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Original Research Article

Porcine intestinal antimicrobial peptide as an in-feed antibiotic alternative improves intestinal digestion and immunity by shaping the gut microbiota in weaned piglets

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ABSTRACT

Antibiotic resistance of pathogens, which is caused by the abuse of in-feed antibiotics, threatens the sustainable development of livestock production. The present study aimed to investigate the efficiency of porcine intestinal antimicrobial peptide (PIAP) as an alternative to in-feed antibiotics in terms of growth performance, intestinal morphology, digestive enzymes and immunity, and microbiota community of the post-weaning piglets. A total of 204 piglets (Duroc × Landrace × Yorkshire, weaned at 28 d age) with a similar body weight of 7.97 ± 1.04 kg were randomly allocated to 4 groups (51 piglets per group): (1) control group: basal diet; (2) AB group: antibiotic, basal diet + chlortetracycline (1000 mg/kg from d 1 to 24; 500 mg/kg from d 25 to 37); (3) P1 group: basal diet + a relatively low dose of PIAP (400 mg/kg from d 1 to 24; 300 mg/kg from d 25 to 37); (4) P2 group, basal diet + a relatively high dose of PIAP (600 mg/kg from d 1 to 24; 500 mg/kg from d 25 to 37). The results showed that serum indicators of hepatocyte damage and relative organ weight were not affected by these treatments ($P > 0.05$). Compared with the AB treatment, the P1 treatment remarkably decreased jejunal crypt depth and increased jejunal and ileal villus height:crypt depth ratio ($P < 0.05$). The values of jejunal maltase, lactase, sucrase, intestinal alkaline phosphatase, and secretory immunoglobulin A (SIgA) in the P1 group were sharply increased compared with those in the control and P2 groups ($P < 0.05$). Compared with the control group, the P1 group decreased serum concentrations of D-lactate, diamine oxidase, and endotoxin ($P < 0.05$), and increased the abundance of *Lactobacillus reuteri* ($P < 0.05$) in the colonic feces. Furthermore, there was a positive correlation between the abundance of *L. reuteri* and the concentrations of maltase, lactase, sucrase, and SIgA ($P < 0.05$). Collectively, dietary supplementation with a relatively low dose of PIAP (400 mg/kg from d 1 to 24; 300 mg/kg from d 25 to 37) demonstrates beneficial effects on intestinal morphology, digestive enzymes, immunity, and permeability by shaping the gut microbiota composition in weaned piglets. This study will provide a valuable reference for using PIAP as an in-feed antibiotic alternative in swine production.

1. Introduction

The emergence and prevalence of antibiotic resistance of pathogens, which is caused by the abuse of in-feed antibiotics, seriously threatens the development of livestock production. In the modern swine industry, piglets are commonly weaned early between 3 and 4 wk of age. Weaning is the biggest challenge in a pig's life because their diet, social association, and environment are suddenly

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changed (Lallès et al., 2007). Piglet weaning leads to multiple stressors, such as anorexia, diarrhea, malnutrition, microbiota imbalance, and performance reduction (Gresse et al., 2017). To solve the negative impacts of weaning, subtherapeutic doses of antibiotics have been routinely added to animal feed for several decades. Unfortunately, the abuse of antibiotics has resulted in the prevalence of antibiotic-resistant genes in pathogens (van Breda et al., 2018; Liu et al., 2016b), residual accumulation in animal food, and heavy environmental pollution through animal manure and wastewater (Manyi-Loh et al., 2018). Consequently, the European Union banned the addition of antibiotics as growth enhancers in animal diets in 2006. China has eliminated growth-promoting antibiotics from pig feed since 2020. However, the banning of antibiotic growth promoters has resulted in outbreaks of diseases and a reduction in growth performance. Therefore, safe and effective alternatives for in-feed antibiotics are needed urgently.

Many materials have been investigated as potential substitutes for antibiotics, such as essential oils and enzymes, but with limited success (Thacker, 2014). Antimicrobial peptides (AMP), also termed host defense peptides, are indispensable components in immune regulation that are naturally abundant in approximately all live beings from microorganisms to animals (Zasloff, 2002). Currently, a total of 3236 AMP have been found in nature (http://aps.unmc.edu/AP/). AMP exert a broad spectrum of biological activities against bacteria, yeast, fungi, and viruses. Furthermore, some AMP even play a therapeutic role in wound healing, endotoxin-neutralizing, and biofilm disruption (Wang et al., 2019a). Unlike conventional antibiotics, AMP exert antimicrobial activities primarily through membrane disruption or by attacking intracellular targets; thus, they reduce the likelihood of inducing antibiotic resistance (Phoenix et al., 2015; Ghosh et al., 2014). Therefore, AMP represent potential alternatives to in-feed antibiotics.

Many different AMP have been reported to attenuate diarrhea and intestinal inflammation, and promote growth, nutrient digestibility, and microbiota composition of weaned pigs (Yu et al., 2017; Wan et al., 2016; Xiao et al., 2015; Wang et al., 2016a). Porcine intestinal antimicrobial peptide (PIAP), which was initially found in the intestine of welfare-friendly (free-range and minimum requirements for antibiotic use) pigs, displayed in vitro antibacterial activity (Liu, 2015) and intestinal antioxidant capacity (Chen et al., 2016). However, few studies have been conducted to evaluate the efficiency of PIAP as an alternative to in-feed antibiotics in swine nutrition. In the present study, PIAP was verified to improve intestinal morphology, digestive enzymes, immunity, and permeability by shaping the gut microbiota composition in weaned piglets, which will provide a valuable reference for using PIAP as an in-feed antibiotic alternative in swine production.

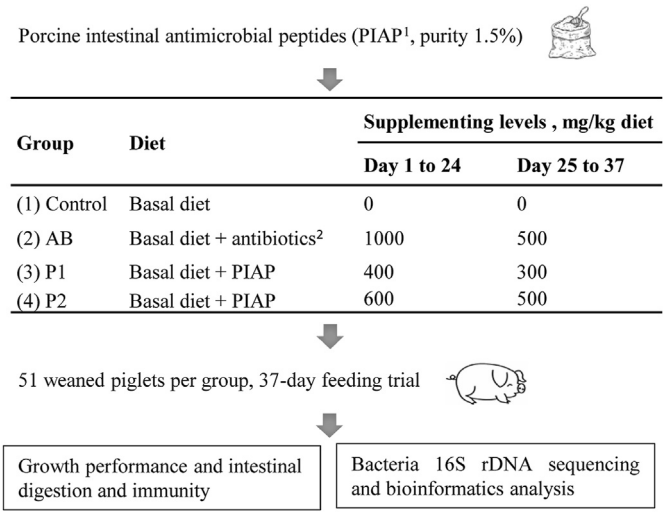
2. Materials and methods

2.1. Animal ethics

All the experiments conducted in this study were reviewed and agreed upon by the Animal Care and Use Committee of Hunan Normal University, Changsha City, Hunan, China.

2.2. Animal and study design

The animal experiment was performed in the nursery pig house of Hainan Agri-Farming Animal Husbandry Group Co., Ltd. The piglets (Duroc × Landrace × Yorkshire) were weaned at 28 d of age. A total of 204 piglets with a similar body weight of 7.97 ± 1.04 kg in a random design were assigned to 4 groups (3 replicates per group, 17 piglets per replicate, Fig. 1). The supplementation levels of PIAP



Group	Diet	Supplementing levels , mg/kg diet	
		Day 1 to 24	Day 25 to 37
(1) Control	Basal diet	0	0
(2) AB	Basal diet + antibiotics[2]	1000	500
(3) P1	Basal diet + PIAP	400	300
(4) P2	Basal diet + PIAP	600	500

Fig. 1. Experiment design. [1] PIAP contained 1.5% peptides, approximately 50% feed grade maifan stone, approximately 25% feed grade silicon dioxide, approximately 15% medium and bacterial cells, less than 7% moisture and some vitamins. [2] Antibiotics, feed grade chlortetracycline, in the form of chlortetracycline calcium salt, contained 20% chlortetracycline, approximately 35% inorganic matter, approximately 30% bacterial protein, and less than 7% moisture. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP. The experiment was separated into phase 1 (d 1 to 24) and phase 2 (d 25 to 37). PIAP = porcine intestinal antimicrobial peptide.

referred to the results of previous experiments (Chen et al., 2016; Liu, 2015). The pigs had 4 meals per day (07:30, 11:30, 15:30, and 19:30) in accordance with the rule"a little each time but many times". Feed and water were freely accessed by all piglets (Wang et al., 2020b). The formula of the basic diet followed NRC (2012) for swine nutrition. The basic formula and nutritional level are presented in Table 1.

