

Effect of pidotimod on growth performance, immune function, intestinal epithelial barriers and microbiota of piglets

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ABSTRACT. Pidotimod (PTD) is a synthetic immunomodulatory dipeptide widely used for immune regulation. This study aimed to investigate the effect of PTD on growth performance, immune function, intestinal epithelial barriers and microbiota in piglets. In total, 120 piglets with a weight of 25.26 ± 5.96 kg and genetic makeup were selected and randomly divided into 4 groups with 3 replicates per group and 10 piglets per replicate. All piglets were weighed, and from 3 piglets from each replicate (9 piglets per group) tissues were collected. Piglets were fed for 44 days basal diet alone or supplemented with 50, 100 or 200 mg/kg PTD. It was found that the PTD addition increased the average daily weight gain of piglets ($P < 0.05$ and $P < 0.01$ for dose 50 and 100 mg/kg, respectively). So, for further studies only dose 50 mg/kg PTD was used. It was found that such dose decreased serum levels of interleukin (IL)-6 ($P < 0.05$) and interferon (IFN)- γ ($P < 0.01$), but increased anti-inflammatory cytokine IL-10 level ($P < 0.05$). Transmission electron micrographs further revealed that supplementation with PTD at a dose of 50 mg/kg enhanced intercellular connectivity, characterized by longer tight junction (TJ) proteins, thicker adhesive tapes (AB) and desmosomes (D) in the jejunum. This phenomenon was validated by the up-regulated protein expression of occludin and ZO-1 in the jejunum. Furthermore, supplementation with PTD at a dose of 50 mg/kg altered the α diversity of intestinal microbiota in piglets (Observed_species, Chao1, PD_whole_tree, Ace). Particularly, the abundance of *Spirochaetes*, *Verrucomicrobia*, *Succinispira* and *Treponema* was decreased, but that of *Lactobacillus* and *Collinsella* was increased. Overall, dietary supplementation with 50 mg/kg PTD improves the growth performance of piglets by enhancing immune function, intestinal epithelial barrier and regulating microbiota composition.

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Introduction

Pidotimod (PTD) is a synthetic dipeptide with biological immunomodulatory and anti-tumour properties (Riboldi et al., 2009). Due to the immunomodulatory activity, PTD is able both to improve

the clinical conditions of patients and to enhance and stimulate their immune cell functions, thereby exerting adaptive and innate immune functions (Ferrario et al., 2015). In comparison with traditional immunomodulators, PTD is very safe, robust and stable (Tian and Zeng, 2005). It was shown that

a higher PTD dose (800 mg/kg oral for rats and 600 mg/kg oral for dogs) induced no adverse reactions in these animals (Coppi et al., 1994). Moreover, the bioavailability of intramuscularly injected PTD was 100% but decreased to just 27% when administered orally (Coppi and Silingardi, 1994). Due to a good thermal stability, it can be administered directly without coating (Fu et al., 2016).

Currently, the immune dipeptide is mainly applied in preventing and treating repeated respiratory and urinary tract infections as well as acute and chronic bronchitis infections both in children and adults (Mahashur et al., 2019). *In vivo* and *in vitro* experiments revealed that PTD can significantly enhance the functions of natural killer (NK) cells, the phagocytic activity of monocytes and the chemotactic roles of neutrophils (Migliorati et al., 1992; 1994). Besides, it can restore the normal expression of immuno-modulated peritoneal macrophages (Capsoni et al., 1992). Pidotimod induces the maturation of human peripheral blood dendritic cells (DC) and up-regulates the expression of human leukocyte DR antigen (HLA-DR) and co-stimulatory molecules such as CD83 and CD86 (Puggioni et al., 2019). Moreover, it promotes the release of several pro-inflammatory molecules such as monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor (TNF)- α , thereby promoting the proliferation and differentiation of T cells into Th1 phenotype (Giagulli et al., 2009). In addition, in the *in vitro* study it was demonstrated that PTD can inhibit the proliferation as well as induce apoptotic death of YAC-1 tumour cells (Migliorati et al., 1993).

In recent years, the increase in intensive farming of highbred animals has resulted in a frequent outbreak of diseases in the farms (Amadori and Zanotti, 2016). This raises the cost of production and lowers the quality of animal products. Therefore, there is an unmet need to develop sustainable disease prevention strategies. It was noted that oral administration of 1 g/l of PTD increased the production of antibodies in broilers, which effectively enhanced resistance of birds against lethal Newcastle disease virus (Li et al., 2016); it was further confirmed by Qu et al. (2017). However, effects of PTD in pigs remained to be validated. Meanwhile, piglet intestinal health is one of the most important factors affecting pigs under intensive production systems. It was reported that in China, the average annual mortality and morbidity of piglets below 30 kg is as high as 10.3 and 46.5%, respectively (Zhong et al., 2005). This underlines the role of intestinal health and diseases limited the expansion of the pig industry

(Zhong et al., 2005). Although piglet intestinal health is often determined by the interaction of multiple factors, intestinal microbes have been shown to be the most fundamental factor. This is attributed to formation of a well-developed immune system promotion (Fulde and Hornef, 2014) and provides sufficient resistance against pathogens (Kamada et al., 2013). It was observed that intestinal microbes can activate regulatory T cells (Treg) to secrete cytokines and inhibit the overgrowth of pathogens (Wang et al., 2015). It is worth mentioning that piglet intestinal health can be improved by improving the immune function in animals. Unfortunately, the application of PTD in pig production is only scarcely reported. Consequently, we explored the effect of PTD on the growth performance, immune function as well as intestinal epithelial barriers and microbiota composition in piglets.

