Int J Clin Exp Med 2015;8(1):1504-1514
www.ijcem.com /ISSN:1940-5901/IJCEM0003152

Original Article

Effect *of Two Macrocephala Flavored Powder* supplementation on intestinal morphology and intestinal microbiota in weaning pigs

Gang Ye[1], Yin Qiu[1], Xiaoli He[2], Ling Zhao[1], Fei Shi[1], Cheng Lv[1], Bo Jing[1], Yinglun Li[1]

[1]College of Veterinary Medicine, Sichuan Agricultural University, Ya'an 625014, Sichuan, China; [2]College of Science, Sichuan Agricultural University, Ya'an 625014, Sichuan, China

Received October 14, 2014; Accepted January 5, 2015; Epub January 15, 2015; Published January 30, 2015

Abstract: A total of 75 pigs were used to investigate effects of feeding *Two Macrocephala Flavored Powder* (TMFP) on small intestinal morphology, intestinal microbiota in weaning pigs. The dietary treatments were: a control diet; control diet + 3 g/kg TMFP; control diet + 0.3 g/kg colistin sulfate (ANT). The results showed that supplementation with TMFP increased ($P < 0.05$) villus height at duodenum, jejunum at 3 time points, increased ($P < 0.05$) crypt depth at duodenum, jejunum at day 14, improved villus height: crypt depth ratio ($P < 0.05$) in jejunum at day 21 as compared with ANT. Supplementation of TMFP and ANT had lower ($P < 0.05$) *E. coli* counts in the ileum, cecum and colon at day 7 as compared with control. Supplementation of TMFP had higher ($P < 0.05$) bifidobacteria counts in the ileum, cecum and colon compared with ANT, except for colon at day 21. No effect ($P > 0.05$) on lactobacilli in colon has been seen with supplementation of TMFP and ANT at 3 time points, while both of supplementations showed increased the number of lactobacilli in cecum at day 14 and day 21. Analysis of DGGE fingerprints indicated that a highest similarity was observed for profiles from samples taken 14 d, 21 d from TMFP. The diversity of DGGE fingerprints of TMFP was higher than those of ANT and control. The results suggest that TMFP is potential to enhancing intestinal morphology and microbiota of weaning pigs, and can be served as an effective and safe dietary additive for weaning pigs.

Keywords: Pig, *Two Macrocephala Flavored Powder* (TMFP), intestinal morphology, intestinal microflora, denaturing gradient gel electrophoresis (DGGE)

Introduction

The weaning period is one of the most stressful phases, including changes in diet composition, the influence of psychology, environment and nutrition stress factors. During this period, pigs show immature bowel function, inadequate secretion of digestive enzymes, incomplete immune system, and poor resistance, which may contribute to digestive upsets, depressions in growth rates, invasion of pathogenic bacteria, diarrhea and an increased risk of disease [1]. For many years, both therapeutic and growth-promoter antibiotics (AGP) have been determined effective in improving the performance of the pigs through a decrease in the detrimental effects caused by macrobiotic [2]. Currently, the possible development of bacterial resistance has motivated the ban of most AGP in animal nutrition in the European Union. Therefore, increasing attention has been paid to discover new feed additives alternative to traditional in animal production such as acidifiers, probiotics, prebiotics, and phytogenic substances [3].

In the past, the research of the effects of drugs or feed additives on pig intestinal microbial community had been studied intensively. However, most attention was paid to traditional plate count method for detecting the number of bacteria. Most intestinal bacterial are difficult to cultivate, thus remaining undetectable by conventional techniques [4, 5], besides, it can't accurately reflect diversity of microbial community. Recent phylogenetic analysis based on the *in vitro* amplification of the 16S r RNA gene and other phylogenetic markers by PCR techniques have revealed dramatically higher diversity than

Table 1. Effect of TMFP supplementation on morphological measurements of villus height, crypt depth and villus height: crypt depth ratio from duodenum, ileum and jejunum of pigs at day 7 after weaning[a]

Items	Control	TMFP	ANT
Duodenum			
Villus height/μm	133.85 ± 17.76[y]	152.45 ± 8.87[y]	55.78 ± 4.96[x]
Crypt depth/μm	60.82 ± 8.34[x]	86.16 ± 12.31[y]	39.30 ± 14.38[x]
V/C[b]	2.20 ± 0.01[x]	1.79 ± 0.18[x]	1.53 ± 0.48[x]
Ileum			
Villus height/μm	102.47 ± 18.96[y]	110.49 ± 5.61[y]	77.15 ± 5.44[x]
Crypt depth/μm	52.13 ± 10.00[y]	52.43 ± 1.47[y]	33.90 ± 4.03[x]
V/C	1.97 ± 0.19[x]	2.11 ± 0.17[x]	2.30 ± 0.34[x]
Jejunum			
Villus height/μm	87.01 ± 3.9[z]	101.14 ± 5.85[y]	60.04 ± 8.62[x]
Crypt depth/μm	48.34 ± 0.63[x]	58.71 ± 16.76[x]	37.16 ± 9.60[x]
V/C	1.80 ± 0.10[x]	1.80 ± 0.40[x]	1.65 ± 0.25[x]

[a]Control = diet of unmedicated; TMFP = control diet + 3 g/kg Two Macrocephala Flavored Powder; ANT = control diet + 0.3 g/kg colistin sulfate. [b]Means villus height: crypt depth ratio. [x,y,z]Means within a row with different letters differ significantly ($P < 0.05$) when tested with Duncan's new multiple-range test following analysis of variance.