Porcine intestinal antimicrobial peptide (product name of natucin P; lot No. 20180316003; purity 1.5%) were purchased from Guangzhou Bestide Bio-Science and Technology Co., Ltd, Guangzhou, China. The peptides were initially isolated from the intestine of free-range pigs in the countryside in Hainan Province, China. *Bacillus amyloliquefaciens* was induced for 30 h to produce antimicrobial substances in the fermenter. Next, the fermentation broth with a content of approximately 600 mg/L was concentrated, adsorbed by silicon dioxide, and spray dried to obtain a crude powder with a content of approximately 6%. Finally, the crude powder was further diluted by the carrier to obtain a peptide product. The PIAP product contained 1.5% peptides, approximately 50% feed grade maifan stone, approximately 25% feed grade silicon dioxide, approximately 15% medium and bacterial cells, less than 7% moisture, and some vitamins. Porcine intestinal antimicrobial peptide, with a relative molecular mass of 5,000 Da and activity of more than 11,000,000 IU/g, was used in the present study.

2.3. Sample collection and preparation

At d 37 of the experiment, 9 pigs per treatment were prepared for blood and tissue samples. The venous blood was incubated at room temperature for 30 min and then centrifuged at 3,000 × *g* for 10 min at 4 °C to obtain serum. The supernatant of each blood sample was stored in an Eppendorf tube at −80 °C prior to analysis. The intestine was segmented to obtain the duodenum, jejunum, and ileum. The jejunal mucosa was gently scraped with a piece of glass. The colonic feces sample was stored in a 1.5 mL sterile tube at −80 °C prior to further analysis.

Table 1
Basic diet composition and nutritional components (%, as-fed basis).

Item	Day 1 to 14	Day 15 to 24	Day 25 to 37
Ingredients			
Corn	42.85	63.90	66.90
Extruded corn	20.00	–	–
Soybean meal	10.00	19.50	20.00
Whey powder	10.00	–	–
Fermented soybean	–	5.00	5.00
Fish meal	3.00	5.00	3.50
Plasma protein powder	5.00	–	–
Soy protein concentrate	2.96	–	–
L-Lysine	0.55	–	–
DL-Methionine	0.12	–	–
Threonine	0.13	–	–
Tryptophan	0.04	–	–
Choline	0.10	–	–
Soybean oil	2.55	2.50	1.50
Dicalcium phosphate	1.00	–	–
Calcium citrate	1.00	–	–
Zinc oxide	0.20	–	–
Antioxidant	0.05	–	–
Vitamin premix[1]	–	0.10	0.10
Mineral premix[2]	0.45	4.00	3.00
Total	100.00	100.00	100.00
Calculated nutrient level			
Metabolizable energy, kcal/kg	3,300	3,250	3,200
Crude protein	18.60	18.50	18.00
Calcium	0.80	0.70	0.65
Total phosphorus	0.40	0.32	0.30
Lysine	1.31	1.21	1.00
Methionine + Cystine	0.72	0.68	0.57
Threonine	0.77	0.78	0.64
Tryptophan	0.21	0.22	0.18
Analyzed nutrient level			
Gross energy, kcal/kg	4,570	4,450	4,320
Crude protein	19.21	19.07	17.65
Calcium	0.94	0.84	0.74
Total phosphorus	0.64	0.59	0.51

[1] One kilogram of multiple vitamin premix contained: 2,200 IU vitamin A, 220 IU vitamin D_3,16 IU vitamin E, 0.5 mg vitamin K_3, 0.0175 mg vitamin B_{12}, 3.5 mg riboflavin, 30 mg niacin, 10 mg D-pantothenic acid, 0.05 mg biotin, 0.3 mg folic acid, 1.0 mg thiamine.
[2] One kilogram of mineral premix contained: 150 mg Fe, 100 mg Zn, 30 mg Mn, 25 mg Cu, 0.5 mg I, 0.3 mg Co, 0.3 mg Se and 4.0 mg ethoxyquin.

2.4. Feed chemical analyses

The chemical analyses were analyzed with 2 replicates and determined as previously described by Wang et al. (2019c). The dry matter was obtained by oven-dried at 105 °C. The gross energy was analyzed by using an isothermal auto calorimeter (5E-AC8018, Kaiyuan Technologies, China). The crude protein percentage was calculated using the factor 6.25 of nitrogen, which was measured using Auto Analyzer 3 flow injection analysis (Seal, Germany). The concentrations of calcium and total phosphorus in feed were determined with a Flame Atomic Absorption Spectrometer (novAA350, Jena, Germany).

2.5. Growth performance, diarrhea rate, serum parameters, and relative organ weight

Each piglet was weighed on d 1, 24, and 37 of the experiment. The daily feed intake of each replicate was recorded. Values for average daily feed intake (ADFI), average daily gain (ADG), and the ratio of feed intake to body weight gain (F:G) were calculated. The diarrhea rate of each replicate was observed daily at a fixed time (10:00 and 15:00 respectively). The diarrhea rate (%) = [(diarrhea piglets number × diarrhea days)/(total number of pigs × experiment days)] × 100. Serum parameters including total protein, albumin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST), were measured via a Cobas c311 Analyzer (Roche Diagnostics Operations, Inc., Switzerland). Visceral organs (liver, heart, and kidney) and small intestine were removed and weighed. The relative weight of each organ = organ weight/final live body weight.

2.6. Intestinal morphology

The intestinal tissues were fixed and immersed in 4% paraformaldehyde and then the fixed samples were followed by dehydration and paraffin-embedding. The paraffin block was cut into a 5-μm thickness section and then stained with hematoxylin-eosin. Stained images were taken using a DM3000 microscope (Leica, Wetzlar, Germany) and measured with Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA). The results were presented as crypt depth (CD), villus height (VH), and villus height to crypt depth ratio (VH:CD), and calculated as the average of double-blind measurements.

2.7. Intestinal cell proliferation and differentiation analysis

The Ki-67 labeled cells in the jejunal crypt, an indicator of cell proliferation, were analyzed by immunohistochemistry according to Wang et al. (2019b). The antibodies of Ki-67 (ab15580, Abcam, Cambridge, UK) were diluted at 1:600. A total of 15 microscopic fields per sample were taken under a light microscope (Leica DM3000, Leica Microsystems, Wetzlar, Germany). The positive cell population in each complete crypt was counted manually using Image-Pro software. In the procedure of chromogranin A (ChgA) immunohistochemistry (labeled enteroendocrine cells), only antibody ab45179 (1:600 dilution; Abcam, Cambridge, UK) differed from Ki-67. Goblet cell staining was performed by immunohistochemistry with Alcian blue-periodic acid-Schiff (AB-PAS, D033-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Histochemical analyses were processed following the standard protocols from the manufacturer. The goblet cell population was counted referring to the Ki-67 procedure.

2.8. Intestinal enzymes and barrier biomarkers

The supernatants were obtained from the jejunal mucosa homogenates after centrifuging (3000 × *g*, 10 min, 4 °C temperature). The protein content was analyzed via commercially available test kits (Lot No.101716170220, Enhanced BCA Protein Assay Kit, Beyotime Biotechnology). Furthermore, the contents of intestinal alkaline phosphatase (IALP, Lot No. 19120141P), maltase (Lot No. 19120134P), lactase (Lot No. 19120165P), sucrase (Lot No. 19120118P) and secretory immunoglobulin a (SIgA, Lot No. 19120181P) were analyzed using enzyme-linked immunosorbent assay (ELISA) method (Shanghai Fanke Industrial Co., Ltd, Shanghai, China). Serum D-lactate (Lot No. 19120103P), diamine oxidase (Lot No. 19120131P), and endotoxin (Lot No. 19120133P) also used the ELISA kits purchased from Shanghai Fanke Industrial Co., Ltd. For each sample, 3-replicate detections were performed.

2.9. Bacteria 16S rDNA sequencing

The sequencing was performed by the Tianjin Novogene Bioinformatic Technology Co., Ltd., Tianjin, China. (1) Extraction of genome DNA: Bacteria DNA was extracted from the colon feces ($n = 9$ per group), their concentrations and purity were monitored, and then diluted to 1 ng/μL. The V3–V4 region of the 16S rDNA gene was amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). (2) Amplicon Generation, PCR Products Identification, and Purification: PCR reactions with 30 μL volume were amplified using thermal cycling. The

reaction products were mixed in isodensity ratios after being detected using electrophoresis on 2% agarose gel and then were purified. (3) Sequencing libraries were generated and assessed using Ion Plus Fragment Library Kit 48 reactions (Thermo Scientific), Qubit@ 2.0 Fluorometer (Thermo Scientific), respectively. And then, the library was sequenced on an IonS5XL platform (Illumina, Inc., San Diego, CA, USA). (4) Bioinformatics analysis: Alpha- and beta-diversity were analyzed by the QIIME software (Version 1.9.1). Alpha diversity included Shannon, Chao1, and observed species. Beta diversity was reflected by the indices of non-metric multidimensional scaling (NMDS), principal coordinate analysis (PCoA), and Wilcox analysis. The graphs were drawn by R package (v3.5.2). The Spearman correlation index between the dominant bacteria and the intestinal measurements was explored by correlation test, then the heatmap function did the visualization work in the heatmap package.

