JAST (Journal of Animal Science and Technology) TITLE PAGE

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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Probiotic *Lactiplantibacillus plantarum* improved growth performance of weaned pigs by enhancing intestinal health and modulating immune responses
Running Title (within 10 words)	Effects of probiotic *Lactiplantibacillus plantarum* in weaned pigs
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Competing interests	SHK, YTJ, and HJM are employed by the CJBIO. All the other authors have no conflict of interest.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This work was supported by the High Value-Added Food Technology Development Program of the Korean Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET), the Ministry for Food, Agriculture, Forestry and Fisheries of the Republic of Korea (321037-05-3-HD030) and the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (RS-2023-00271355).
Acknowledgements	We acknowledge supports from CJBIO, CJ CheilJedang, Suwon, South Korea.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
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	Writing - review & editing: Kyoung H, Kang Y, Kim Y, Kim Y, Ahn J, Nam J, Shin I, Kim SH, Jang YT, Moon HJ, Kim SW, Song M.
Ethics approval and consent to participate	The experimental protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, South Korea (approval #CNU-01092).

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Abstract

The objective of this study was to evaluate the effects of dietary probiotic *Lactiplantibacillus plantarum* (*Lactobacillus plantarum*) on growth performance, nutrient digestibility, immune responses, and intestinal health of weaned pigs. In a randomize complete block design (block: initial body weight), a total of 40 weaned pigs (initial body weight: 6.93 ± 0.27 kg) were assigned to 2 dietary treatments (1 pig/pen; 10 replicates/treatment): 1) a basal weaner diet based on corn and soybean meal (CON) and 2) CON + 0.1% dietary probiotic *L. plantarum* (PRO). Pigs fed PRO had increased ($p < 0.05$) body weight, average daily gain, and gain to feed ratio during the overall period than those fed CON. Pigs in PRO had greater ($p < 0.05$) apparent ileal digestibility of crude protein than those in CON. However, there were no differences on apparent total tract digestibility of dry matter, crude protein, and energy between dietary treatments. The PRO group decreased ($p < 0.05$) crypt depth in the duodenum compared with the CON group, but increased ($p < 0.05$) number of goblet cells in the ileum. Pigs fed PRO had lower ($p < 0.05$) serum cortisol on day 7 and number of white blood cells on day 21 than those fed CON, but higher ($p < 0.05$) serum interleukin-6 on day 21. The PRO group upregulated ($p < 0.05$) claudin-2, claudin-3, claudin-4, occludin, and mucin-1 genes expression in the ileum compared with the CON group. Pigs fed PRO had altered gut microbial composition by increasing ($p < 0.05$) the relative abundance of genera *Enterococcus* and *Pediococcus* in fecal microbiota on day 7, genera *Lactobacillus* and *Streptococcus* in fecal microbiota on day 21, and genera *Streptococcus* and *Turicibacter* in jejunal microbiota on day 35 compared with pigs fed CON. Our findings suggest that supplementation of probiotic *L. plantarum* in weaner diets can enhance the growth performance of weaned pigs via modified intestinal health by improving intestinal morphology, upregulating tight junction protein genes expression, and altering microbial communities. Furthermore, the dietary probiotic *L. plantarum* modulated systemic immune responses and local inflammatory cytokine genes expression in the ileum of weaned pigs.

Keywords: growth performance, gut microbiota, ileal gene expression, immune responses, probiotics, weaned pigs

Introduction

The weaning period is one of the crucial stages in the growth and development of pigs. This is because weanling piglets are suddenly faced with complex and diverse stressors [1,2]. These challenges disrupt the physiological, immunological, and microbiological interactions that maintain gut health, leading to growth retardation, increased morbidity, and even mortality. Furthermore, incidence of post-weaning diarrhea (PWD) caused by *Escherichia coli* is increased due to changes in the gut ecosystem following the diet transition at weaning, and it remains a challenge on the gut health of pigs during the immediate post-weaning period [3,4]. Thus, targeted nutritional interventions to reduce colonization with pathogenic *E. coli* through the establishment of beneficial microbes is important for the prevention of PWD. Historically, there has been usage of in-feed antibiotics for prophylactic or therapeutic treatment of the disease [5,6]. However, the overuse of in-feed antibiotics for the purpose of growth promotion was banned in the many countries due to public health concerns about development of antimicrobial resistance that can be transferred to animals and humans [7]. Therefore, there is a need for nutritional strategies that can replace in-feed antibiotics.