Table 2. Effect of TMFP supplementation on morphological measurements of villus height, crypt depth and villus height: crypt depth ratio from duodenum, ileum and jejunum of pigs at day 14 after weaning[a]

Items	Control	TMFP	ANT
Duodenum			
Villus height/μm	128.51 ± 6.09[y]	125.00 ± 13.83[y]	73.50 ± 3.79[x]
Crypt depth/μm	71.48 ± 9.37[y]	56.74 ± 8.92[x,y]	43.35 ± 2.08[x]
V/C[b]	1.81 ± 0.16[x]	2.22 ± 0.25[y]	1.70 ± 0.07[x]
Ileum			
Villus height/μm	89.44 ± 17.11[y]	108.54 ± 10.66[y]	63.32 ± 7.25[x]
Crypt depth/μm	51.93 ± 12.20[x]	51.98 ± 7.97[x]	36.04 ± 5.53[x]
V/C	1.74 ± 0.18[x]	2.10 ± 0.15[y]	1.77 ± 0.86[x]
Jejunum			
Villus height/μm	99.65 ± 6.10[y]	99.74 ± 21.82[y]	53.94 ± 9.05[x]
Crypt depth/μm	46.88 ± 3.44[y]	49.70 ± 1.57[y]	32.33 ± 2.13[x]
V/C	2.13 ± 0.21[x]	2.00 ± 0.38[x]	1.66 ± 0. 17[x]

[a]Control = diet of unmedicated; TMFP = control diet + 3 g/kg Two Macrocephala Flavored Powder; ANT = control diet + 0.3 g/kg colistin sulfate. [b]Means villus height: crypt depth ratio. [x,y,z]Means within a row with different letters differ significantly ($P < 0.05$) when tested with Duncan's new multiple-range test following analysis of variance.

described previously by cultivation [6, 7]. To this end, molecular approaches based on PCR can introduce different types of primers, a combination of PCR and fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), has led to new insights into the microbial diversity. The objective of this study is to investigate the effects of feeding traditional Chinese herb *Two Macrocephala Flavored Powder* (TMFP) on intestinal morphology, intestinal microbiota in newly weaning pigs.

Material and methods

Plant material

The Two Macrocephala Flavored Powder (TMFP) was composed of five dried Chinese herbs, including Rhizoma atractylodis macrocephalae, Rhizoma atractylodis, Pericarpium citri reticulatae, Radix bupleuri and Salvia miltiorrhiza. All the herbal materials were purchased from Ya'an Pharmaceutical Co (Sichuan, China) in 2012 and verified by Qiao-Jia Fan (Sichuan Agriculture University of Chinese Materia Medica, China). The herbal materials were crushed and sieved using a grinder and passed through a 100-mesh sieve, packed in hermetical bags, and stored at room temperature before test.

Test animals and sample collection

75 pigs (Duroc × Landrace × Yorkshire) were selected from the swine herd at Sichuan Agriculture University Farm. This facility has a closed, minimal disease herd. All pigs had ad libitum access to a standard finishing diet and water. Pigs were cared for under the guidelines of the China Animal protection association.

Pigs were weaned at 30 days (6.45 ± 1.21 kg BW) and randomly divided into three groups, and diets that met or exceeded NRC [8] recommendations for required nutrients: control diet; control diet + 3 g/kg TMFP; control diet + 0.3 g/kg colistin sulfate (Qiankun animal pharmaceutical co, Sichuan, China).

Table 3. Effect of TMFP supplementation on morphological measurements of villus height, crypt depth and villus height: crypt depth ratio from duodenum, ileum and jejunum of pigs at day 21 after weaning[a]

Items	Control	TMFP	ANT
Duodenum			
Villus height/μm	101.20 ± 11.70[x]	146.25 ± 11.39[y]	114.14 ± 3.99[x]
Crypt depth/μm	59.56 ± 9.37[x]	75.02 ± 8.89[x]	68.85 ± 12.14[x]
V/C[b]	1.75 ± 0.39[x]	1.97 ± 0.32[x]	1.70 ± 0.37[x]
Ileum			
Villus height/μm	120.49 ± 9.93[x]	121.00 ± 4.55[x]	106.98 ± 5.46[x]
Crypt depth/μm	64.75 ± 5.20[x]	66.30 ± 10.2[x]	57.63 ± 14.60[x]
V/C	1.86 ± 0. 07[x]	1.85 ± 0.22[x]	1.96 ± 0.64[x]
Jejunum			
Villus height/μm	87.01 ± 3.97[y]	101.14 ± 5.85[z]	60.04 ± 8.62[x]
Crypt depth/μm	43.67 ± 2.19[x]	42.23 ± 0.91[x]	51.82 ± 22.91[x]
V/C	2.22 ± 0. 19[y]	2.27 ± 0.24[y]	1.48 ± 0.28[x]

[a]Control = diet of unmedicated; TMFP = control diet + 3 g/kg Two Macrocephala Flavored Powder; ANT = control diet + 0.3 g/kg colistin sulfate. [b]Means villus height: crypt depth ratio. [x,y,z]Means within a row with different letters differ significantly ($P < 0.05$) when tested with Duncan's new multiple-range test following analysis of variance.