2.10. Statistical analysis

The results were presented as means and standard error of the mean (SEM). A normal distribution test for the data was performed before analysis. All data were analyzed by SPSS v. 22.0 software (IBM-SPSS Inc., Chicago, IL, USA), using one-way ANOVA procedures. Significant differences were evaluated by Tukey's multiple comparisons test when the variance and sample size of each group is the same. The diarrhea rate was calculated using the chi-square test. If there were significant differences between groups, Pearson's chi-square for multiple comparisons was applied. Differences between means were considered highly significant when $P < 0.01$, significant when $P < 0.05$, and a statistical tendency when $0.05 < P < 0.10$. Metastatic and Linear discriminant analysis coupled with effect size (LEfSe) were used to analyze the taxonomic differences. Finally, the correlation between the intestinal parameters and the bacteria abundance was assessed using Spearman's correlation coefficient.

3. Results

3.1. Growth performance, diarrhea rate, serum parameters, and relative organ weight

Compared with the AB group, the P1 group showed a tendency to increase ADFI during d 1 to 24 ($P = 0.075$; Table 2). However, the results did not show differences ($P > 0.05$) in the body weight, ADG, F:G, diarrhea rate of each stage, and ADFI during d 25 to 37 and d 1 to 37 among the 4 groups. In addition, pigs in the 4 groups had no differences in serum biochemical indices, including total protein, albumin, ALT, and AST, and relative organ weights ($P > 0.05$, Table 3).

3.2. Intestinal morphology, cell proliferation, and differentiation

The duodenal morphological structure among the 4 groups was not affected ($P > 0.05$, Fig. 2). The jejunal CD in the P1 group was sharply decreased ($P = 0.0001$) compared with the other 3 groups, and the jejunal VH:CD ratio in the P1 group was increased ($P = 0.002$) compared with that in the control and AB groups. The ileal VH:CD ratio in the P1 group was greater ($P = 0.035$) than that

Table 2
Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on growth performance and diarrhea rates of weaned piglets[1].

Item	Control	AB	P1	P2	*P*-value
Day 1 BW, kg	7.94 ± 1.63	7.97 ± 0.85	7.96 ± 1.55	8.02 ± 0.46	1.000
Day 24 BW, kg	12.05 ± 1.66	11.88 ± 1.03	12.07 ± 1.64	11.88 ± 0.51	0.996
Day 37 BW, kg	18.78 ± 1.83	18.88 ± 0.95	19.08 ± 2.21	18.19 ± 0.99	0.910
Day 1 to 24					
ADG, g/d	171.41 ± 12.34	163.13 ± 10.61	171.24 ± 12.37	160.70 ± 2.06	0.497
ADFI, g/d	315.35 ± 25.38	273.95 ± 11.52	320.15 ± 23.11	300.25 ± 15.16	0.075
F:G	1.84 ± 0.14	1.68 ± 0.04	1.87 ± 0.11	1.87 ± 0.12	0.181
Diarrhea rates, %	10.29 ± 1.12	10.07 ± 1.92	11.35 ± 1.75	11.44 ± 1.97	0.601
Day 25 to 37					
ADG, g/d	517.59 ± 32.46	538.21 ± 6.27	538.87 ± 51.51	485.59 ± 48.40	0.360
ADFI, g/d	770.53 ± 45.99	795.01 ± 18.39	806.11 ± 78.83	740.88 ± 53.58	0.495
F:G	1.49 ± 0.04	1.48 ± 0.06	1.50 ± 0.06	1.53 ± 0.08	0.740
Diarrhea rates, %	5.76 ± 2.25	3.84 ± 2.19	3.95 ± 1.68	4.20 ± 1.96	0.302
Day 1 to 37					
ADG, g/d	293.04 ± 19.38	294.91 ± 5.41	300.41 ± 17.87	274.85 ± 17.89	0.310
ADFI, g/d	475.28 ± 31.60	457.03 ± 13.66	490.89 ± 21.23	455.07 ± 9.04	0.190
F:G	1.62 ± 0.07	1.55 ± 0.03	1.64 ± 0.05	1.66 ± 0.08	0.195
Diarrhea rates, %	8.80 ± 1.42	7.96 ± 1.43	9.03 ± 1.65	8.98 ± 1.77	0.641

BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F:G = average daily feed intake to average daily gain ratio.
[1] Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP.

Table 3
Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on serum biochemical parameters and relative organ weight of weaned piglets[1].

Item	Control	AB	P1	P2	*P*-value
Total protein, g/L	52.99 ± 3.12	56.48 ± 3.12	57.39 ± 5.50	55.68 ± 5.96	0.239
Albumin, g/L	29.16 ± 3.84	30.31 ± 2.98	30.02 ± 3.25	28.04 ± 5.41	0.626
ALT, U/L	39.77 ± 7.83	43.26 ± 9.23	41.20 ± 9.42	41.63 ± 5.56	0.840
AST, U/L	59.11 ± 27.63	60.33 ± 16.42	67.75 ± 28.66	65.00 ± 28.71	0.890
Heart, g/kg	5.21 ± 0.38	5.35 ± 0.81	5.27 ± 0.69	4.85 ± 0.45	0.327
Liver, g/kg	27.11 ± 3.17	25.75 ± 2.62	26.36 ± 3.14	24.16 ± 1.53	0.144
Kidney, g/kg	4.60 ± 0.62	4.71 ± 0.40	4.81 ± 0.47	4.46 ± 0.45	0.484
Small intestine, g/kg	43.22 ± 4.08	42.92 ± 4.58	41.74 ± 2.37	42.70 ± 3.15	0.841

ALT = alanine aminotransferase; AST = aspartate aminotransferase.
[1] Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP.