Probiotics (PRO) are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [8]. In general, PRO is known to support growth performance of animals via their modulating effects on gut microbiota. Furthermore, PRO can support gut health promotion by ameliorating diarrhea incidence, nutrient digestibility, and gut integrity as well as immune modulation [9,10]. Because the proportion of lactic acid-producing bacteria (LAB) is decreased after weaning, providing LAB in weaner diets may be beneficial for intestinal balance of weaned pigs [11]. Among different PRO, *Lactobacillus* spp. are decreased in the weaning period and are known to have positive effects on gut health, which play an important role in establishing a potential targeted therapy for weaned pigs with intestinal microbial imbalance [12]. *Lactobacillus* spp. secrete antimicrobial substances such as bacteriocins to suppress the growth and adhesion to intestinal epithelium of potential pathogenic strains, thereby regulating the intestinal microbial ecosystem [13,14]. In addition, based on this competitive exclusion, *Lactobacillus* strains stimulate digestive enzymes activity by fermenting available digested nutrients [15,16] and improve the morphological development

of the intestinal epithelial cells [17,18]. Moreover, it regulated inflammatory cytokines by interacting with intestinal immune cells through immunomodulatory effects [19,20]. However, information is lacking on whether microbial shifts by supplementation *Lactiplantibacillus plantarum* (*Lactobacillus plantarum*) strain, contributes to gut health and immune responses, and whether it can modulate gut microbiota in weanling pigs. Thus, it was hypothesized that dietary PRO addition in weaner diets will enhance intestinal health by modulating gut microbiota and immune responses, thereby promoting the growth performance in post-weaning piglets. Therefore, the objective of this study was to evaluate the effects of dietary PRO (*L. plantarum* CJLP 243/475) on growth performance, nutrient digestibility, immune responses, and intestinal health of weaned pigs.

Materials and Methods

Animal ethics

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, South Korea (approval: #202003A-CNU-036).

Experimental animals, design, and diets

Forty newly weaned pigs [(Landrace × Yorkshire) × Duroc; initial body weight (BW) of 6.93 ± 0.27 kg; 28 days of age)] obtained from a commercial farm (CJ CheilJedang Corp., Yeongam-gun, South Korea) were allotted to one of two dietary treatments based on a randomized complete block design (block: initial BW). Pigs were fed either 1) a basal weaner diet based on corn and soybean meal (CON) and 2) CON added with 0.1% dietary PRO (3×10^9 CFU/g of *L. plantarum* CJLP 243/475, Immprober-S, CJ CheilJedang Corp., Seoul, South Korea) for 5 weeks. Each dietary treatment was applied to 20 replicate pens of individually housed pigs (equal ratio of barrows and gilts per pen). Energy and nutrient content of the basal diet were formulated based on the nutrient requirements for weaned pigs (Table 1) as recommended by the National Research Council [21]. Dry matter, gross energy, crude protein, total calcium, and total phosphorous content of the basal diet were analyzed according to the Association of Official Agriculture Chemists (AOAC) [22] procedures at the Institute

of Agricultural Science, Chungnam National University (Daejeon, South Korea). All pigs were provided a mash form of dietary treatments and fresh water ad libitum in the same sized pen (232 × 175 cm; width × length) throughout the study. In addition, pigs were housed in ambient temperature-controlled pens at 25 to 28℃ and the humidity was set at 50 to 65%. The lighting program was maintained at light/dark intervals of 12 h. During the last week of study, chromic oxide (Daejung Chemicals & Metals Co. Ltd., Siheung-si, Gyeonggi-do, South Korea) was added to the diets, as an indicator for nutrient digestibility analysis at a concentration of 2 g/kg.