Material and methods

Reagents

Pidotimod was synthesized by Shandong Qilu Pharmaceutical Co., Ltd. Production (Jinan, China); batch number: 201009001. Different amounts of PTD were mixed with the feed directly before the experiment.

Animals, diet and management

All procedures of this study were in accordance with the Chinese guidelines for animal welfare. All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Zhejiang Province, China), permission number: ZJU20160416.

In total, 120 healthy piglets (Duroc \times Landrace \times Yorkshire) with a weight of 25.26 ± 5.96 kg were fed the same basal diet in a closed pigsty with consistent environmental conditions and feeding management at a suitable temperature (~ 23 – 25 °C). To compare the effects of different doses of PTD on the growth performance, piglets were randomly divided into 4 groups: control group (CON) was fed basal diet, and treatments 1 to 3 were supplemented with 50, 100 and 200 mg/kg of PTD, for 44 days. Each group has 3 replicates, 10 piglets per replicate, 5 barrows and 5 gilts. Feed and water were given *ad libitum*. Basal diet was formulated according to the nutritional requirements of the National Research Council (NRC, 2012). Its nutrient composition is shown in Table 1. Piglets were weighed at the beginning and the end of the trial. Before weighing,

Table 1. Composition and nutrient level of the basal diet, % as fed basis

Ingredients	Content, %	Nutrient levels ²	Content, %
Maize	61.25	Digestible energy, MJ/kg	14.11
Soyabean meal (44% crude protein (CP))	15.79	Calcium	0.80
Extruded soyabean (36% CP)	10.00	Total phosphorus	0.63
Imported fish meal (65% CP)	5.00	Available phosphorus	0.40
Wheat bran	3.00	Lysine	1.15
Soyabean oil	1.74	Methionine + Cysteine	0.67
Premix ¹	1.00	Threonine	0.77
Limestone	0.98	Tryptophan	0.22
CaHPO ₄	0.78	CP	19.00
Salt	0.37	Crude ether extract	7.26
Lysine-HCl	0.09	Crude fibre	2.30
		Ash	6.27

¹ provided per kg of diet: mg: Zn (ZnO) 50, Cu (CuSO₄) 20, Mn (MnO) 55, Fe (FeSO₄) 100, I (KI) 1, Co (CoSO₄) 2, Se (Na₂SeO₃) 0.3; vitamin B₁ 2, vitamin B₂ 4, pantothenic acid 15, vitamin B₆ 10, vitamin B₁₂ 0.05, vitamin PP 30, folic acid 2, vitamin K₃ 1.5, biotin 0.2, choline chloride 800, vitamin C 100; IU: vitamin A 8255, vitamin D₃ 2000, vitamin E 40;

² calculated values

pigs were fasted for 12 h and coaxed for 10 min and weighed after excretion of urine to calculate the daily weight gain. The daily feed intake was also recorded. According to feed intake and daily weight gain, the feed conversion ratio was calculated as:

$$\text{feed conversion ratio} = \frac{\text{total feed consumption (kg)}}{\text{total weight gain (kg)}}$$

Based on the analysis of daily weight gain and feed conversion ratio, the supplementation with PTD at a dose of 50 mg/kg resulted in the best growth performance among the three groups, and so was used in the subsequent investigations.

Sample collection

At the beginning and the end of the feeding test, all piglets were weighed after deprivation of feed overnight. Then, 3 piglets from each replicate (9 piglets per group) were electrically stunned, exsanguinated, and scalded to enable to collect tissues. The spleen and liver were collected, and the spleen was weighed after removing the adherent fatty tissue. The spleen index was calculated as:

$$\text{spleen index (g/kg)} = \frac{\text{spleen weight}}{\text{live weight before slaughter}}$$

The serum was collected for the ELISA test. Caecum contents were collected, stored at -80°C for DNA extraction. The mid-jejunum segments were fixed in 2.5% glutaraldehyde, rinsed with 0.1 M, pH 7.0 phosphate buffer, and next fixed with

1% OsO₄ buffer for transmission electron microscopy (TEM). Spleen and liver samples were fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining. The jejunum mucosa was gently scraped, stored at -80°C for Western blot analysis.

Hematoxylin and eosin (H&E) staining

Liver and spleen samples of piglets fed diet supplemented with PTD at a dose of 50 mg/kg were fixed in 4% paraformaldehyde, embedded in paraffin, sliced, dehydrated, and stained with hematoxylin and eosin. Images were captured using an Olympus microsystem (Tokyo, Japan).

Transmission electron microscopy (TEM)

Specimens of piglets fed diet supplemented with PTD at a dose of 50 mg/kg were first fixed with 2.5% glutaraldehyde and then fixed with 1% OsO₄ buffer. The sample was then dehydrated with a gradient of ethanol solution. After infiltration and embedding, middle jejunum samples were ultrathin sectioned and observed under a Hitachi Model H-7650 transmission electron microscope (Tokyo, Japan).

Serum cytokines

Blood samples from piglets fed diet supplemented with PTD at a dose of 50 mg/kg were collected from the vena cava anterior and then centrifuged for 10 min at 4°C (3000 g, Centrifuge 5804R, Eppendorf, Hamburg, Germany) to obtain serum samples. Levels of inflammatory factors (interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)- α), Th1 cytokines (IL-12 p40, interferon (IFN)- γ), Th2 cytokines (IL-4), anti-virus-related cytokines (IFN- α) and anti-inflammatory factors (IL-10) in serum were determined by porcine enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (eBioscience, Santa Clara, CA, USA). The standard curves were made according to the instructions ($R^2 > 0.99$), concentrations of cytokines were then calculated from the standard curves.