Table 4. Effect of TMFP supplementation on Intestinal microbial population in contents of ileum, cecum and colon of pigs exposed to different dietary treatments at days 7 after weaning (log 10 cfu/g of wet contents)[a]

Segment	Bacteria	Diet		
		Control	TMFP	ANT
Ileum	*E. coli*	6.11 ± 0.01[x]	7.49 ± 0.10[y]	7.41 ± 0.01[y]
	Lactobacilli	8.46 ± 0.05[y]	8.17 ± 0.04[x]	8.64 ± 0.01[z]
	Bifidobacteria	8.76 ± 0.12[y]	8.34 ± 0.01[x]	8.86 ± 0.01[y]
Cecum	*E. coli*	6.21 ± 0.04[x]	7.89 ± 0.08[y]	7.52 ± 0.01[y]
	Lactobacilli	8.37 ± 0.10[x]	8.55 ± 0.01[x]	8.38 ± 0.06[x]
	Bifidobacteria	7.43 ± 0.06[x]	8.16 ± 0.02[z]	7.56 ± 0.01[y]
Colon	*E. coli*	7.61 ± 0.03[z]	7.39 ± 0.01[y]	7.14 ± 0.01[x]
	Lactobacilli	8.68 ± 0.08[x]	8.79 ± 0.05[x]	8.62 ± 0.01[x]
	Bifidobacteria	7.67 ± 0.06[x]	8.16 ± 0.01[y]	7.63 ± 0.01[x]

[a]Control = diet of unmedicated; TMFP = control diet + 3 g/kg Two Macrocephala Flavored Powder; ANT = control diet + 0.3 g/kg colistin sulfate. [x,y,z]Means within a row with different letters differ significantly ($P < 0.05$) when tested with Duncan's new multiple-range test following analysis of variance.

Sample handling

Five pigs of each treatment were sacrificed to evaluate gut morphology and intestinal microflora on day 7, 14 and 21, respectively. The tissue samples of small intestine were cut open longitudinally along the mesenteric attachment and fixed in a 10% (vol/vol) formalin solution until histological measurements were taken. Contents of the duodenum, cecum and colon were sampled aseptically. Samples of intestinal contents were divided immediately into aliquots for DNA and plate count analyses, stored on dry ice, and transported to the laboratory within 30 min. Samples for DNA analysis were stored at -70°C until analyzed. Viable plate counts were performed immediately upon receipt of the samples.

Intestinal morphology

The samples of small intestine tissue were dehydrated and embedded in paraffin wax. The samples were cut into 3-μm slices and were stained with hematoxylin and eosin before microscopic examination. The villus height was measured from the tip to the base, and then the crypt depth was measured from the base of the villus to the base of the crypt. The villus height: crypt depth ratio was also calculated. The 10 longest and straightest villus and their associated crypts from each segment were measured. Mean villus heights, crypt depths, and VCR within each segment were calculated for statistical analysis.

Microbiological examination

The samples of duodenum, cecum and colon were cultured in different media for determined. For bacterial assays, 10-fold serial dilutions were made from 1-g aliquots of contents, using PBS as a diluent. One hundred microliters of each dilution was spread on Petri dishes containing growth media specific for each bacterial type. Total *E. coli* were determined by growth on EMB agar. Total lactobacilli were determined by growth on MRS ager. Total bifidobacteria were determined by growth on TPY agar. Cultures were returned to the incubator until enumeration was performed. Escherichia coli were incubated aerobically at 37°C for 24 h. Anaerobic

Table 5. Effect of TMFP supplementation on Intestinal microbial population in contents of ileum, cecum and colon of pigs exposed to different dietary treatments at days 14 after weaning (log 10 cfu/g of wet contents)[a]

Segment	Bacteria	Diet		
		Control	TMFP	ANT
Ileum	*E. coli*	6.63 ± 0.25^{x}	7.93 ± 0.04^{y}	6.48 ± 0.23^{x}
	Lactobacilli	8.34 ± 0.15^{y}	8.10 ± 0.04^{x,y}	7.90 ± 0.04^{x}
	Bifidobacteria	7.41 ± 0.13^{x}	8.75 ± 0.02^{y}	7.48 ± 0.02^{x}
Cecum	*E. coli*	7.70 ± 0.06^{y}	7.82 ± 0.07^{y}	7.34 ± 0.13^{x}
	Lactobacilli	8.30 ± 0.03^{y}	8.86 ± 0.04^{z}	7.71 ± 0.08^{x}
	Bifidobacteria	7.79 ± 0.16^{x}	8.72 ± 0.14^{z}	8.31 ± 0.02^{y}
Colon	*E. coli*	7.29 ± 0.01^{y}	6.64 ± 0.07^{x}	7.21 ± 0.01^{y}
	Lactobacilli	8.66 ± 0.05^{x}	8.75 ± 0.17^{x}	8.92 ± 0.01^{x}
	Bifidobacteria	7.82 ± 0.04^{x}	8.57 ± 0.04^{z}	8.15 ± 0.34^{y}

[a]Control = diet of unmedicated; TMFP = control diet + 3 g/kg Two Macrocephala Flavored Powder; ANT = control diet + 0.3 g/kg colistin sulfate. ^{x,y,z}Means within a row with different letters differ significantly ($P < 0.05$) when tested with Duncan's new multiple-range test following analysis of variance.

Table 6. Effect of TMFP supplementation on Intestinal microbial population in contents of ileum, cecum and colon of pigs exposed to different dietary treatments at days 21 after weaning (log 10 cfu/g of wet contents)[a]

Segment	Bacteria	Diet		
		Control	TMFP	ANT
Ileum	*E. coli*	7.29 ± 0.21^{y}	6.80 ± 0.14^{x,y}	6.27 ± 0.33^{x}
	Lactobacilli	8.54 ± 0.25^{y}	8.52 ± 0.18^{y}	6.66 ± 0.18^{x}
	Bifidobacteria	8.83 ± 0.13^{y}	8.44 ± 0.05^{y}	7.28 ± 0.17^{x}
Cecum	*E. coli*	7.45 ± 0.15^{y}	6.90 ± 0.01^{x}	7.46 ± 0.01^{y}
	Lactobacilli	8.08 ± 0.01^{y}	8.73 ± 0.04^{z}	7.54 ± 0.01^{x}
	Bifidobacteria	8.95 ± 0.01^{y}	8,84 ± 0.16^{y}	8.38 ± 0.01^{x}
Colon	*E. coli*	7.43 ± 0.06^{x}	7.63 ± 0.09^{x,y}	7.88 ± 0.13^{y}
	Lactobacilli	8.62 ± 0.03^{x}	8.91 ± 0.07^{x}	8.73 ± 0.16^{x}
	Bifidobacteria	8.21 ± 0.06^{x}	8.37 ± 0.15^{x}	8.18 ± 0.10^{x}

[a]Control = diet of unmedicated; TMFP = control diet + 3 g/kg Two Macrocephala Flavored Powder; ANT = control diet + 0.3 g/kg colistin sulfate. ^{x,y,z}Means within a row with different letters differ significantly ($P < 0.05$) when tested with Duncan's new multiple-range test following analysis of variance.

culture included bifidobacteria, lactobacilli, which were incubated at 37°C for 48 h. Bacteria were enumerated by visual count, disregarding atypical colonies.