Data collection and sampling

The BW of the weaned pigs and their diet residuals after feeding were recorded on day 1, 7, 14, 21, and 35 and used to calculate the average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F; feed efficiency). The fecal score of each pig was visually monitored for the first 2 weeks after weaning by two independent evaluators. The fecal score ranged from 1 to 5 (1 = hard and dry feces, 2 = soft feces, 3 = moist feces, 4 = mild diarrhea, and 5 = watery severe diarrhea). The diarrhea frequency was presented as a percentage by calculating the number of pen days with a pen average diarrhea score of 3 or higher [23]. Blood samples (5 mL each) were collected from the jugular vein of pigs in each pen using vacuum tubes (BD Vacutainer Systems, Franklin Lakes, NJ, USA) with (on day 1, 7, 21, and 35) and without (on day 1, 7, and 21) ethylenediaminetetraacetic acid (EDTA). Serum samples were obtained from non-EDTA tubes by using centrifugation (1580R, LaboGene, Lynge, Denmark) at 3,000 × g for 15 min at 4℃ and stored at -20℃ for further immune responses analysis. Fecal samples for fecal microbial analysis were collected from six randomly pigs per dietary treatments on day 1, 7, and 21 via rectal stimulation using a sterile swab and stored at -80℃ until metagenomic analysis [24]. For apparent total tract digestibility (ATTD) analysis, fecal samples were collected by daily rectal palpation during 3 days of final week of the experiment after 4 days of adaptation to chromium oxide-containing diets and kept at -80℃ until analysis [25]. On the last day, six randomly selected pigs per dietary treatment were anesthetized by 2 mL suxamethonium chloride (Succicholine Inj., Goyang-si, Gyeonggi-do, South Korea) via intramuscular injection. After anesthesia, the pigs were

euthanized by exposure to carbon dioxide. Jejunal digesta samples for microbial analysis were collected at the middle part of jejunum from the pigs and stored at -80℃ until metagenomic analysis. Ileal digesta samples for apparent ileal digestibility (AID) analysis were collected between the distal ileum and ileocecal junction and stored at -80℃ until chemical analysis. To analyze intestinal morphology, segments approximately 3 cm in length were collected from the duodenum, jejunum, and ileum and washed with distilled water, placed in a 50 mL conical tube and fixed with 10% neutral buffered formalin solution (BBC Biochemical, Mount Vernon, WA, USA) until microscopy analysis. Other ileal segments were scraped for gene expression analysis, stabilized in a 1.5 mL microtube with RNA later reagent (QIAGEN GmbH, Hilden, Germany) for 24 h at room temperature, and stored at -80℃ until analysis.

Nutrient digestibility analysis

Diet and frozen fecal samples were forced-air dried in an oven (FC-PO-91, LabHouse, Pocheon-si, Gyeonggi-do, South Korea) at 65℃ for 72 h. Frozen ileal digesta samples were freeze-dried at a chamber pressure of 5 mTorr at room temperature for 48 h using a freeze dryer (Bondiro, Ilshin, Seoul, South Korea). After drying, all samples were finely ground using a coffee grinder (Electric Coffee Grinder, Hamilton Beach, USA) for chemical analysis. The ground samples were analyzed for dry matter (DM), energy by a bomb calorimeter (C2000, IKA Works Inc., Staufen, Germany), and crude protein (CP) using Kjeldahl method (VAPOXX, Gerhardt Ltd., IdarOberstein, Germany) based on the AOAC [22]. Chromium concentrations of all grinded samples were estimated by absorption spectrometry (Hitachi Z-5000 Absorption Spectrophotometer, Hitachi High-Technologies, Tokyo, Japan). The AID and ATTD of DM, energy, and CP were summarized for each dietary treatment according to previous reports [26].

Intestinal morphology analysis

For microscopy, the fixed duodenum, jejunum, and ileum tissues were implanted in paraffin, cut into thin sections, stained with hematoxylin and eosin (H&E), and sealed on slide glass. Fifteen villi

and their associated crypts were selected from the H&E slides by a fluorescence microscope (TE2000; Nikon, Tokyo, Japan) and NIS-Elements software (v. 3.00; NIS Elements, Nikon, Tokyo, Japan) to measure villus height, width, area, crypt depth, villus height to crypt depth ratio (VH:CD), and number of goblet cells.