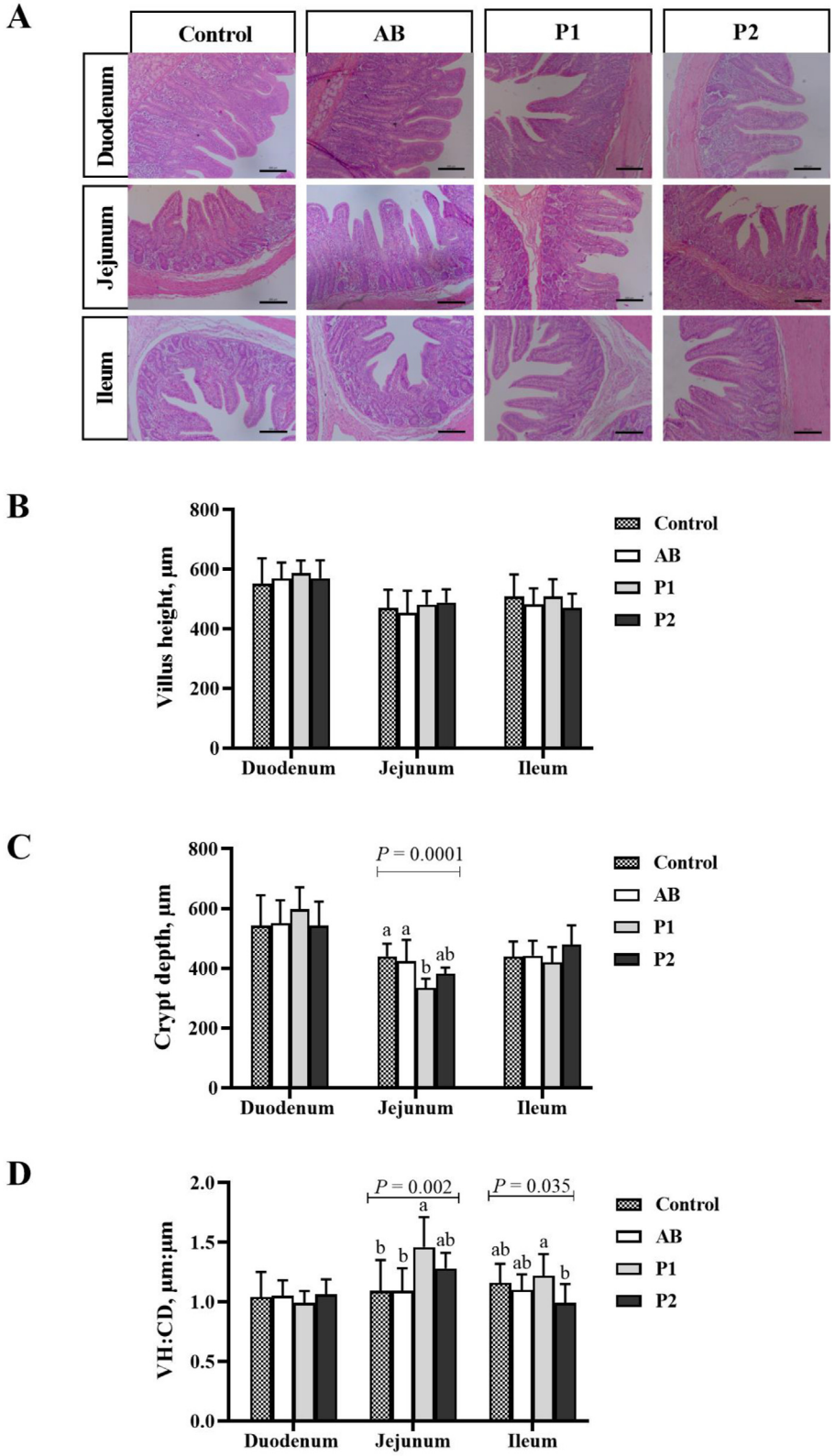


Fig. 2. Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on small intestinal morphology of weaned piglets. (A) Representative morphology images (100× magnification; scale bar = 200 μm) in the duodenum, jejunum, and ileum. (B) Villus height, (C) crypt depth, and (D) villus height to crypt depth ratio. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP. The data are expressed as means ± SEM, each group $n = 9$. [a,b] Bars without a common letter differ signicantly.

in the P2 group. The P1 group tended to show more ($P = 0.070$) Ki-67 labeled cells in the jejunal crypt than the AB group (Fig. 3). However, AB-PAS (goblet) cells and ChgA (enteroendocrine) cells did not show any differences ($P > 0.05$) among the 4 groups.

3.3. Jejunal digestive enzymes, SIgA, and biomarkers of intestinal barrier function

The values of IALP, sucrase, and lactase in the jejunal mucosa were greater ($P < 0.001$) in the P1 and AB groups than those in the P2 group; additionally, the values of maltase and SIgA in the jejunal mucosa in the P1 group were greater ($P < 0.05$) than those in the other 3 groups (Fig. 4). Compared with the control group, the P1 group significantly decreased the serum concentrations of D-lactate, endotoxin, and diamine oxidase ($P < 0.05$; Fig. 5A–C). The AB group also significantly decreased the values of serum D-lactate and diamine oxidase ($P < 0.05$; Fig. 5A–B).

3.4. Alpha- and beta-diversity of colonic microbiota

The alpha diversity indices, including Shannon, observed species, and Chao1, are shown in Fig. 6A–C. No differences in the alpha diversity indices were found among the 4 treatments. The beta diversity indices, such as PCoA, NMDS and weighted Wilcoxon analysis, are shown in Fig. 6D–F. The minimum value in beta diversity was observed in the AB group compared with the other 3 groups ($P < 0.05$; Fig. 6F); and the diversity in the P1 and P2 groups did not differ from that in the control group ($P > 0.05$; Fig. 6F).

3.5. Microbial composition of bacteria in the colon

Metastatic analysis for the main significant taxonomic differences among the groups in weaned piglets is shown in Fig. 7. The control and P1 groups showed a greater abundance of Verrucomicrobia in the phylum level than that in the AB group (corrected $P < 0.05$; Fig. 7A). At the genus level, the P2 group had a higher abundance of *Bacteroides* (corrected $P < 0.05$; Fig. 7B) and lower abundances of *Terrisporobacter* and unidentified *Clostridiales* (corrected $P < 0.05$; Fig. 7C and D) than those in the AB group. At the species level, the P2 group showed a greater abundance of species *Alloprevotella* sp feline oral taxon 309 than that in the AB group (corrected $P < 0.05$; Fig. 7E). However, compared with the AB group, the P2 group had lower abundances of *C. butyricum* , *Clostridium disporicum*, and *Eubacterium hallii* (corrected $P < 0.05$; Fig. 7F–H).

The top 10 phyla, genera, and species in the relative abundance of the microbiota present in weaned piglets are shown in Fig. 8. The gut microbiota majorly comprises Firmicutes and Bacteroidetes, which accounted for more than 90% of the total flora at the phylum level (Fig. 8A). *Lactobacillus,* unidentified *Clostridiales,* unidentified *Ruminococcaceae*, *Agathobacter*, and *Terrisporobacter* were the most dominant genera and accounted for nearly 45% of the total sequences (Fig. 8B). At the species level, *Lactobacillus amylovorus*,

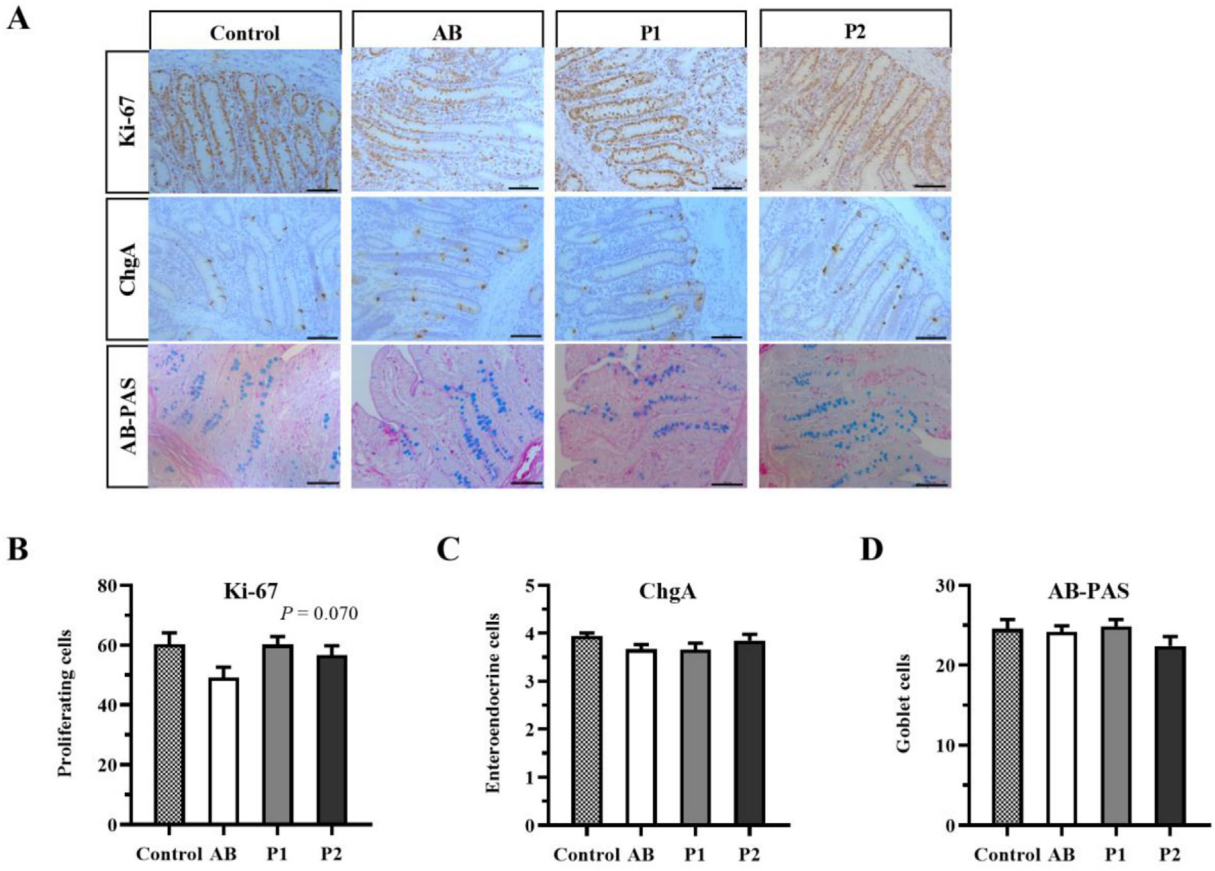


Fig. 3. Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on the jejunal cell proliferation and differentiation of piglets. (A) Representative immunohistochemical images (200× magnification; scale bar = 100 μm) of piglets in the jejunum; (B) proliferating cells with a brown color labeled by Ki-67; (C) enteroendocrine cells with a brown color labeled by chromogranin A (ChgA); and (D) goblet cells with a blue color stained by Alcian blue-periodic acid–Schiff (AB-PAS). Control = basal diet; AB, basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP. The data are expressed as means ± SEM, each group $n = 9$.

