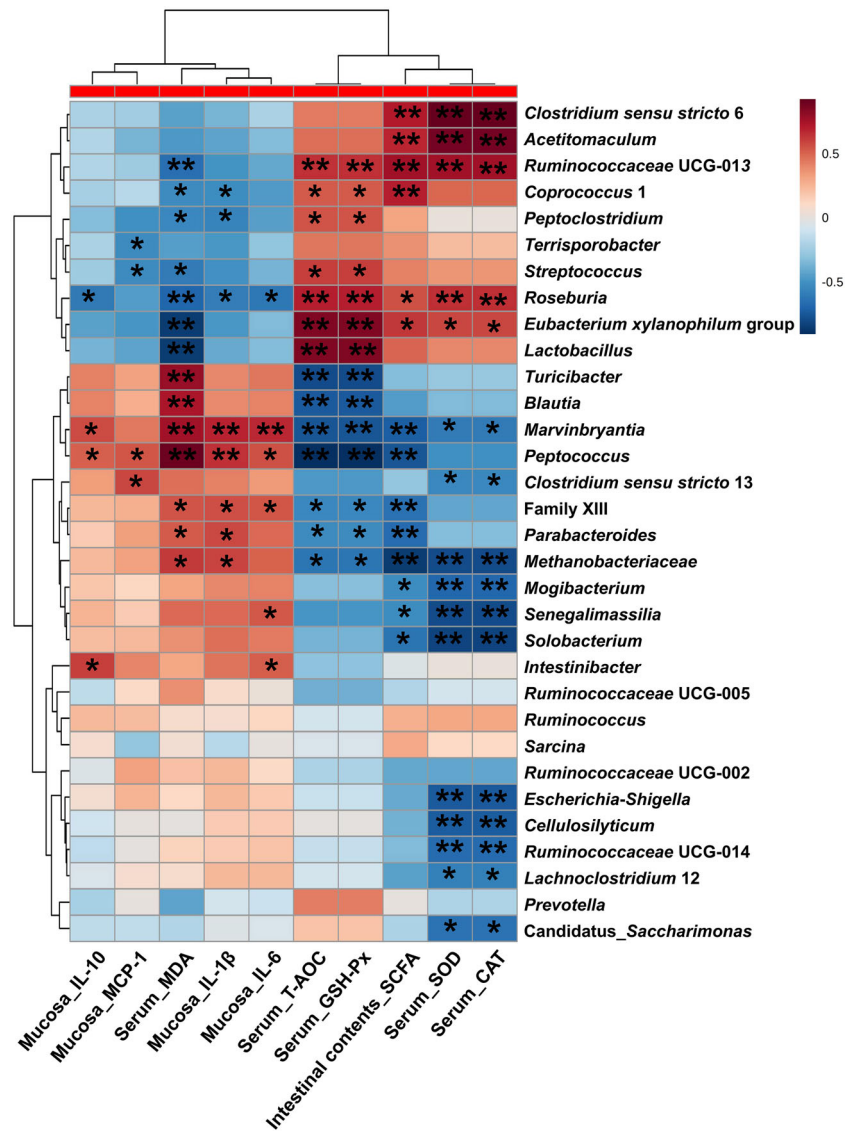
Sample ID	OTU	ACE	Chao1	Simpson	Shannon	Coverage
Con_A	594 ± 10 c	716.85 ± 4.12 c	729.67 ± 9.11 c	0.03 ± 0.003	4.50 ± 0.07 a	0.9920
Con_B	741 ± 57 b	854.71 ± 55.96 b	861.58 ± 45.67 b	0.03 ± 0.005	4.86 ± 0.17 bc	0.9918
Ant_A	674 ± 34 b	768.70 ± 24.98 c	779.60 ± 19.90 c	0.02 ± 0.002	4.66 ± 0.08 abd	0.9945
Ant_B	862 ± 38 a	952.07 ± 14.36 a	954.48 ± 10.86 a	0.02 ± 0.003	5.03 ± 0.05 c	0.9937
MMFP_A	667 ± 26 bc	789.39 ± 37.58 bc	789.76 ± 36.61 c	0.03 ± 0.014	4.62 ± 0.27 ab	0.9929
MMFP_B	801 ± 35 a	890.02 ± 35.12 ab	891.39 ± 41.45 ab	0.02 ± 0.001	4.94 ± 0.03 cd	0.9949