Western blotting

The jejunum mucosal samples collected from piglets fed diet supplemented with PTD at a dose of 50 mg/kg were collected and homogenized in radio-immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The protein concentration was determined using a BCA kit (Beyotime, Shanghai, China). After denaturation, proteins from each sample were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes

(Roche, Mannheim, Germany). The membranes were blocked by 5% skimmed milk then incubated with primary antibody overnight at 4 °C. Primary antibodies occludin, claudin-1 and ZO-1 (ab222691, ab129119, ab214228) were purchased from Abcam (Cambridge, UK), while β -actin antibody (AA128) was purchased from Beyotime (Shanghai, China). After incubation with secondary antibody (A0208, A0216, Beyotime, Shanghai, China) for 1 h at room temperature, the immunoreactive bands were detected by an ECL system (Tanon, Shanghai, China). The relative band density was determined by an ImageJ 1.52 software (Schneider et al., 2012).

Microbial analysis

The caecal microbial genomic DNA of piglets fed diet supplemented with PTD at a dose of 50 mg/kg was extracted by a TIANamp Stool DNA Kit (Tiangen, Beijing, China) following the manufacturer's instructions. The V3-V4 hypervariable region of the 16S rDNA was amplified using the primer pair 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). The PCR procedure was set according to our previous study (Xu et al., 2018). Then the sequencing was performed by the MiSeq platform (Illumina Inc., San Diego, CA, USA). Sequences were filtered and clustered into operational taxonomic units (OTU) at 97% similarity by a QIIME software (ver. 1.9.1) (Caporaso et al., 2010). Bacterial OTU representative sequences were assigned to a taxonomic lineage by Ribosomal Database Project (RDP) classifier based on the Greengenes database (13_8 release). Alpha diversity and beta diversity between samples were also analyzed by QIIME software using 'alpha_diversity.py' and 'beta_diversity_through_plots.py'. Beta diversity was displayed by principal components analysis (PCA) using the 'ggplot2' package of R software and permutational multivariate analysis of variance (PERMANOVA) was calculated to determine significant differences in the microbial community using the 'vegan' package (based on the Bray-Curtis distance matrices).

Statistical analysis

Growth performance data were analyzed by a one-way ANOVA followed by the Tukey's test. Others were analyzed for 2 groups by a two-tailed t-test using SPSS 22 (SPSS Inc., Chicago, IL, USA) for Windows and results were expressed as mean \pm standard deviation (SD). Data were significantly different when $P < 0.05$ (*) and $P < 0.01$ (**). Data were visualized by an Origin 9.0 software (Origin Lab Corporation, Northampton, MA, USA).

Results

Growth performance

As shown in Table 2, in comparison with control, PTD supplementation significantly increased the average daily weight gain of piglets ($P < 0.05$ and $P < 0.01$ for dose of 50 and 100 mg/kg, respectively).

Table 2. Effect of pidotimod on the growth performance of piglets

Indices	Control	Pidotimod, mg/kg		
		50	100	200
Initial body weight, kg	24.60 \pm 5.48	25.15 \pm 5.19	24.48 \pm 3.11	26.82 \pm 8.86
Final body weight, kg	47.74 \pm 5.83	51.93 \pm 5.17	52.08 \pm 4.2	50.5 \pm 8.41
Average daily feed intake, kg/day	1.21 \pm 0.11	1.28 \pm 0.03	1.40 \pm 0.15	1.37 \pm 0.21
Average daily gain, kg/day	0.55 \pm 0.01 ^{ab}	0.61 \pm 0.01 ^a	0.64 \pm 0.03 ^a	0.5 \pm 0.02 ^b
Feed conversion ratio	2.19 \pm 0.19	2.10 \pm 0.07	2.20 \pm 0.15	2.50 \pm 0.45

values are mean \pm SD; data were analyzed by a one-way ANOVA followed by Tukey test (n = 30 in each group), ^{ab} – values with different superscripts for each parameter are significantly different at $P < 0.05$

Comparatively, the lower feed conversion ratio was stated in piglets fed diet supplemented with PTD at a dose of 50 mg/kg conferred the greatest feed conversion performance ($P > 0.05$), without inducing apparent undesirable side effects on the spleen and liver (Figure 1). Moreover, there were no differences in spleen index between the control and the PTD group. Accordingly, PTD dose of 50 mg/kg was used in the subsequent investigations.

Cytokines level

As shown in Figure 2, in comparison with the control group, supplementation with PTD at a dose of 50 mg/kg significantly reduced serum levels of pro-inflammatory cytokine IL-6 ($P = 0.0415$), IFN- γ ($P = 0.0041$), and had no effects on TNF- α , IL-8, IL-4, IL-12 p40 and IFN- α cytokines ($P > 0.05$). However, it remarkably up-regulated serum levels of IL-10 ($P = 0.0374$).