Analysis of intestinal samples by PCR-DGGE

Bacterial genomic DNA extraction: DNA was extracted from 500 mg of intestinal contents using a DNA extraction kit (TIANNampTIANGEN, China) according to the manufacturer's instructions.

PCR amplification: The PCR amplifications of the total bacterial community DNA were undertaken using the primers gc338f (5'-CGC CCG GGG CGC GCC CCG GGG CGG GGC GGG GGC GCG GGG GG CCT ACG GGA GGC AGC AG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') [9]. 50 microliters of each PCR reaction mixture contained 2 μL of template DNA, 20 mM of each primer, 3.2 μL of 2.5 mM deoxyribonucleotide triphosphate, 5 μL of 10 × PCR buffer, and 0.4 μL of 5 U/μL Taq polymerase. PCRs were run under the following cycling conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 45 s, 72°C for 1 min, and the final primer extension was carried out at 72°C for 10 min.

PCR-DGGE: After visual confirmation of the PCR products with agarose gel electrophoresis, DGGE was performed using the Bio-Rad D-code system as described previously [10]. To separate PCR fragments, 35 to 55% linear DNA-denaturing gradients (100% denaturant is equivalent to 7 mol/L urea and 40% deionized acrylamide) were formed in 8% polyacrylamide gels using a Bio-Rad Gradient Former. Bacterial V3 16S PCR products were loaded in each lane and electrophoresis performed at 60°C at 150 V for 5 h. After electrophoresis, gels were silver-stained and scanned using a Gel-Doc2000 gel imagining system (Bio-Rad). Each individual amplicon was then visualized as a distinct band representing at least one bacterial species on the gel. Representative bands were sterile excised from DGGE gels, re-amplified as previously described and sequenced for identification. Sequence data were analyzed using Sequencher 3.0 software, and a BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search was performed for phylogenetic identification.

Estimates of microbial richness and diversity

Discovery Series Diversity Database software (Bio-Rad) (version 2.1) was used to analyze banding patterns by measuring the migration distance and intensity of the bands within each

Table 7. Identification of predominant bands of DGGE fingerprints

Band no.	Closest species found in the GenBank database	GenBank accession no.	Similarity	Bacteriophyta
Band 1	Prevotella oralis	NR_042841	98	Bacteroidetes
Band 2	Lactobacillus acidophilus	NR_075049	100	Firmicutes
Band 3	Uncultured bacterium	EU778531	100	environmental samples
Band 4	Lactobacillus acidophilus	NR_075049	99	Firmicutes
Band 5	Alistipes putredinis	NR_025909	97	Bacteroidetes
Band 6	Corynebacterium_hansenii	NR_042703	100	Actinobacteria
Band 7	Prevotella amnii	NR_042587	90	Bacteroidetes
Band 8	Helicobacter pullorum	NR_043053	99	Proteobacteria
Band 9	Lactobacillus_johnsonii	NR_075064	100	Firmicutes
Band 10	Coprococcus_catus	NR_024750	97	Firmicutes
Band 11	Campylobacter canadensis	NR_044319	97	Proteobacteria
Band 12	Mogibacterium neglectum	NR_027203	91	Firmicutes
Band 13	Ethanoligenens harbinense	NR_042828	95	Firmicutes
Band 14	Roseburia faecis	NR_042832	99	Firmicutes
Band 15	Aminobacterium colombiense	NR_074624	94	Synergistetes

gel lane. This information was then used to analyze banding patterns via measurement of community diversity parameters, including band number, Sorenson's pairwise similarity coefficient (Cs), and Ward's algorithm. Clustering analysis by un-weighted pair group with mathematical averages (UPGMA), and microbial richness and diversity were evaluated by Shannon-Wiener (H), Margalef Index (S).

Statistical analysis

Statistical analysis the SPSS version 17.0 for windows was used for statistical tests. Results were statistically analyzed using one way analysis of variance. Duncan's multiple range test was used to compare differences among the treatment group. The differences at $P < 0.05$ were considered.

Results

Intestinal morphology

Morphological measurements of the small intestinal mucosa of pigs are presented in **Tables 1-3**. At weaning 7 d, supplementation with TMFP and control had higher ($P < 0.05$) villus height at the duodenum, ileum than that of the antibiotic group. Supplementation with TMFP had higher ($P < 0.05$) villus height at the jejunum as compared with control and the antibiotic group. There was no effect of dietary treatments on crypt depth at the intestinal morphology ($P > 0.05$). And as the same case with the villus height: crypt depth radio. At weaning 14 d, supplementation with TMFP and control had higher ($P < 0.05$) villus height at the intestinal than that of the antibiotic group. Supplementation with TMFP had higher ($P < 0.05$) crypt depth at the jejunum as compared with control and the antibiotic group. Supplementation with TMFP had higher ($P < 0.05$) villus height: crypt depth radio at the duodenum and ileum as compared with control and the antibiotic group. There was no effect of dietary treatments on villus height: crypt depth radio in the jejunum ($P > 0.05$). At weaning 21 d, for ileum, there was no effect of dietary treatments on the intestinal morphology. Supplementation with TMFP had higher ($P < 0.05$) villus height at the duodenum than the control and the antibiotic group. There was no effect of dietary treatments on crypt depth and villus height: crypt depth radio ($P > 0.05$). And as the same with the villus height: crypt depth radio. Supplementation with TMFP had higher ($P < 0.05$) villus height and villus height: crypt depth radio at the jejunum as compared with control and the antibiotic group. There was no effect of dietary treatments on crypt depth in the jejunum ($P > 0.05$).