Data represent mean values ± SEM (three fecal samples from each group on day 0 and day 49 respectively). Student's *t* test was used to calculate *p* values. a, b, c, d: Different letters mean significant difference ($p < 0.05$)

about the beneficial effects of probiotics-fermented Chinese medicines on weaning piglets. Our study confirmed that MMFP treatment increased ADFI and ADG while reducing diarrhea incidence and mortality. On the one hand, MMFP contains various digestive enzymes (Xu et al. 2013), and is

usually used to promote food digestion and nutrient absorption. Herbal formulas containing MMF promote gastric emptying and intestinal transit (Xu et al. 1998). On the other hand, MMFP is fermented by probiotics. Dietary supplementation with probiotics, such as *Bacillus* strain and *Lactobacillus*

Fig. 7 Heatmap of correlations between the metabolic profile and the relative abundance of bacteria in intestinal contents. Analysis is based on Spearman's correlation coefficient. * $p < 0.05$; ** $p < 0.01$



rhamnosus GG, has reduced diarrhea and promoted growth in pigs (Loh et al. 2014; Mao et al. 2016). Taken together, the data suggest that the efficacy of MMFP is founded on the synergy between MMF and probiotics.

Weaning can induce acute and long-lasting damage to the intestinal physiology, including reduction of villus height and extension of crypt depth (Boudry et al. 2004; McCracken et al. 1999; Montagne et al. 2007). These dramatic alterations can impact the ability of digestion, absorption, and secretion and ultimately intestinal barrier function. Once disrupted, the increased permeability of the barrier will allow toxins, bacteria, or feed-associated antigens to cross the epithelium, which results in inflammation and diarrhea (Pie et al. 2004). The activation of the immune system will reduce the growth rate, feed intake, and lean tissue deposits (Williams et al. 1997). Clearly, to ameliorate weaning stress, it is critical to reduce the degeneration of the intestinal structure and activation of the immune system during the weaning period.

In the current research, villus height and mucosal thickness were increased in MMFP treatment, which were beneficial to intestinal function. The increased villus height raises the amount of contact and the number of active sites between digesta and epithelium, which benefits the absorption of nutrients. In addition, our results reveal that there were no goblet cells or Paneth cells in the intestine of the Ant group, and antibiotic treatment reduced the ratio of villus height to crypt depth and mucosal thickness of the cecum and colon. A previous study reported that dietary supplementation with antibiotics, such as bacitracin zinc, reduced the intestinal tract weight of broiler chicks (Henry et al. 1987). These results suggest that antibiotics may have adverse effects on the development of the GI tract. In contrast, MMFP promotes the development of the GI tract and improves intestinal barrier function.

The over expression of intestinal pro-inflammatory cytokines has adverse effects on the integrity of the intestinal mucosa and allow increased tissue penetration of antigens (Al-Sadi et al. 2013; Liu et al. 2008). The inhibition of cytokines may prevent the propagation of certain pathogens, which contributes to the stability of the intestinal micro-ecosystem (Walsh et al. 2013). In this study, dietary supplementation with MMFP reduced MCP-1, IL-1 β , IL-6, and IL-10 in the intestinal mucosa, which might be one of the reasons why MMFP reduced diarrhea incidence and improved the growth rate. Antioxidant capacities in the serum reflect the host's capacity to respond to endogenous oxidative damage. Improving the antioxidant capacities has positive effects on weaning stress (Fan et al. 2017; Wei et al. 2016). In this study, MMFP increased the levels of T-AOC, SOD, GSH-Px, and CAT and decreased the level of MDA in serum, which suggests that MMFP ameliorates oxidative stress during the weaning period in piglets.

Organic acids play important roles in regulating body's growth and homeostasis. Acetate could inhibit pathogen

proliferation and invasion of the intestinal epithelium (Asahara et al. 2009; Fukuda et al. 2011) and increase feed intake and obesity (Perry et al. 2016). From this, we infer that increased acetate in the MMFP group might have reduced the risks of pathogen proliferation and infection, and it also contributed to weight gain in weaning piglets. Butyrate is preferentially used as an energy source by gut epithelial cells. Propionate is mainly metabolized in the liver through gluconeogenesis. Butyrate and propionate inhibit the activity of histone deacetylases (HDACs) in colonocytes and immune cells, which leads to the down-regulation of pro-inflammatory cytokines in colonic macrophages (Louis et al. 2014). Dietary supplementation with sodium butyrate decreased the diarrhea incidence of weaned piglets and enhanced immune function (Fang et al. 2014). The present data demonstrate that MMFP increased the concentrations of butyrate and propionate in the intestinal contents, which indicated that MMFP may contribute to the intestinal immune system and improve energy acquisition from food. In the MMFP group, the concentration of lactate in the cecal contents was higher than that in the colonic and rectal contents, whereas acetate, propionate, and butyrate were lower in the cecal contents. This might confirm that lactate is used as a metabolic substrate for SCFA biosynthesis. The lactate-related trophic chain is considered one of the principal metabolic pathways in mammalian gut ecosystems (Valeriano et al. 2017). Lactate could be utilized to form propionate and pyruvate, and then pyruvate can be used for acetate and butyrate biosynthesis (Flint et al. 2015; Louis et al. 2014). Consequently, in the MMFP group, the increased lactate might have facilitated SCFA biosynthesis. In summary, increased concentrations of organic acids in the MMFP group might have contributed to intestinal homeostasis.