TEM observation and expression of tight junction proteins in the jejunum of piglets

Transmission electron microscopy further revealed that in comparison with the control group, PTD dose of 50 mg/kg increased the intercellular connectivity, characterized by longer tight junction (TJ) proteins as well as denser adhesive tapes (AB) and desmosomes (D) in jejunal cells of piglets

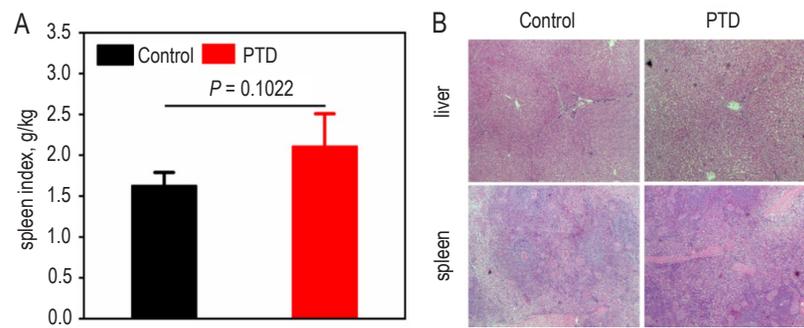


Figure 1. Effects of pidotimod on spleen index (A) and histopathology of organs in piglets (B)

Control group was fed basal diet, pidotimod (PTD) group was fed basal diet supplemented with PTD at a dose of 50 mg/kg; (A): no significant variation has been observed on piglet spleen index: spleen index (g/kg) = spleen weight / live weight before slaughter, values are mean \pm SD ($n = 9$); (B): hematoxylin and eosin staining, 40 \times magnification, scale bar: 200 μ m; the histomorphology of organs is the same between the two groups; the structure of the liver and spleen is clear, and there is no obvious chronic inflammatory cell infiltration in the portal area.

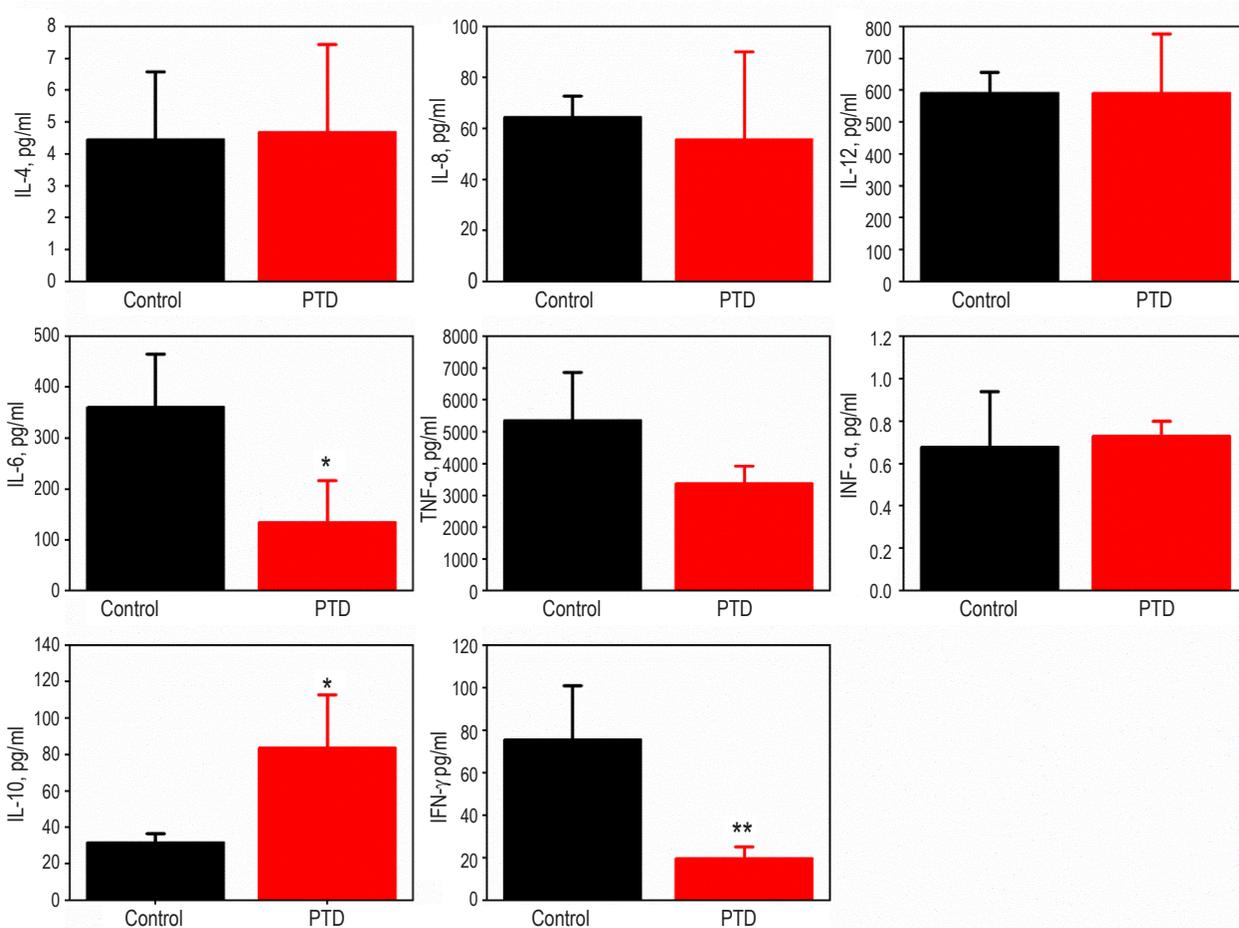


Figure 2. Effects of pidotimod on serum cytokines levels in piglets

Control group was fed basal diet, pidotimod group (PTD) was fed basal diet supplemented with PTD at a dose of 50 mg/kg; values are mean \pm SD ($n = 9$); * – $P < 0.05$, ** – $P < 0.01$; IL-4 – interleukin-4, IL-8 – interleukin-8, IL-12 – interleukin-12, IL-6 – interleukin-6, TNF- α – tumor necrosis factor- α , INF- α – interferon- α , IL-10 – interleukin-10, IFN- γ – interferon- γ .