Blood profiles and immune responses analysis

Blood profiles of weaned pigs were measured from whole blood samples in EDTA tubes using an automated hematology analyzer calibrated by porcine blood (scil Vet abc hematology analyzer; scil animal care company, Altorf, France) for total white blood cell (WBC) count, red blood cell (RBC) count, and hematocrit (HCT) level. The immune response of the pigs was measured from serum samples using enzyme-linked immunosorbent assay kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Serum concentrations of cortisol, tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta1 (TGF-β1), interleukin-1beta (IL-1β), and interleukin-6 (IL-6) were estimated using a microplate reader at 450nm (Epoch microplate spectrophotometer, BioTek instruments Inc., Winooski, VT, USA).

Ileal gene expression analysis

Expression of tight junction (TJ)-related protein and inflammatory cytokine genes [caludin-1 (*CLND1*), claudin-2 (*CLDN2*), claudin-3 (*CLND3*), claudin-4 (*CLDN4*), occludin (*OCLN*), mucin-1 (*MUC1*), interferon-gamma (*INF-γ*), *TNF-α*, *IL-1β*, *IL-6*, and monocyte chemoattractant protein-1 (*MCP1*)] from the ileal mucosa of the pigs was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from ileal mucosa samples using a commercial kit (HiGene Total RNA Prep kit, BIOFACT, Daejeon, South Korea) and the concentration measured with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA). cDNA was synthesized using a QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). The qRT-PCR analysis was performed with a StepOnePlus RT-PCR system (Applied Biosystems, Foster

City, CA, USA) using SFCgreen I (BIOFACT, Daejeon, South Korea), and gene-specific primers (Table 2). The 18S rRNA was used as an internal control for normalization of target gene cycle threshold (Ct) value. The relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method [27].

Gut microbiota analysis

Total genomic DNA was extracted from jejunal digesta and fecal samples using DNeasy PowerSoil kit (QIAGEN, Hilden, Germany) following their protocol. The V3-V4 regions of the 16S rRNA gene were amplified with primer set by Bakt 341F and Bakt 805R [28]. The 16S rRNA gene amplicons were sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) at a biotechnology company (Macrogen Inc., Seoul, South Korea). Paired-end reads were merged using FLASH v. 1.2.11 [29]. Low quality reads, ambiguous reads, and chimeric reads, were identified and removed using CD-HIT-OUT v. 4.5.4 [30]. Sequences were clustered into operational taxonomic units (OTUs) at 3% different distance cut-offs [31]. Taxonomic assignment of each OTU was performed using BLASTN v. 2.9.0 with reference to the NCBI 16S microbial database. Taxonomic composition was generated using QIIME-UCLUST. Comparison of various microbial communities was performed using QIIME (v. 1.9) based on the OTUs abundance and taxonomy information. Microbial data were normalized by data scaling using the total sum scaling before statistical comparison. Alpha diversity including observed OTUs, Chao1, Shannon, and Simpson indices were measured for within-sample richness and evenness. Beta diversity was measured between dietary treatments based on principal coordinate analysis (PCoA) plots using Bray–Curtis dissimilarity.

Statistical analyses

Data were analyzed using the GLM procedure of SAS (v. 9.4; SAS Inst. Inc., Cary, NC, USA), except diarrhea frequency, ileal gene expression, and gut microbiota, in a randomized complete block design (block: initial BW). The experimental unit was the pig. Diet was a main effect and initial BW was a covariate. Frequency of diarrhea was analyzed using the Proc Freq in SAS. The *t*-test was used

for a comparison of ileal gene expression between dietary treatments. The MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) webtool was used for analysis of the gut microbial diversities statistics (alpha diversity, Kruskal-Wallis test; beta diversity, PERMANOVA) of gut microbiota. Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis [32] was used to identified taxonomic biomarkers with effect size at the 2.0 LDA score threshold using Galaxy online platform (https://huttenhower.sph.harvard.edu/galaxy/). Results were presented as means ± SEM, excluding the microbial alpha diversity, which was presented as means ± SD. Statistically differences were considered significant and tendency between dietary treatments at $p < 0.05$ and $0.05 \leq p < 0.10$, respectively.