Dietary supplementation with MMFP altered the microbial composition of the intestinal contents, as shown in Fig. 5 and Fig. S2c. The relative abundances of *Lactobacillus* and *Streptococcus* were increased in the MMFP group compared with the Con and Ant groups. This had beneficial effects on the health of the host. *Lactobacillus* is one of "core microbiota" in the GI tract of mammals (Katouli and Wallgren 2005), which can prevent the diarrhea and intestinal infections caused by bacteria and viruses (Konstantinov et al. 2006; Li et al. 2012; Mao et al. 2016). Bacteria of the *Lactobacillus* group are often decreased during the weaning transition (Gresse et al. 2017), so supplementation of *Lactobacillus* spp. may decrease the risks of disease. *Streptococcus thermophilus* has been widely used as probiotic to enhance the immune system and inhibit the proliferation of pathogens (Feng et al. 2017). *Acetivomaculum* is an acetate-producing bacterium (Greening and Leedle 1989). *Roseburia* (Duncan et al. 2002; Louis and Flint 2009), *Coprococcus* (Van den Abbeele et al. 2013), *Eubacterium xylanophilum* group (Van Gylswyck and van der Toorn 1985), *Clostridium sensu stricto* (Varel and Pond 1992), and *Ruminococcus* (Claesson

et al. 2012) can produce butyrate. The increased relative abundances of above bacteria in the MMFP group explained the increase of organic acids in the intestinal contents. Correlation analysis showed that the relative abundances of *Clostridium sensu stricto* 6, *Acetitomaculum*, *Coprococcus* 1, *Streptococcus*, *Roseburia*, *Eubacterium xylanophilum* group, and *Lactobacillus* were positively correlated with the antioxidant activities in serum and negatively correlated with the levels of MDA and inflammatory cytokines, which meant these bacteria have positive effects on host health. Dietary supplementation of MMFP increased the relative abundance of cellulolytic and SCFA-producing bacteria, indicating a shift toward a more adult pig-like intestinal environment associated with increased functional ability for carbohydrate degradation.

The long-term use of subtherapeutic doses of antibiotics, or taking antibiotics for bacterial infections will increase the risks of pathogen proliferation and infection (Fouhse et al. 2016; Mu et al. 2017). A similar pattern appeared in the Ant group in this study. *Peptococcus* has been significantly enriched in oral squamous cell carcinoma samples (Zhao et al. 2017). *Turicibacter* has been isolated from a blood culture of a febrile patient with acute appendicitis (Bosshard 2002). *Blautia* was notably increased in the fecal samples of hyperlipidemic patients compared with the samples from normal volunteers (Wang et al. 2017). The relative abundances of *Turicibacter*, *Blautia*, *Marvinbryantia*, and *Peptococcus* were increased in the colonic and rectal contents of Ant group (Fig. 6b). Furthermore, the abundance of these bacteria was positively correlated with the levels of MDA in serum and inflammatory factors in intestinal mucosa, and negatively correlated with the antioxidant capacities in serum and the concentrations of organic acids in intestinal contents (Fig. 7). These results suggest that antibiotics may increase the risks of infections and oxidative damage.

The pro-inflammatory bacterium *Mogibacterium* is more abundant in feces of patients with adenomatous polyps (Hale

et al. 2017) and intestinal mucosa of colorectal cancer patients (Candela et al. 2014). *Solobacterium moorei* can cause bacteremia (Lau et al. 2006), and pathogenic *Escherichia-Shigella* can cause piglet diarrhea (Gresse et al. 2017). The decreased relative abundances of *Mogibacterium*, *Solobacterium*, and *Escherichia-Shigella* in the intestinal contents of the Ant and MMFP groups reflected that a reduced diarrhea incidence in Ant and MMFP group might occur partially through the inhibition of certain pathogenic bacteria. Methane is a major greenhouse gas. Some evidences show that methanogenesis is associated with slower intestinal and colonic transit (Pimentel et al. 2006; Soares et al. 2005), which may result in constipation (Chatterjee et al. 2007) and dyspepsia. A previous study showed that acidification of the gut lumen caused by high levels of SCFAs inhibited methanogenic activity (Ong et al. 2010). In this study, high levels of SCFAs in the intestinal contents of MMFP group might have inhibited methanogenesis, which had positive effects on food digestion and environment.