(Figure 3). Western blot analysis further revealed that PTD dose of 50 mg/kg supplemented to piglets diet up-regulated the protein expression of occludin ($P < 0.05$) and ZO-1 ($P < 0.05$) in jejunal mucosa (Figure 4).

Pidotimod influence on intestinal microbiota

Alpha diversity analysis showed that PTD supplemented at a dose of 50 mg/kg significantly ($P < 0.05$) decreased the Observed_species, Chao1, PD_whole_tree and Ace indices (Figure 5). Principal

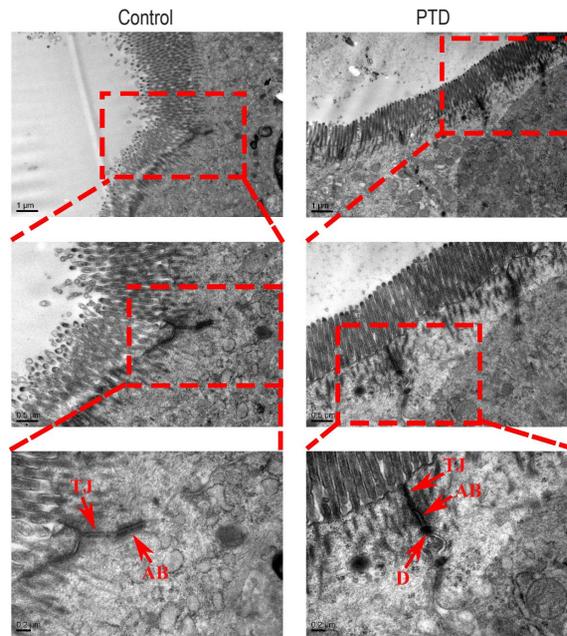


Figure 3. Transmission electron micrographs (TEM) of the jejunum microvilli of piglets

Control group was fed basal diet, pidotimod group (PTD) was fed basal diet supplemented with PTD at a dose of 50 mg/kg. Images were observed at 30,000× magnification, scale bar: 1, 0.5 and 0.2 μm; PTD group had increased intercellular connectivity, longer tight junctions (TJ), denser adhesive belt (AB) and desmosomes (D) in the jejunum of piglets.

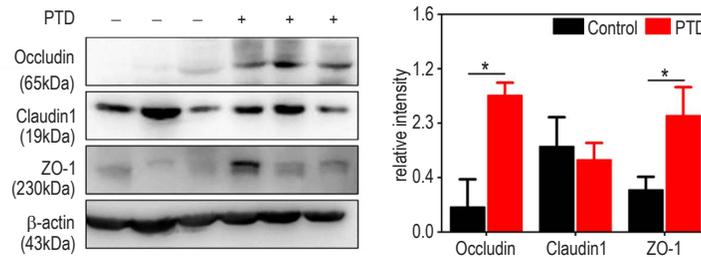


Figure 4. Effects of pidotimod on the expression of tight junction proteins in jejunal mucosa of piglets

Control group was fed basal diet, pidotimod group (PTD) was fed basal diet supplemented with PTD at a dose of 50 mg/kg. Protein lysates from jejunal mucosa were examined by Western blot for occludin, claudin 1 and ZO-1 protein levels. The ratio of Occludin, Claudin 1 and ZO-1 to β-actin was analyzed using ImageJ software (Schneider et al., 2012); values are mean ± SD (n = 3); * – P < 0.05.

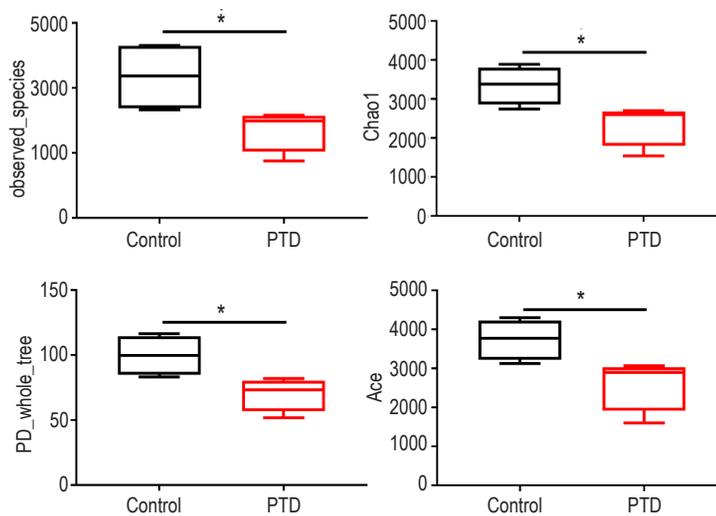


Figure 5. α diversity estimates for the samples

Control group was fed basal diet, pidotimod group (PTD) was fed basal diet supplemented with PTD at a dose of 50 mg/kg; * P < 0.05; n = 4.