Microbiological examination

E. coli, Lactobacillus and Bifidobacteria in the intestinal contents of each treatment were

Figure 1. DGGE from bacteria of piglet.

determined at 3 different time points (d 7, 14 and 21) during the study (**Tables 4**-**6**).

No difference was detected in E. coli counts of ileum and cecum among group with supplementation of TMFP ($P > 0.05$) and antibiotic ($P > 0.05$) at day 7, day 14. Pigs offered diets supplemented TMFP showed a significantly lower population of lactobacilli in the ileum compared with the antibiotic ($P < 0.05$) and control group at day 7. However, the number of lactobacilli had increased and was significantly higher than that of the antibiotic ($P < 0.05$) group at day 14, day 21. In addition, the number of bifidobacteria was lower in the ileum than that of the antibiotic ($P < 0.05$) and control group at day 7, but had increased and significantly higher than that of the antibiotic ($P < 0.05$) and control group at day 14, day 21. The counts of cecum *E. coli* were higher ($P < 0.05$) at day 7 in TMFP group as compared with the antibiotic and control group, but it was turned out to be lower at day 14, day 21 on TMFP group. On contrast, the counts of cecum lactobacillus were lower ($P < 0.05$) at day 7 on TMFP group as compared with the antibiotic and control group ($P < 0.05$), but it was higher at day 14, day 21 on TMFP group ($P < 0.05$). The number of cecum bifidobacteria was higher than the antibiotic group at 3 different time points ($P< 0.05$). No treatment effects were observed on the counts of lactobacillus ($P > 0.05$) in colon content at all 3 time points. The supplementation of TMFP had a higher bifidobacteria number in colon contents as compared to the antibiotic and control group ($P < 0.05$) at day 7, day 14.

DGGE-PCR analysis

To study the diversity of bacteria in the cecum digesta of weaned pigs, DGGE fingerprinting of 16S r RNA genes (V2-V3 region) was used. Comparison between the bands from sample lanes with bands from the marker lane and direct sequencing of the dominant bands (**Table 7**) revealed the presence of some bacteria (**Figure 1**) after weaning in all animals.

According to the DGGE fingerprinting, several bands were common in all samples. In addition, there were daily variations in band intensities within pig samples and some appearance or disappearance of bands. However, for the samples from control and TMFP group, the pattern was sufficiently stable to observe shifts in individual bands representing temporal changes in bacterial populations. To obtain an objective interpretation of the electrophoretic patterns of DGGE fingerprint, the samples were subjected to a numerical analysis based on the Dice similarity coefficient, followed by cluster analysis. Dietary supplementation of TMFP and antibiotic respectively could observe a dramatic increase or decrease in the intensity of the bands, while the intensity of other bands did not change obviously. Initially (at weaning 7 days) band 9 (Lactobacillus johnsonii) dominated in three group, while later (14 days), band 9 got more intense of TMFP group, while antibiotic and control group were strongly reduced in intensity (**Figure 1**). The number of DGGE bands was shown in **Figure 2**. At weaning 7 d, the number of bands of TMFP group was one of the most; the number of bands of antibiotic group was less than the TMFPF group, but more than

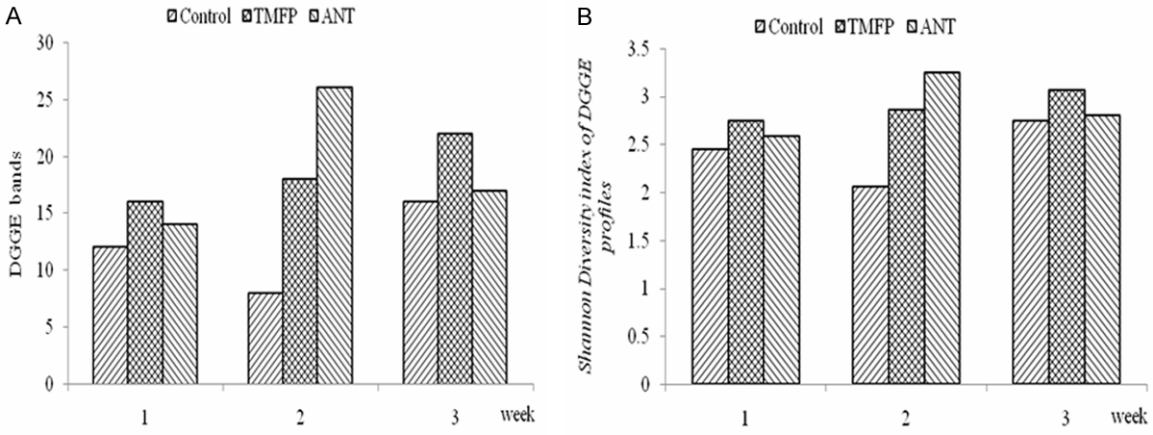

Figure 2. The number of DGGE bands (A) and Shannon diversity (B), H' of DGGE profiles of cecum samples of newly weaning pigs.