It is worth noting that the microbial composition of feces was barely affected by Ant or MMFP treatment at the end of the experiment. The most remarkable changes were that MMFP diminished the reduction of *Lactobacillus* caused by weaning and reduced the relative abundance of *Campylobacter*. *Campylobacter* is a typical enteric pathogen. *Campylobacter* is abundant in the gut of pigs with dysentery (Burrough et al. 2017), and humans may be infected with *Campylobacter* through various animal sources (Rosner et al. 2017). The reduction of *Campylobacter* in the feces of the Ant and MMFP groups suggested that antibiotics and MMFP may decrease the risks of dysentery and diffusion of pathogens. The microbial composition of feces had no correlation with the growth performance or metabolic profiles in our study. These results reveal that the microbiota in the GI tract, instead of in the feces, was closely related to the homeostasis of host, which is in accordance with a previous study (Zhao et al. 2015).

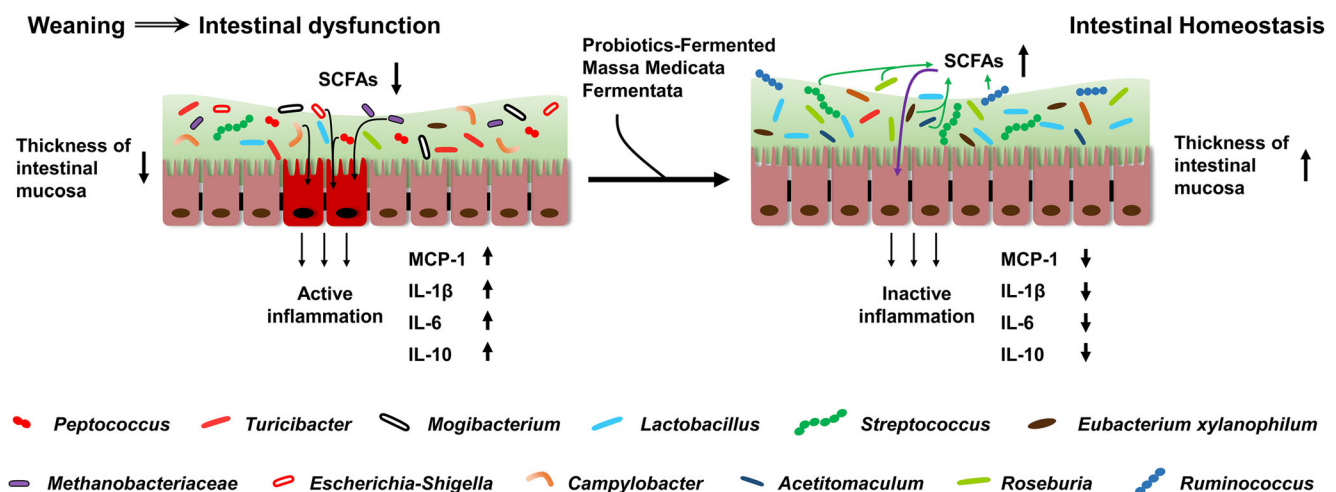


Fig. 8 Mechanisms of MMFP enhancing host intestinal homeostasis

In conclusion, dietary supplementation with MMFP improved the growth rate and average daily feed intake and reduced the diarrhea incidence and mortality of piglets. These results confirmed that MMFP can effectively alleviate weaning stress in piglets. Analysis of morphology and ELISA showed that MMFP promoted the development of intestinal histology and decreased the levels of inflammatory factors in the intestinal mucosa. In addition, MMFP elevated the antioxidant capacities of serum and the concentrations of organic acids in the intestinal contents. Correspondingly, the relative abundances of lactic acid and SCFA-producing bacteria were dramatically increased in the intestinal contents of the MMFP group. Dietary supplementation with MMFP reduced the relative abundances of pathogens in the intestinal contents and feces. These results demonstrate that MMFP enhanced host intestinal homeostasis by modulating the composition of the gut microbiota, thereby ameliorating weaning stress in piglets (Fig. 8). Obviously, the growth-promoting mechanism of MMFP is different from that of antibiotics. Furthermore, correlation analysis between the relative abundances of microbiota and host metabolic profile showed that the microbiota in the GI tract, not feces, modulates host metabolism. More investigations are still needed to explore the effects and mechanisms of probiotics-fermented Chinese medicine. We hope that this report could contribute to the development of new and efficient feed additives, and serve as a reference and a case for discussion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The animal experiment was approved by the Institutional Animal Care and Use Committee of Jilin University (IACUC). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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