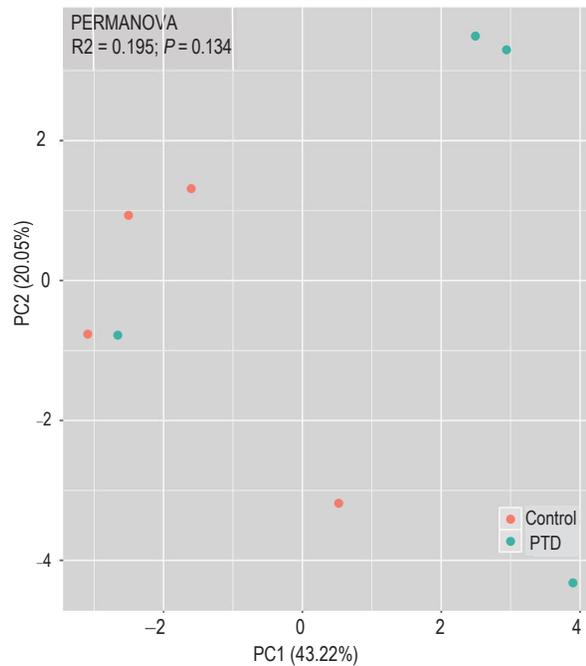


Figure 6. Principal component analysis (PCA) of intestinal microbial communities between the two groups based on Bray-Curtis distance. Control group was fed basal diet, pidotimod group (PTD) was fed basal diet supplemented with PTD at a dose of 50 mg/kg; n = 4.

component analysis (PCA) based on Bray-Curtis distance revealed that PDT had no significant effect on the beta diversity of microbial communities ($R^2 = 0.195$, $P = 0.134$) (Figure 6).

In total, 24 phyla were identified in both control and PTD groups, there were no difference in the microbe number of the control group and PTD group at the phylum level. *Firmicutes* and *Bacteroidetes* are the most dominant phyla in the caecum of piglets, accounting for more than 90% (Figure 7) of the total microbiota. LDA Effect Size (LEfSe) analysis (Figure 8) showed that *Bacilli*, *Lactobacillales*,

Streptococcus and *Streptococcaceae* were the most abundant taxa in the experimental group, converse to taxa *Spirochaetales*, *Spirochaetaceae*, *Treponema*, *Peptococcaceae* and *rc4_4* in the control group.

In comparison with control diet, PTD decreased the relative abundance of *Spirochaetes* ($P = 0.051$) and *Verrucomicrobia* ($P = 0.059$) at phyla level and that of *Succinispira* ($P = 0.024$) and *Treponema* ($P = 0.048$) at the genus level. However, there was a substantial increase in the abundance of *Lactobacillus* ($P = 0.082$) and *Clinsella* ($P = 0.074$) (Figure 9) at the genus level.

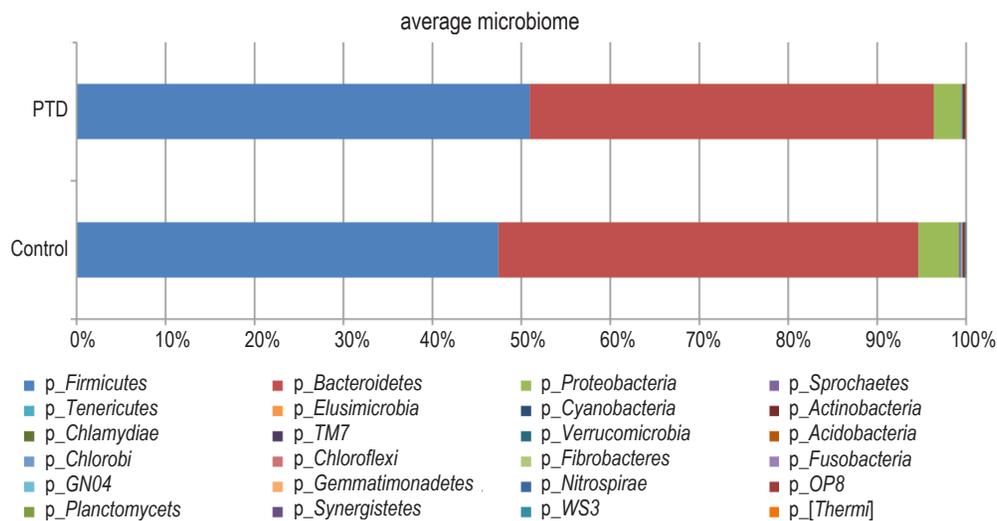


Figure 7. Phylum distributions of intestinal microbiota

Control group was fed basal diet, pidotimod group (PTD) was fed basal diet supplemented with PTD at a dose of 50 mg/kg; n = 4.