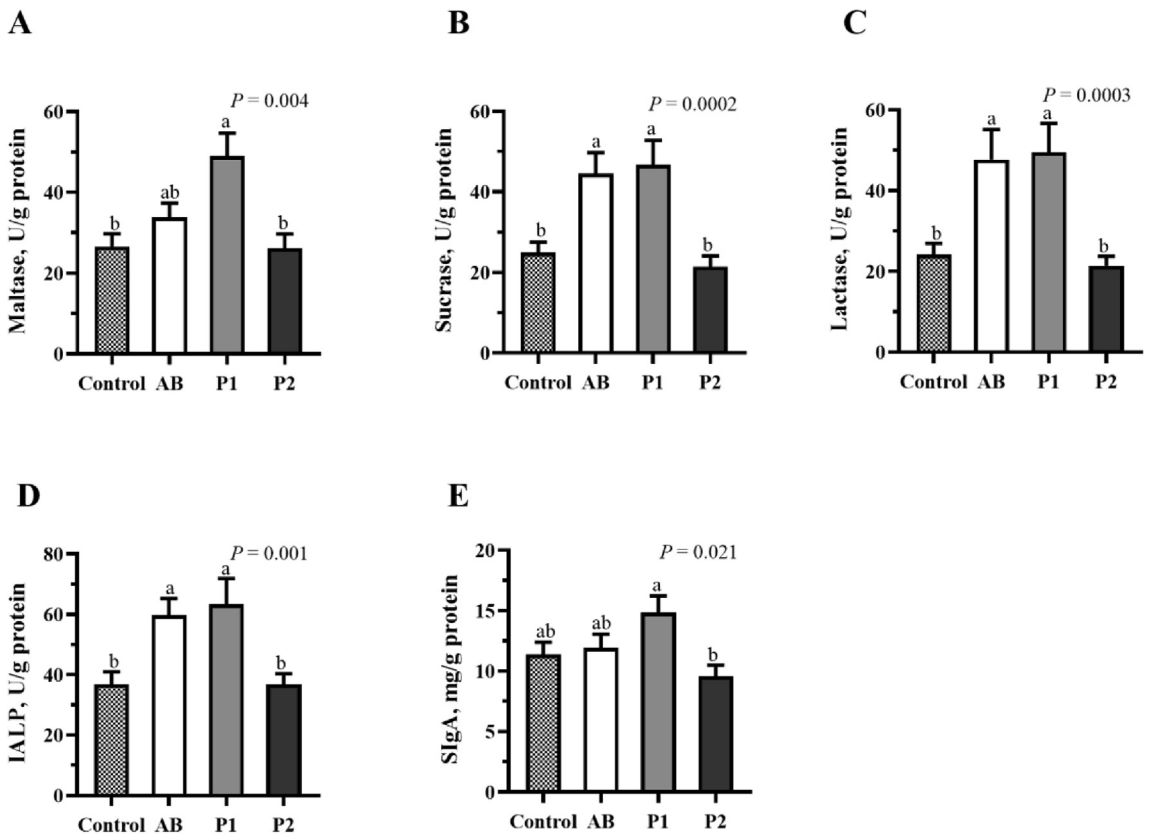


Fig. 4. Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on concentrations of digestive enzymes and secretory immunoglobulin A (SIgA) in the jejunal mucosa of piglets. (A) Maltase, (B) sucrase, (C) lactase, (D) intestinal alkaline phosphatase (IALP), and (E) SIgA. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP. The data are expressed as means ± SEM, each group $n = 9$. [a,b] Bars without a common letter differ signicantly.

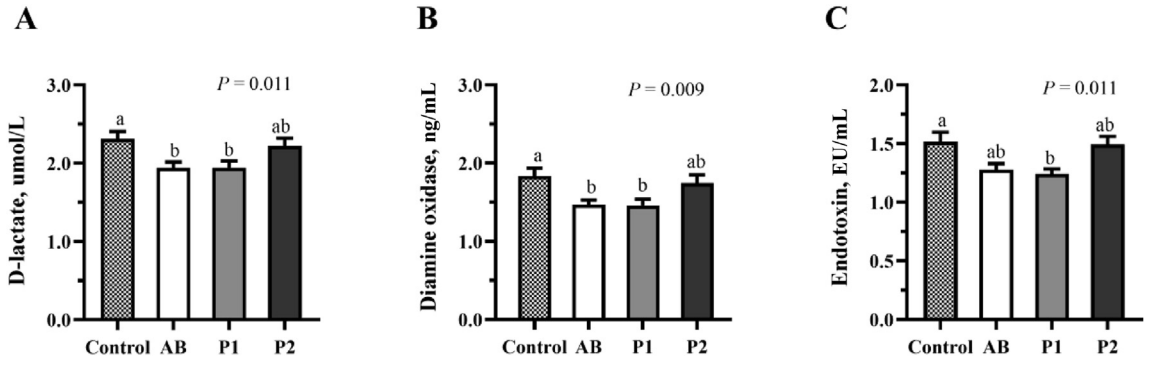


Fig. 5. Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on serum concentrations of intestinal permeability biomarkers in piglets. (A) D-Lactate, (B) diamine oxidase, and (C) endotoxin. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP. The data are expressed as means ± SEM, each group $n = 7$. [a,b] Bars without a common letter differ signicantly.

C. disporicum, and *Lactobacillus reuteri* (*L. reuteri*) dominated and accounted for approximately up to 25% of the total sequences (Fig. 8C).

The bacterial biomarkers are shown with LEfSe in Fig. 9. Compared with the control group, the P1 group had significant enrichment of species *L. reuteri* (Fig. 9A), and the P2 group showed significant enrichment of the family Ruminococcaceae (Fig. 9B). Compared with the P2 group, the genera unidentified *Clostridiales* and *Terrisporobacter* and the species *C. disporicum* were increased in the AB group (Fig. 9C), corroborating the metastatic analysis data. Furthermore, the genus unidentified *Clostridiales* was increased in the P1 group compared with the P2 group (Fig. 9D).

3.6. Spearman correlation analysis

The correlation showed that there was a negative correlation between the relative abundance of the genus unidentified *Ruminococcaceae* and the concentrations of jejunal maltase, lactase, sucrase, and IALP ($P < 0.05$; Fig. 10A). Additionally, the relative abundance of the species *L. reuteri* was positively correlated with

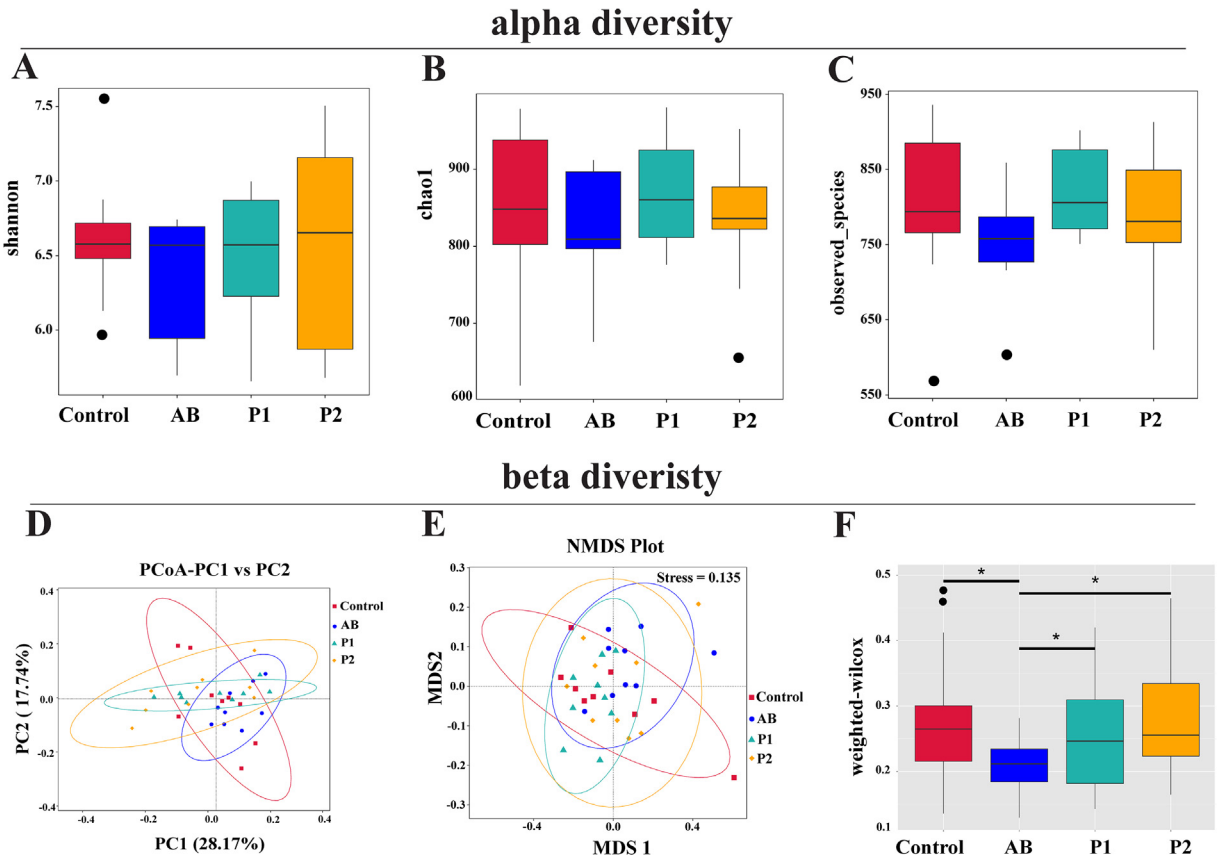


Fig. 6. Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on colonic microbiota diversity in piglets. Alpha diversity indices include: (A) Shannon, (B) Chao 1, and (C) observed species. Beta diversity indices include: (D) PCoA cluster map based on the weighted UniFrac distance, (E) non-metric multidimensional scaling (NMDS) diagram based on the Bray–Curtis distance (analysis was credible when stress < 0.2), and (F) the weighted Wilcoxon analysis result. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP. The data are expressed as means ± SEM, each group $n = 9$. *$P < 0.05$.

the content of maltase, lactase, sucrase, and SIgA in the jejunal mucosa ($P < 0.05$; Fig. 10B). Furthermore, there was a positive correlation between species *Alloprevotella* sp feline oral taxon 309 and intestinal SIgA ($P < 0.05$; Fig. 10B).