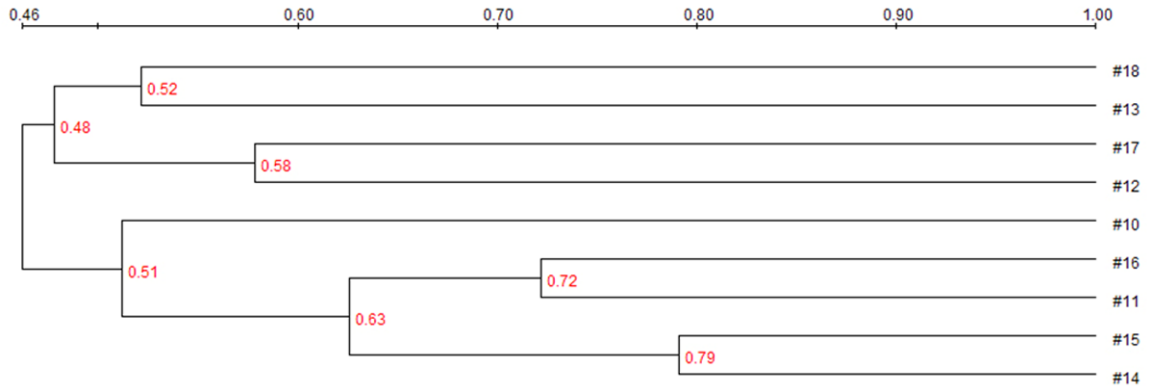

Figure 3. Similarity of DGGE profiles of samples of f ileum and cecum.

control. At weaning 14 d and 21 d, the number of bands of TMFP group was increase gradually. While control group, the number of bands was reduced at weaning 14 d, but it was strongly increased at weaning 21 d. Compared with control, the number of bands of antibiotic group was increased at weaning 14 d, but it was strongly reduced at weaning 21 d.

The similarity was visualized using the unweighted pair group method with averaging algorithm. There was a high similarity of the profiles between each treatment throughout the collection period (**Figure 3**). A comparison of inter-age similarity of the DGGE fingerprints of each treatment revealed a constant drop in the similarity between profiles with age, while the exception of TMFPF group. The highest similarities, of 79.1%, were observed for profiles from samples taken 14 d, 21 d from TMFP group. In contrast, similarity was significantly lower of antibiotic group of 3 weeks. TMFP group had a high similarity to control at 14 d (60.1%) and 21 d (64.3%).

The diversity of DGGE fingerprints of TMFP group determined as the Shannon index, shown in **Figure 2**, was higher at weaning 7 d, and remained increasing at weaning 14 d, 21 d. The diversity of DGGE fingerprints of antibiotic and control group were increased at 14 d, but decreased at 21 d. Taken together, the results obtained after DGGE analysis demonstrated that the bacterial compositions in the cecum of pigs were modulated by the TMFP diet from day 14.

Several diet-specific and common (dominant bands) were excised from the DGGE gels for sequencing and are listed in **Table 7** with their percent similarity to sequences in GenBank. The results of clones showed that the bacterial

corresponding with bands had high similarity to GenBank. Five clones, with 100% similarity, to known sequences in GenBank. The other clones were also closely related to GenBank. Only one clone represented uncultured bacterium, which was from environment samples. In contrast, sequencing several diet-specific and common bands from DGGE gel were corresponded to GenBank were mainly come from Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria.

Discussion

Small intestinal morphology has been used as an estimate of intestinal health in pigs typically [11]. The adverse stressful effects of weaning which may be due to psychological, environmental or nutritional factors [12], are presented in the digesta and can lead relatively quick changes in the intestinal morphology. A shortening of the villus decreases the surface area for nutrient absorption. The crypt is the area where stem cells divide to permit the renewal of the villus, and a large crypt indicates fast tissue turnover and a high demand for new tissue. Villus height reduces generally whilst increase is seen on crypt depth in control group, which may explain the increased occurrence of diarrhiea and reduced growth after weaning [13]. While villus height increases and crypt depth reduces in TMFP and ANT groups. These results were consistent with previous studies in broilers [14], rats [15] and pigs [16]. Mechanism for the improved intestinal morphology is unclear. It can be hypothesized that TMFP has the potential to promote intestinal morphology through cell proliferation [17]. In the present study, a significant increase in villus height, crypt depth and villus: crypt ratio at the small intestinal of the pigs supplemented with TMFP were observed at weaning 7 day, while at weaning 21 day, there were only numerically increase on crypt depth and villus: crypt depth ratio in the duodenum and also in the ileum compared with the control group and ANT. This may explain that TMFP could promote the development of small immediately after weaning and may be conducive to adapt to the weaning stressors. Furthermore, this data suggests supplementation with TMFP can enhance greater intestinal development and promote homeostasis during the time of weaning challenges.

Martin et al [18] suggested that dietary organic acid can influence the intestinal microbiota by changing the intestinal environment to less appropriate conditions for pathogenic bacteria growth or killing some pathogens. Hammer et al [19] reported that herb extracts such as lemongrass, oregano, hay, and thyme have antimicrobial activity. Kong et al [20] regulates Chinese medicine can improve the metabolism of amino acids and glucose and enhance the anti-oxidant activity in the small-intestinal mucosa. Alternatively, the dietary supplementation with the Chinese herbs enhances the intestinal immune function, thereby reducing inflammation in the small-intestinal mucosa that often occurs in weanling pigs [21].