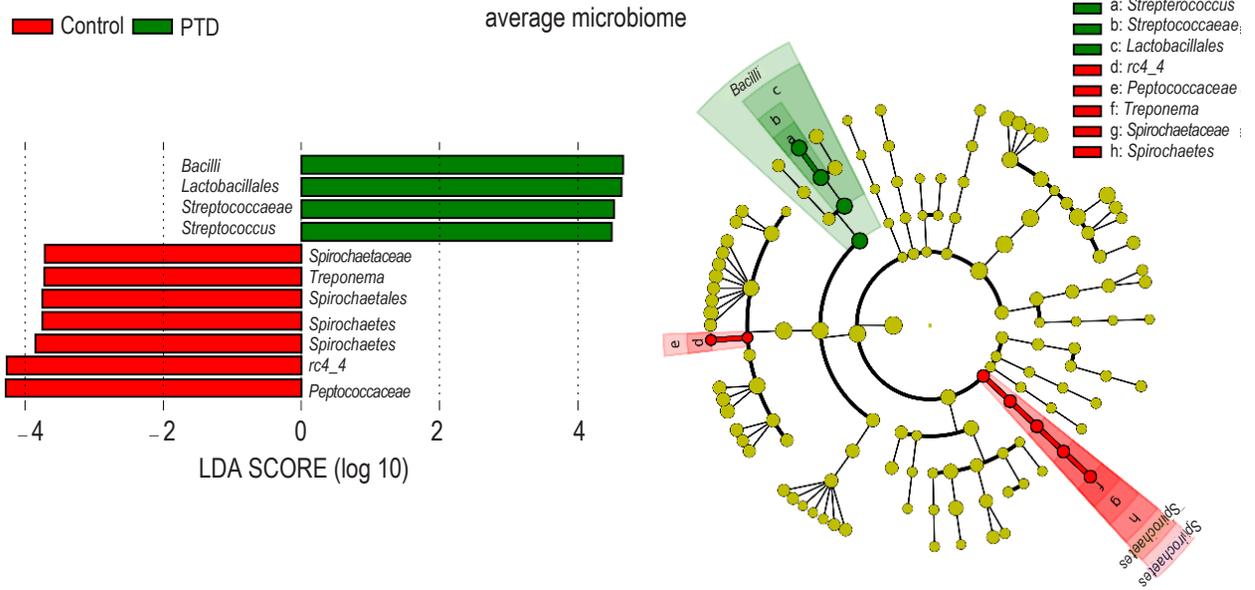


Figure 8. LEfSe analysis of intestinal microbiota

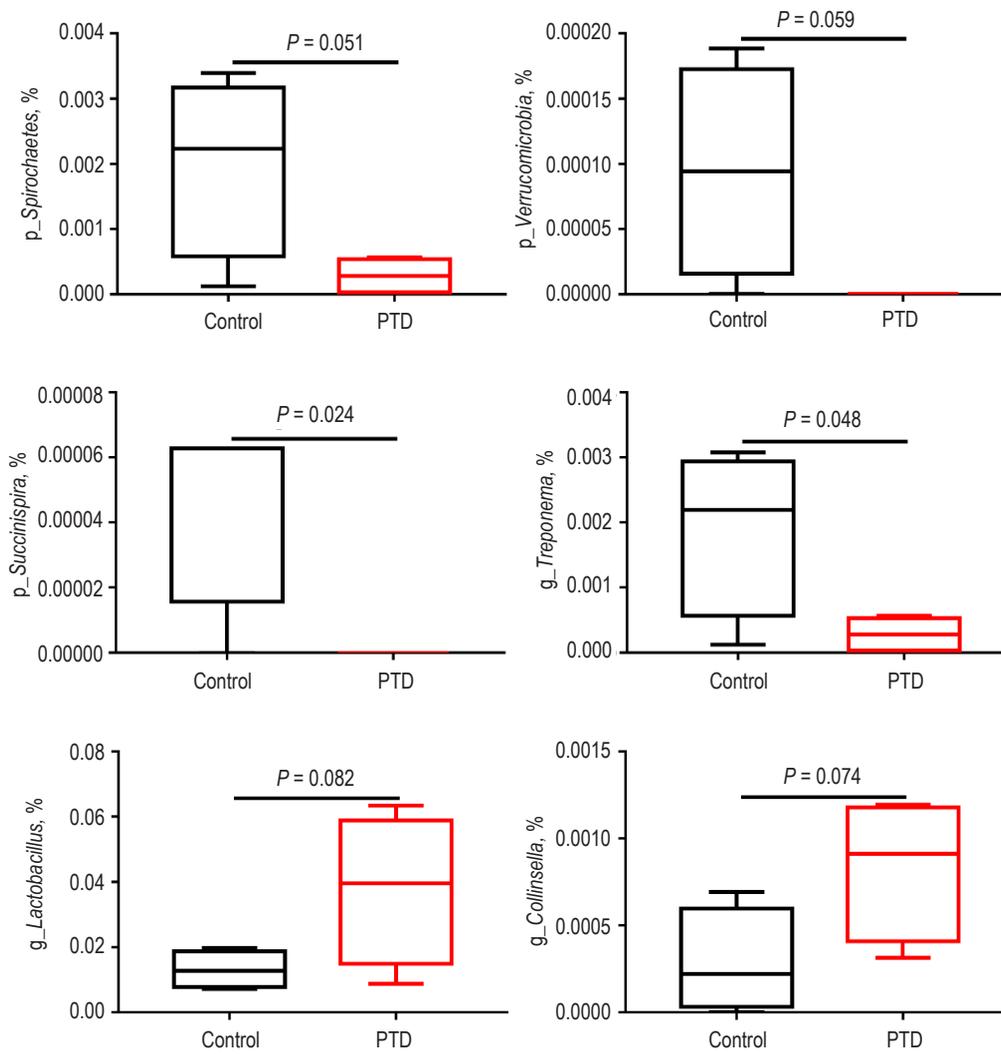


Figure 9. Differentially abundant features between control and pidotimid groups at the phylum and genus levels. Control group was fed basal diet, pidotimid group (PTD) was fed basal diet supplemented with PTD at a dose of 50 mg/kg.

Discussion

Immunomodulators can improve the growth and immune performance of weaned piglets, thereby enhancing their disease resistance capacity (Zhong et al., 2016). At the same time, it was shown that PTD did not induce teratogenic effects and reproductive toxicity in animals (Caramia et al., 1994; Clemente et al., 1994). In this study, in comparison with the control group, the spleen index of piglets from group fed diet supplemented with PTD at a dose of 50 mg/kg did not differ significantly and there were no undesirable side effects in the spleen and liver according to H&E staining. The spleen is an important immune organ, and the index of the spleen was found to decrease in the case of injury (Wu et al., 2020). Our finding provided strong evidence for the safety of PTD. Moreover, based on the average daily weight gain that was measured, PTD accelerated the growth of piglets. Based on the characteristics of PTD as an immunomodulator, we speculate that this may be due to the improvement of immune function and intestinal health.