4. Discussion

The intensive animal breeding industry is heavily dependent on safe and effective feed additive replacements for antibiotics to control infectious pathogens and improve production efficiency. Antimicrobial peptides have a lower likelihood of inducing antibiotic resistance (Wang et al., 2019a), and therefore are a major research subject in developing alternatives to in-feed antibiotics. Our observation shows that PIAP might be an effective antibiotic alternative for weaned piglets in terms of improving intestinal morphology, digestive enzymes, and mucosal immunity and shaping the gut microbial community.

4.1. Growth performance, serum parameters, and relative organ weight

Many studies have reported the positive effects of AMP on improving animal growth performance and decreasing the diarrhea rate (Xu et al., 2020; Xiao et al., 2015; Wang et al., 2016a). For example, synthetic plectasin and porcine β-defensin 2 exhibited improvements in the ADFI and ADG and a reduction in the diarrhea incidence of postweaning piglets (Wan et al., 2016; Peng et al., 2016). However, weaning piglets administered 90 mg/kg of AMP displayed lower values of the final body weight, ADG, and ADFI than those in the antibiotic control (Yoon et al., 2012). In the present study, the PIAP supplementation in feed did not affect the final body weight, ADG, ADFI, F:G, and diarrhea rate of piglets, when compared with the AB group. The different peptides and animal models might explain the discrepancies. Additionally, compared with PIAP, feed-grade chlortetracycline as an in-feed antibiotic has a slightly bitter flavor, which may cause relatively poor palatability and reduction in feed intake in the AB group. Pigs' gut health status and intestinal responses to the feed additives could be reflected by serum biochemical profiles. Changes in serum biochemical parameters, such as ALT and AST, can indicate hepatocyte damage (Jakimiuk et al., 2015). The greater relative organ weight often consumes a larger portion of body energy and oxygen, and also reduces the growth efficiency in other tissues (Elefson et al., 2021). Our findings showed that the blood biochemical parameters and relative organ weight in pigs were not affected by PIAP supplementation, suggesting that PIAP may have no negative effects on visceral organ development.

4.2. Intestinal morphology, cell proliferation and differentiation

Early weaning stress often results in decreased VH and increased CD (Wang et al., 2008; Hu et al., 2013). Therefore, the VH,

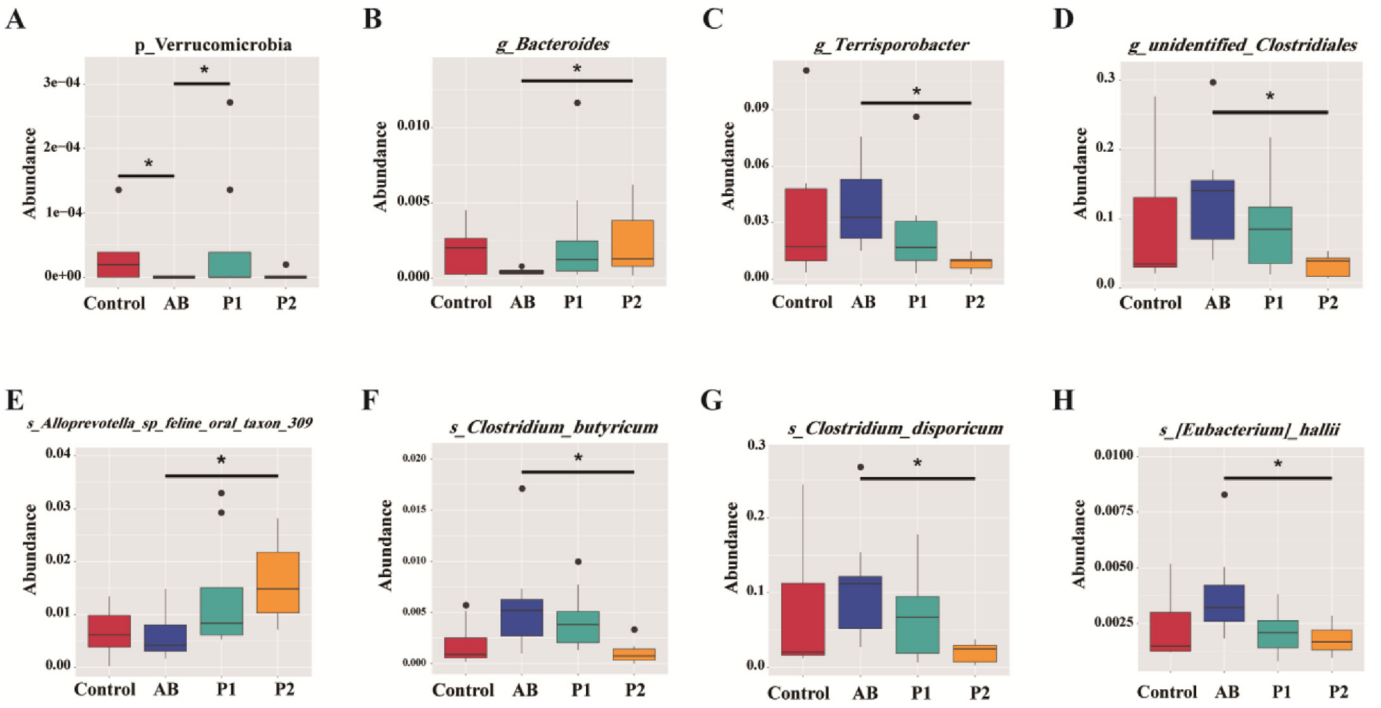


Fig. 7. Metastatic analysis for the taxonomic differences of the colonic bacteria in piglets. Remarkably different bacteria were shown in (A) phylum Verrucomicrobia, (B-D) genera *Bacteroides, Terrisporobacter,* and unidentified *Clostridiales*, respectively, and (E-H) species *Alloprevotella* sp feline oral taxon 309, *Clostridium_butyricum*, *Clostridium_disporicum*, and *Eubacterium hallii*, respectively. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP; PIAP = porcine intestinal antimicrobial peptide. The data are expressed as means ± SEM, each group $n = 9$. * Corrected $P < 0.05$.

CD, and their ratio were proposed as common indicators to evaluate the injuries of intestinal morphology. In swine and broiler, dietary supplementation with a series of antimicrobial peptides (porcine beta-defensin-2, cathelicidin-WA, cecropin, and plectasin or microcinJ25) enhanced mucosal development, such as increasing the VH:CD ratio and decreasing the CD (Tang et al., 2016; Peng et al., 2016; Yi et al., 2016; Xie et al., 2020; Wang et al., 2020a). Consistent with previous studies, weaned piglets treated with a relatively low dose of PIAP showed a greater VH:CD ratio and shallower CD than those in the control and AB groups.

The complete structure of the intestinal mucosa depends on the constant renewal and differentiation of intestinal cells along the villus–crypt axis (Yang et al., 2013). The Ki-67 positive cell, a key index reflecting cell proliferation (Goodlad, 2017), was positively associated with VH, CD, and lactase activity in weaned piglets (Wang et al., 2019b). Intestinal stem cells have a remarkable ability to differentiate into multiple cell types, including absorptive columnar epithelial cells and secretory lineage (Paneth, goblet, and enteroendocrine cells). Our findings showed that dietary supplementation with a relatively low dose of PIAP tended to increase the number of Ki-67 labeled cells but did not change the numbers of goblet and enteroendocrine cells, suggesting that PIAP may improve intestinal development.