E. coli was observed to decrease in pigs weaned at 7 d of age in control group in ileum, cecum, colon segment, but increased at 21 d of age after weaning. *E. coli* were decreased in pigs weaned at 21 d of age in diet supplement with TMFP in 3 intestinal segments, however, in good accordance with previous study. Namkung et al [22] reported that the counts of fecal coliform bacteria were lower ($P < 0.05$) at day 4 and 14 post-weaning in pigs on feeding blends of organic acids and herbal extracts as compared to the non-medicated control treatment. It was hypothesized that the bacteriostasis active ingredients of the Chinese herbal medicine may inhibite the growth of *E. coli*. There was no significant effect on colon in 3 time points. Supplement with TMFP increased the Lactobacilli population in ileum and cecum at 21 day after weaning compared with 7 day after weaning. This is in line with data from a previous study [23], showing that an increase in small intestinal concentrations of lactate were found 4 and 10 days after weaning in pigs fed a mixture of different fermentable carbohydrates (inulin, lactulose, wheat starch, and sugar beet pulp), whereas this difference was not significant 1 day after weaning. This may indicate that pigs weaned at older ages are more adept at dealing with changes over the course of weaning [24]. Bifidobacteria population was increased in cecum and colon compare to the control and ANT group at 7, 14 days after weaning and no significant difference at 21 days. The apparent inverse relationship between anaerobes and aerobes may indicate a competitive interaction between these bacteria. While the beneficial bacteria have beneficial effects on animal health and show antagonistic action toward pathogenic bacteria, their fast proliferation may be of vital importance for

weaned pigs [25]. These results suggest that inclusion of ANT in the diet reduced the proliferation of both of potentially harmful bacteria and potentially beneficial lactobacilli in the pigs' gut, while supplement with TMFP appeared to increase beneficial bacteria and reduce proliferation of potentially harmful bacteria. Supplement with TMFP have beneficial effects on animal health.

Many studies have focused on the changes in microbial communities in weaned pigs and investigate the effects of additives, like antibiotic [26], prebiotics [23], organic acids [27], plant extracts [22], during the weaning period could alter the diversity or composition of microbiota in the gastrointestinal tract. The PCR-DGGE technology is useful than traditional microbiological approaches and could be similarly applied to evaluate other dietary-, drug- or disease associated alterations of intestinal microbial populations, and screen shifts in microbial populations, differentially expressed bands can be cloned and sequenced to allow an objective identification of organisms whose appearance or loss is associated with diet or disease [28]. In this study, the PCR-DGGE technique was used to determine the microbial diversity and banding patterns in the intestinal tract of pigs. The PCR-DGGE approach is not used to quantify bacterial species, but can provide information on shifts in the bacterial community by showing changes in the predominant bacterial species [29].

In the present study, the results of PCR-DGGE indicated that the total bacteria increased in the cecum of weaning pigs in diet of TMFP. A similar phenomenon was investigate in previous results that plant extract showed increases in lactobacilli numbers and increase in the lactobacilli: enterobacteria ratio in the jejunum of weaned pigs [30]. While the total bacteria was also increased in diet of antibiotic group, which was different to Collier et al. [31], using a similar PCR methodology described a decrease in the total bacteria in the ileum of growing pigs after 2 wk of treatment with 40 ppm of tylosin. While Mccracken reported that Cephalosporins methoxyl toxin could not influence the diversity of mice [28], which maybe the selectivity function of antibiotic on intestinal and the specific susceptibility of bacteria to different antibiotic.

In the present study, individual pigs had stable and repeatable bacterial specific banding patterns over time and there were clear differences between individual patterns, which were unique for each animal. These results indicate that each individual exhibits a unique bacterial community as demonstrated by stable and repeatable banding patterns. Diversity as measured by Shannon's index was increased in pigs fed with TMFP and antibiotic as compared with the control group. The increased number of bands in pigs fed with TMFP indicating increased numbers of species as well as increased overall diversity. The intestinal bacterial community diversity changed greatly at day 7 and day 14, DGGE profiles stability until day 21, which was similar to Zhu report [32]. The diversity changed greatly during 3 time points. It was mostly likely that the antibiotic influence the intestinal bacterial until 21 days and the results concur with that of Collier study [31], who observed that adding tylosin in growing pigs could influence the intestinal bacterial until 28 days. The results suggest that supplement with TMFP has changes in the diversity of complex bacterial communities and promote the stable of intestinal bacterial during weaning period. In order to identify changes in the bacterial diversity detected by DGGE analysis, the 16S rRNA genes from the cecum samples of pigs at 3 time points were amplified and cloned sequenced (**Table 4**). The result showed that supplement with TMFP not only increase the proliferation of beneficial bacteria, but also promote the appearance of some bacterial, like *Coprococcus catu*, *Campylobacter Canadensis*. On the contrary, inclusion of antibiotic in the diet reduced the proliferation of both of potentially harmful bacteria and potentially beneficial lactobacilli in the pigs' gut.

In summary, dietary supplementation with TMFP can enhance greater intestinal development and enhance gut health by regulating the microbiota composition, maintaining a normal morphology in weanling pigs and change the diversity of intestinal bacterial community, promote the stability of intestinal bacterial microbiota in weaning pigs, thereby decreasing the incidence of diarrhea resulting from weaning stress. TMFP as a traditional Chinese herb additive in dietary can serve as an alternative to in-feed antibiotics for weaning pigs.

Acknowledgements

We gratefully acknowledge Beijing Bio-ulab Co., Ltd for DGGE determination and financial sup-

port provided by Sichuan science project, Sichuan Agriculture University Farm.

Disclosure of conflict of interest

None.

Address correspondence to: Yinglun Li, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an 625014, Sichuan, China. Tel: +86-1890-8162655; Fax: +86-0835-2885614; E-mail: liyinglun01@126.com

References

[1] Lalles JP, Bosi P, Smidt H and Stokes CR. Nutritional management of gut health in pigs around weaning. Proceedings of the Nutrition Society 2007; 66: 260-268.

[2] Visek W. The mode of growth promotion by antibiotics. Journal of Animal Science 1978; 46: 1447-1469.