Pidotimod promotes various aspects of the specific and non-specific immunity of the host (Tian and Zeng, 2005). For instance, PTD (0.5 g/l) promotes growth, lymphocyte proliferation, antibody production and increases the CD4/CD8 cell ratio also in poultry (Qu et al., 2017). In addition, PTD enhances the production of IL-2 in lymphocytes (Chiarenza et al., 1994) as well as up-regulates the expression of IL-8 level in individuals with Tourette's syndrome (Li et al., 2015). IL-6 regulates chronic inflammation (Murakami et al., 2019). Together with IL-6, IFN- γ and IL-8 are a predominant pro-inflammatory cytokines (Zelová and Hošek, 2013; Kim and Moudgil, 2017). However, the up-regulated IL-8 in another study was not found this time, and down-regulated pro-inflammatory cytokines including IL-6 and IFN- γ were observed. It might be that no inflammation occurred in our research because there was no infection by a pathogen. Pathogen infection tends to stimulate the production of pro-inflammatory cytokines including IL-8, IL-6 leading to a generation of an inflammatory response (de Jong et al., 2006; Kobasa et al., 2007). In a previous study, PTD up-regulated the expression of IL-8 and IL-6, thereby enhancing the activity of the immune system for a long time (Esposito et al., 2015; Trabattoni et al., 2017). In addition, our PTD treatment increased serum levels of IL-10. Given that IL-10 and IL-2 modulate anti-inflammation

(Wojdasiewicz et al., 2014), PTD can inhibit inflammatory responses by regulating the expression of both pro-inflammatory and anti-inflammatory cytokines. This result was also observed in a previous study by Ucciferri et al. (2020), who also found a decreased level of pro-inflammatory cytokines in individuals infected with HIV. This implies that PTD exerts an anti-inflammatory effect in piglets through down-regulated pro-inflammatory cytokines and up-regulated anti-inflammation cytokines.

Besides being sites for digestion and food absorption, the intestines are the largest immune organs in the body. Meanwhile, the physiological functions of the intestines are based on the morphological integrity of the organ. Damage to the intestinal barrier disrupts their defence functions (Caramia et al., 1994). The intestinal epithelium is the first line of defence of the intestinal mucous system. It is comprised of connected tight junction (TJ) proteins which form a physical barrier against the entry of pathogens. The TJ is composed of occludin, claudins and ZO-1 proteins located in the cell. These three proteins are tightly bound together to form a TJ barrier that prevents the entry of antigenic substances (Swamy et al., 2010). In this study, we found that PTD improved the morphological structure of intestinal mucosa by strengthening the physical barrier at TJ and reducing swelling as well as cavitation of epithelial cells. Defects in barrier integrity caused chronic inflammation of the intestine (Suzuki, 2020), the maintenance and protection of the TJ barrier could be effective for preventing diseases and promoting growth. In addition, PTD significantly up-regulated the expression of jejunal TJ proteins such as occludin and ZO-1. TNF- α and interleukins may affect paracellular permeability by altering the expression of proteins in the TJ, thereby disrupting the barrier for antigens and bacteria (Camilleri et al., 2012). In this study, the reducing levels of pro-inflammatory cytokines were found. Combined with the up-regulated expression of TJ proteins, we thought that PTD could improve intestinal barrier function by anti-inflammatory effect, thereby enhancing the intestinal anti-infection ability which is beneficial to maintain intestinal health. Overall, PTD can effectively improve the intestinal epithelium integrity and intestinal physical barrier function in piglets.

The animal intestinal microbial communities are interconnected, with some exhibiting antagonistic relationships. The dynamic intestinal micro-

bial interactions affect the health of the host (Duncan and Edberg, 1995). We found *Firmicutes* and *Bacteroidetes* to be the predominant bacteria in the pig gut (>90%), which is consistent with the previous study (Kim et al., 2011). Pidotimod significantly reduced the relative abundance of the *Spirochaetes*, *Verrucomicrobia*, *Succinispira* and *Treponema* but increased those of the *Lactobacillus* and *Collinsella*. Research shows that colonization of *Spirochetes* in the large intestine can cause several intestinal diseases in animals (Shears, 1997). On the other hand, a high relative abundance of *Verrucomicrobia* in the intestines induced *Fusobacterium nucleatum* infection and promoted colonic tumorigenesis (Li et al., 2017). Also, certain *Treponema* species are associated with digital dermatitis in dairy cattle (Beninger et al., 2018). Reducing the relative abundance of these harmful bacteria PTD can improve microbial composition. It was observed that the digestibility of neutral detergent fibre positively correlated with the abundance of *Collinsella* (Niu et al., 2019). Consequently, higher intestinal *Lactobacillus* and *Collinsella* levels could positively improve the intestinal health of animals. It is worth mentioning that intestinal microbes have been reported to alter epithelial permeability indirectly through effects on host immune cells and the release of cytokines, which can regulate barrier function (Camilleri et al., 2012). *Lactobacillus* was associated with barrier function integrity *in vivo* and *in vitro* (Anderson et al., 2010; Mujagic et al., 2017). Thus, the increased *Lactobacillus* level after PTD treatment in our finding might contribute to intestinal health. Combined with previous research, it has been demonstrated that PTD improves the composition of intestinal microbiota by reducing the relative abundance of these harmful microbes and increasing the relative abundance of these beneficial microbes.

Conclusions

Pidotimod at a dose of 50 mg/kg may play a beneficial role in improving growth performance and intestinal health by enhancing immune function, intestinal epithelial barrier, and regulating intestinal ones.

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Conflict of interest

The authors declare that there is no conflict of interests.

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