4.3. Intestinal digestive enzymes and immunity

A complete intestinal morphology often corresponds to a healthy digestive and immune system (Camilleri et al., 2012; Khoshbin and Camilleri, 2020). In the present study, PIAP was observed to increase the contents of sucrase, maltase, and lactase, which is in accordance with a previous study (Wan et al., 2016). Additionally, the parameters concerning immunity, such as SIgA and IALP, were analyzed. SIgA, the major immunoglobulin secreted by mucosa, constitutes the first immunological barrier that effectively defends the epithelium against the invasion of various toxins, pathogenic microorganisms, and food antigens (Mantis et al., 2011). Thus, SIgA functions in the immune response, intestinal microbiota, and host-commensal homeostasis (Hooper et al., 2012; Pietrzak et al., 2020). IALP plays various vital roles in intestinal homeostasis. For example, IALP can detoxify bacterial lipopolysaccharides and free nucleotides, regulate intestinal surface pH and lipid absorption, modulate gut microbiota and localize the ZO-1 and occludin proteins (Lallès, 2014; Liu et al., 2016a). We observed that the concentrations of SIgA and IALP were increased in the P1 group, indicating that the additions of PIAP to the diet enhanced the mucosal immunity function of piglets.

4.4. Serum biomarkers of intestinal permeability

Weaning also reduced the intestinal barrier integrity of piglets, as indicated by increased paracellular permeability (Hu et al., 2013). D-Lactate and endotoxin are the main metabolites of intestinal bacteria. In pigs, elevated serum concentrations of D-lactate and endotoxin can reflect increased intestinal permeability and bacterial translocation, respectively (Hollander and Kaunitz, 2020, Schoultz and Keita, 2020). Diamine oxidase is present at the apical end of mature villus cells and is commonly considered a marker of small intestinal integrity and maturity (Celi et al., 2019). Xiao et al. (2013) reported that an AMP mixer repaired intestinal injury in piglets exposed to deoxynivalenol, as indicated by decreased serum D-lactate and diamine oxidase levels. Additionally, Microcin J25 was a synthesized AMP using fecal *Escherichia coli* that was observed to decrease serum levels of these bacterial metabolites compared with the control group in pigs (Yu

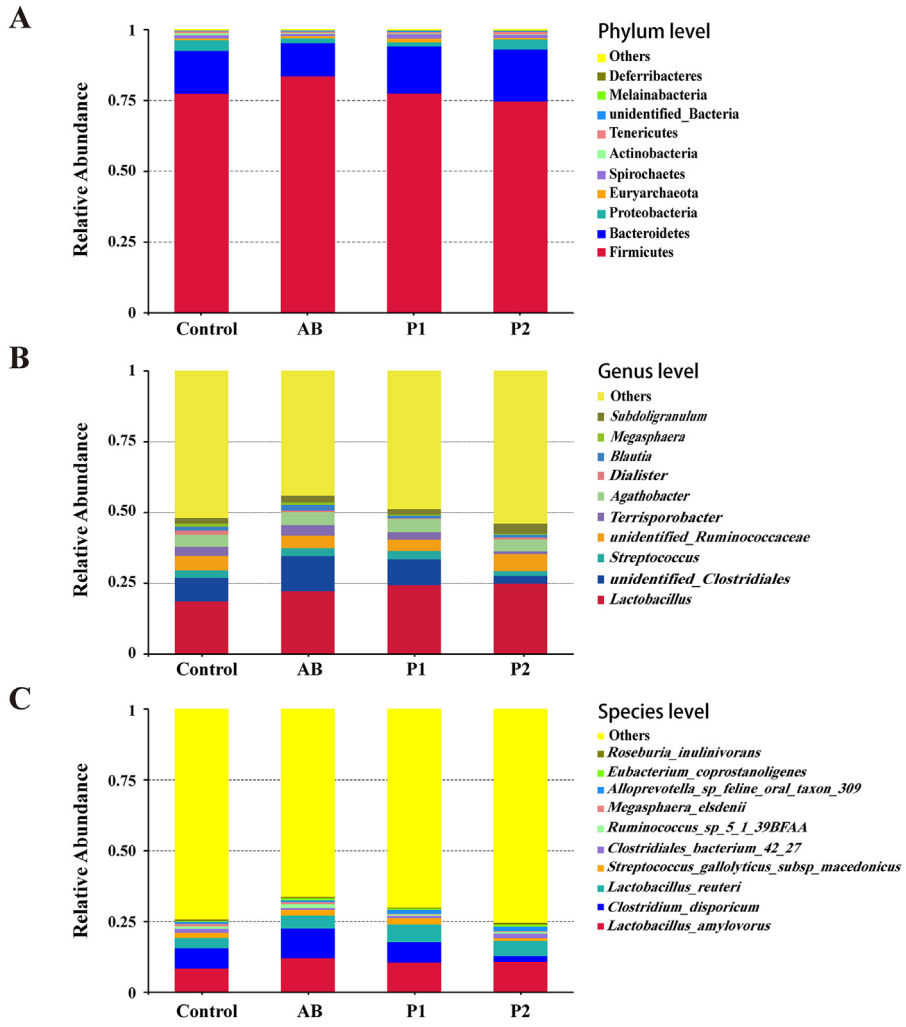


Fig. 8. Effects of dietary porcine intestinal antimicrobial peptide (PIAP) on the microbial community of the colon in piglets. Distribution of the colonic microbiota at multiple levels, including (A) phylum level, (B) genus level, and (C) species level. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP. The data are expressed as means ± SEM, each group $n = 9$.

et al., 2017). In this study, the P1 and AB groups reduced D-lactate, diamine oxidase, and endotoxin levels compared with those in the control group. This result indicated that piglets in the P1 group had an advantage in decreasing intestinal permeability, which is consistent with their higher intestinal morphology and digestive enzymes.

4.5. Fecal microbiota community and correlation analysis

The gut microbiome functions as an important coordinator in preserving gut homeostasis (Xiang et al., 2020) in piglets. Weaning contributed to the dramatic changes in intestinal flora composition (Guevarra et al., 2019), such as reductions in the genus *Lactobacillus* (Pajarillo et al., 2014; Yang et al., 2019) and diversity (Hu et al., 2016). Our study pigs fed the PIAP diet increased beta diversity. However, piglets treated with 5 g/kg of the recombinant porcine β-defensin 2 decreased their cecal diversity indices and bacterial pathogens compared with those in the antibiotics group (Peng et al., 2016). The reason for this discrepancy should be further investigated. In a previous study, dietary supplementation with Microcin J25 resulted in a decrease in pathogenic *E. coli* numbers and increases in *Lactobacillus* and *Bifidobacterium* numbers in pig feces (Yu et al., 2017). Besides, cathelicidin-WA, an AMP that originated from the genus *Bungarus fascia*, showed the same changes in *E. coli* and *Lactobacillus* in diarrhea piglets (Yi et al., 2016).

In the present study, the metastatic analysis revealed that the species *Alloprevotella* sp feline oral taxon 309 was significantly increased, while the abundances of species *C. disporicum,* and *C. butyricum* were decreased in the P2 group, when compared with the AB group. *Alloprevotella* feline oral taxon 309, an acetic and succinic acid producer (Downes et al., 2013), functions in anti-inflammation in a mouse model (Li et al., 2020). *C. disporicum* is an opportunistic bacterium with saccharolytic properties that can produce ursodeoxycholic acids (Horn, 1987); the available report on this was associated with higher disease (McBride et al., 2017). Furthermore, the abundance of *C. disporicum* was negatively associated with growth parameters and the cecal butyrate concentration in growing-finishing pigs (Torres-Pitarch et al., 2020). This evidence indicated that the P2 group might potently alter the microbial composition in the host gut by increasing the beneficial and decreasing the opportunistic bacteria abundance. Although no significant differences of *Alloprevotella* feline oral taxon 309, *C.*