[3] Kamel C. Plant extracts in an integrated approach. Feed Mix 2001; 9: 14-16.

[4] Vaughan EE, Schut F, Heilig H, Zoetendal EG, de Vos WM and Akkermans AD. A molecular view of the intestinal ecosystem. Curr Issues Intest Microbiol 2000; 1: 1-12.

[5] Tannock GW. Molecular assessment of intestinal microflora. Am J Clin Nutr 2001; 73: 410s-414s.

[6] Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M and Møller K. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol 2002; 68: 673-690.

[7] Hill JE, Seipp RP, Betts M, Hawkins L, Van Kessel AG, Crosby WL and Hemmingsen SM. Extensive profiling of a complex microbial community by high-throughput sequencing. Appl Environ Microbiol 2002; 68: 3055-3066.

[8] Nutrition NRCSoS. Nutrient requirements of swine. National Academies Press. 1998.

[9] Ercolini D. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J Microbiol Methods 2004; 56: 297-314.

[10] Simpson JM, McCracken VJ, White BA, Gaskins HR and Mackie RI. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J Microbiol Methods 1999; 36: 167-179.

[11] Oliver WT, Mathews S, Phillips O, Jones E, Odle J and Harrell R. Efficacy of partially hydrolyzed corn syrup solids as a replacement for lactose in manufactured liquid diets for neonatal pigs. J Anim Sci 2002; 80: 143-153.

[12] Funderburke D and Seerley R. The effects of postweaning stressors on pig weight change, blood, liver and digestive tract characteristics. J Anim Sci 1990; 68: 155-162.

[13] Pluske J, Williams I and Aherne F. Villous height and crypt depth in piglets in response to increases in the intake of cows' milk after weaning. Animal Science 1996; 62: 145-158.

[14] Sharifi S, Dibamehr A, Lotfollahian H and Baurhoo B. Effects of flavomycin and probiotic supplementation to diets containing different sources of fat on growth performance, intestinal morphology, apparent metabolizable energy, and fat digestibility in broiler chickens. Poultry Science 2012; 91: 918-927.

[15] Li S, Wang C, Peng X and Wu X. Effects of Aqueous Extracts and Essential Oil from Cinnamomum cassia on Rat Colonic Mucosal Morphology and Bacteroidales. Journal of Food and Nutrition Research 2013; 1: 7-12.

[16] Nyachoti C, Kiarie E, Bhandari S, Zhang G and Krause D. Weaned pig responses to Escherichia coli K88 oral challenge when receiving a lysozyme supplement. J Anim Sci 2012; 90: 252-260.

[17] Walsh A, Sweeney T, Bahar B, Flynn B and O'Doherty J. The effect of chitooligosaccharide supplementation on intestinal morphology, selected microbial populations, volatile fatty acid concentrations and immune gene expression in the weaned pig. Animal 2012; 6: 1620-1626.

[18] Verstegen MW and Williams BA. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Animal Biotechnology 2002; 13: 113-127.

[19] Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol 1999; 86: 985-990.

[20] Kong X, Wu G, Liao Y, Hou Z, Liu H, Yin F, Li T, Huang R, Zhang Y and Deng D. Effects of Chinese herbal ultra-fine powder as a dietary additive on growth performance, serum metabolites and intestinal health in early-weaned piglets. Livestock Science 2007; 108: 272-275.

[21] Nabuurs M. Microbiological, structural and functional changes of the small intestine of pigs at weaning. Pig News and Information 1995; 16.

[22] Namkung H, Li J. Gong M, Yu H, Cottrill M and de Lange C. Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs. Canadian Journal of Animal Science 2004; 84: 697-704.

[23] Konstantinov SR, Awati A, Smidt H, Williams BA, Akkermans AD and De Vos WM. Specific response of a novel and abundant Lactobacillus amylovorus-like phylotype to dietary prebiotics in the guts of weaning piglets. Appl Environ Microbiol 2004; 70: 3821-3830.

[24] Franklin M, Mathew A, Vickers J and Clift R. Characterization of microbial populations and volatile fatty acid concentrations in the jejunum, ileum, and cecum of pigs weaned at 17 vs 24 days of age. J Anim Sci 2002; 80: 2904-2910.
[25] Pieper R, Janczyk P, Zeyner A, Smidt H, Guiard V and Souffrant WB. Ecophysiology of the developing total bacterial and Lactobacillus communities in the terminal small intestine of weaning piglets. Microbial ecology 2008; 56: 474-483.
[26] La-Ongkhum O, Pungsungvorn N, Amornthewaphat N and Nitisinprasert S. Effect of the antibiotic avilamycin on the structure of the microbial community in the jejunal intestinal tract of broiler chickens. Poultry Science 2011; 90: 1532-1538.
[27] Gong J, Yu H, Liu T, Li M, Si W, de Lange CF and Dewey C. Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. Livestock Science 2008; 116: 318-322.
[28] McCracken VJ, Simpson JM, Mackie RI and Gaskins HR. Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. J Nutr 2001; 131: 1862-1870.
[29] Muyzer G and Smalla K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 1998; 73: 127-141.
[30] Manzanilla E, Perez J, Martin M, Kamel C, Baucells F and Gasa J. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. J Anim Sci 2004; 82: 3210-3218.
[31] Collier C, Smiricky-Tjardes M, Albin D, Wubben J, Gabert V, Deplancke B, Bane D, Anderson D and Gaskins H. Molecular ecological analysis of porcine ileal microbiota responses to antimicrobial growth promoters. J Anim Sci 2003; 81: 3035-3045.
[32] Zhu W, Yao W and Mao S. Development of bacterial community in faeces of weaning piglets as revealed by denaturing gradient gel electrophoresis]. Wei Sheng Wu Xue Bao 2003; 43: 503.