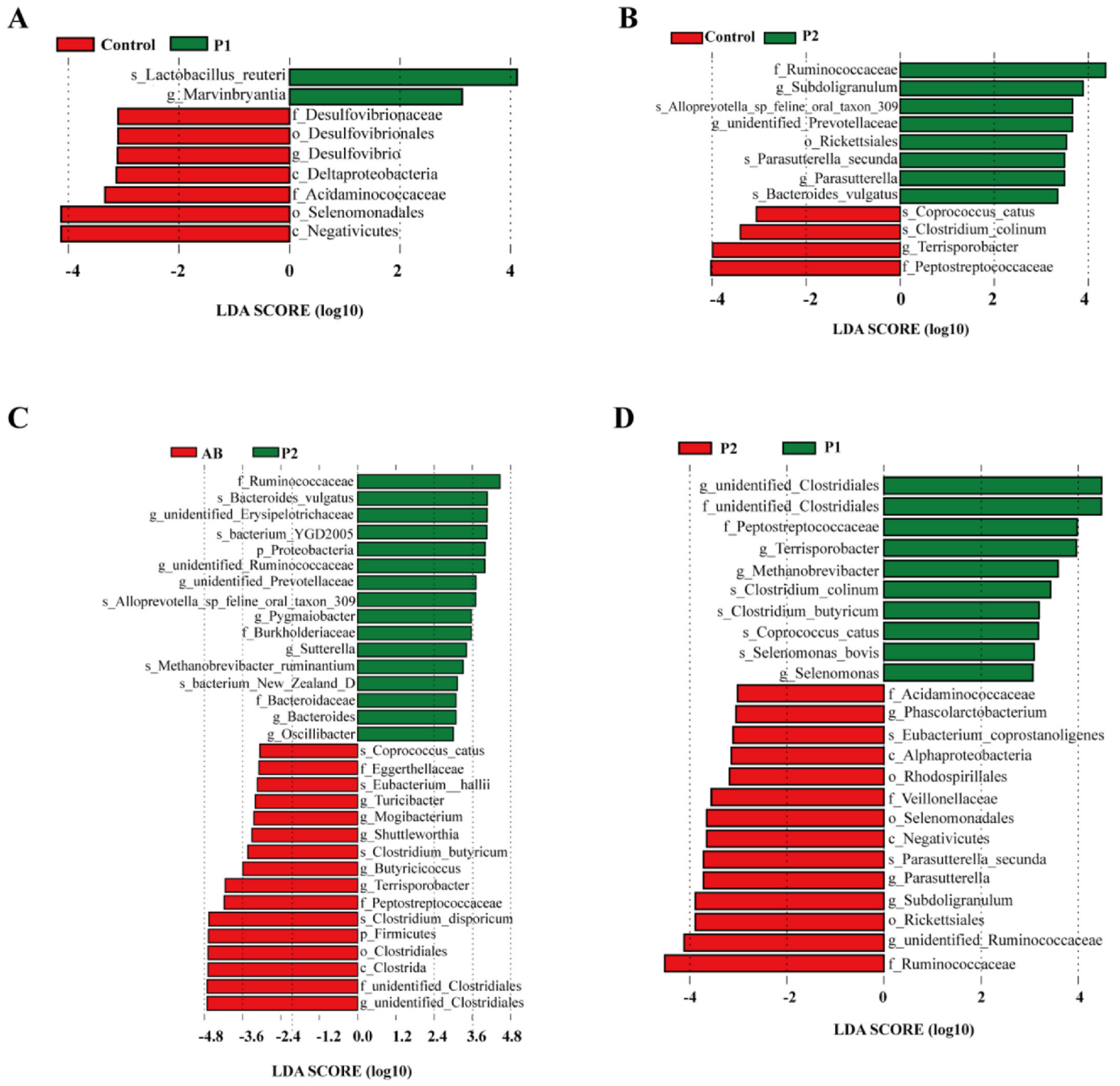


Fig. 9. Linear discriminant analysis (LDA) coupled with effect size for microbiota enrichment in piglets between the 2 groups. (A) Control and P1 groups, (B) Control and P2 groups, (C) AB and P2 groups, and (D) P2 and P1 groups. Control = basal diet; AB = basal diet plus antibiotics; P1 = basal diet plus a low dosage of PIAP; P2 = basal diet plus a high dosage of PIAP; PIAP = porcine intestinal antimicrobial peptide. The data are expressed as means ± SEM, each group $n = 9$.

disporicum, and C. butyricum were observed between the P1 and AB groups, a relatively obvious fluctuation of these bacteria in the P1 group might have contributed to an improvement in intestinal permeability.

LEfSe analysis identified unique biomarkers at different levels of the bacterial community that further confirmed these findings. We found that the species *L. reuteri* and *Alloprevotella* sp feline oral taxon 309 were significantly enriched in the P1 and P2 groups, respectively. *L. reuteri* has been reported to show active adhesion to the porcine intestinal mucosa (Li et al., 2008), inhibit the growth of pathogenic bacteria, and enhance mucosal immunity (Wang et al., 2016b; Hou et al., 2015). Furthermore, Spearman's correlation analysis showed that there was a positive correlation between the abundance of *L. reuteri* and concentrations of maltase, lactase, sucrase, and SIgA. The correlation indicated that pigs in the P1 group modulated mucosal immunity and digestion by shaping the microbial community. In addition, the P1 treatment demonstrated positive effects on intestinal morphology, digestive enzymes, immune substance IALP and SIgA, and permeability. These results revealed that PIAP at dosages of 400 and 300 mg/kg from d 1 to 24 and d 25 to 37, respectively, could potentially serve as an alternative to in-feed antibiotics in weaned piglets.

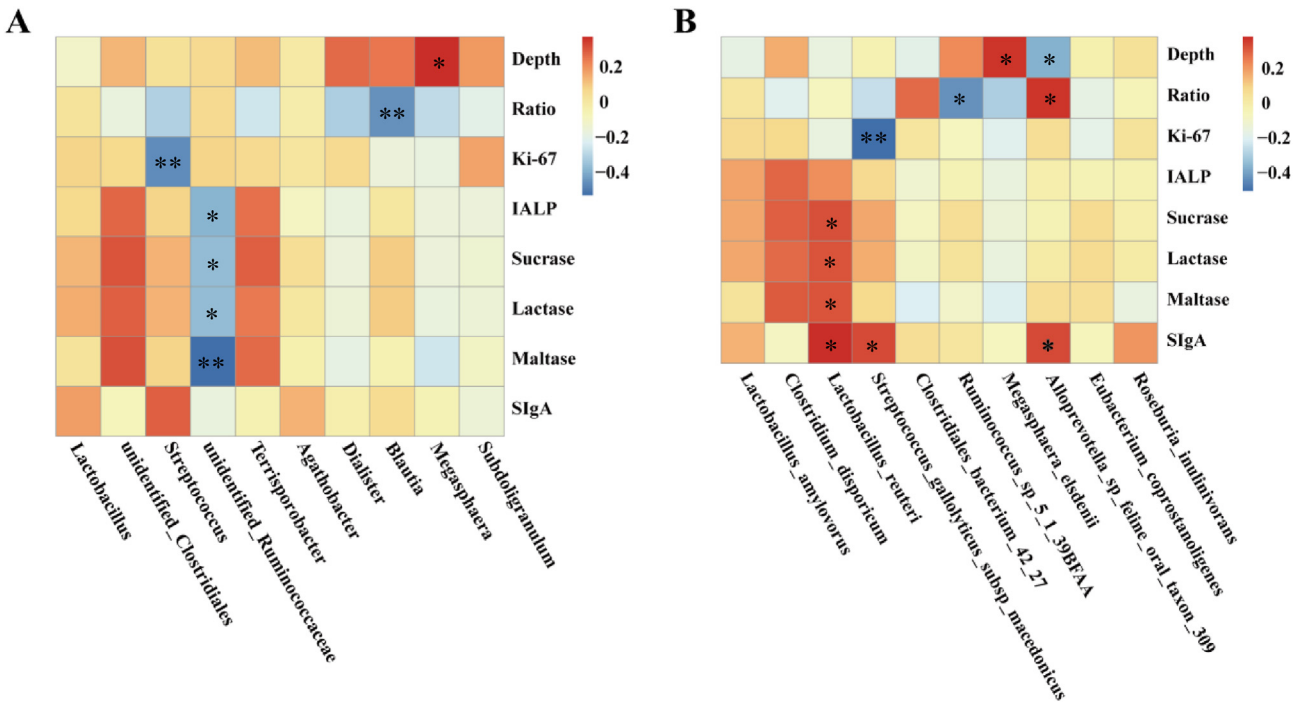


Fig. 10. Correlation between the dominant bacteria in the colon and measurements in the jejunal mucosa. (A) Genus level, (B) species level. The blue and red grids indicate negative and positive correlation (*$P < 0.05$, **$P < 0.01$), respectively. Ki-67 = proliferating cells labeled by Ki-67; IALP = intestinal alkaline phosphatase; SIgA = secretory immunoglobulin A.

5. Conclusion

Collectively, weaned piglets supplemented with a relatively low dose of PIAP (400 mg/kg from d 1 to 24; 300 mg/kg from d 25 to 37) demonstrated beneficial effects on intestinal morphology, digestion, immunity, and permeability by shaping the microbiota community. This study will provide a valuable reference for using PIAP as an alternative to conventional antibiotics in swine production.

Data availability statement

The datasets of 16S rRNA gene sequence presented in this research have been uploaded to the online repositories of NCBI. The accession number is PRJNA784417.

Author contributions

Huansheng Yang: Conceptualization, Methodology, Supervision, Funding acquisition, Writing–review and editing. **Fengjie Ji**: Investigation, Data curation, Writing–Original draft preparation. **Qiye Wang**: Visualization. **Jianzhong Li**: Project administration. **Hanlin Zhou**: Investigation. **Shengmin Liu**: Animals and equipment.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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