Results

Growth performance and diarrhea frequency

During the overall period of the study, the pigs fed PRO had increased ($p < 0.05$) ADG and G:F compared with those fed CON (Table 3). Dietary PRO increased ($p < 0.05$) ADG and ADFI from day 8 to 14 compared with CON. In addition, the PRO group tended to have a higher ADG ($p < 0.10$) and G:F ($p < 0.05$) from day 22 to 35 than CON group. There were no differences on diarrhea frequency between dietary treatments for the first 2 weeks after weaning.

Nutrient digestibility and intestinal morphology

Dietary PRO increased ($p < 0.05$) AID of CP compared with CON (Table 4), but there were no differences on ATTD of DM, CP, and energy between dietary treatments. The result of intestinal morphology was shown in Table 5. In the duodenum, the pigs fed PRO had lower ($p < 0.05$) crypt depth and tended to have higher ($p < 0.10$) VH:CD than those fed CON. In addition, the PRO group tended to have an increase ($p < 0.10$) villus area in the jejunum compared with the CON group. In the ileum, the PRO diet increased ($p < 0.05$) the number of goblet cell counts compared with the CON diet, but there was no difference on the number of goblet cell counts in the duodenum and jejunum.

Blood profiles and immune responses

Pigs fed PRO had lower ($p < 0.05$) number of WBC counts on day 21 than those fed CON, but tended to have higher ($p < 0.10$) number of RBC on day 35 (Table 6). However, there was no difference on HCT level between CON and PRO. As shown in Table 7, the pigs fed PRO had lower serum concentrations of cortisol on day 7 ($p < 0.05$) and TNF-α on day 21 ($p < 0.10$) than those fed CON, but had higher ($p < 0.05$) serum concentration of IL-6 on day 21. There were no differences on serum concentrations of TGF-β1 and IL-1β.

Ileal gene expression

The PRO had upregulated ($p < 0.05$) relative expression of TJ-related protein genes in the ileum such as *CLDN1*, *CLDN2*, *CLDN3*, *CLDN4*, *OCLN*, and *MUC1* compared with the CON (Fig. 1). Furthermore, the PRO had upregulated ($p < 0.05$) relative expression of inflammatory cytokine genes such as *TNF-α*, *IL-1β*, *IL-6*, and *MCP1* compared with the CON.

Gut microbial alpha and beta diversity

Alpha diversity indices are presented in Table 8. In fecal microbiota, there were no differences on alpha diversity indices between dietary treatments. However, in the jejunal microbiota, dietary PRO increased ($p < 0.05$) Shannon and Simpson indices on day 35 compared with CON. Beta diversity was presented in the PCoA plot based on Bray–Curtis distance and shown in Figure. 2. There were no differences (Fig. 2A) on Bray–Curtis distance in fecal microbial communities on day 1 between dietary treatments. However, differences on clustering of fecal samples were determined on d 7 ($r^2 = 0.32$, $p < 0.05$; Fig. 2B) and day 21 ($r^2 = 0.26$, $p < 0.05$; Fig. 2C) between CON and PRO. In addition, clustering of Bray–Curtis distance in jejunal microbial communities was different on day 35 between dietary treatments ($r^2 = 0.41$, $p < 0.05$; Fig. 2D).

Gut microbial taxonomic composition

The relative abundance and differences of the gut microbiota between dietary treatments are shown in Fig. 3 and Fig. 4, respectively. In fecal microbiota, phylum Firmicutes (CON, 65.23%; PRO,

66.30%) was the most dominant in both dietary treatments at day 1, followed by Proteobacteria (CON, 24.74%; PRO, 29.18%; Fig. 3A). On day 7, Firmicutes was increased in relative proportion in both CON (90.37%) and PRO (91.51%) compared with at the beginning of the study, but Proteobacteria was decreased (CON, 4.04%; PRO, 1.46%). On day 21, Firmicutes was the most dominant phylum in both dietary treatments (CON, 95.73%; PRO, 95.51%), followed by Actinobacteria (CON, 1.79%; PRO, 1.91%). At the genus level (Fig. 3B), *Escherichia* was the most predominant fecal microbiota in both CON (23.73%) and PRO (28.23%) on day 1. In the CON group, genera *Pediococcus* (14.39%), *Lactobacillus* (7.41%), *Enterococcus* (6.89%), and *Clostridium* (3.47%) were followed. However, genera *Pediococcus* (14.82%), *Enterococcus* (6.30%), *Staphylococcus* (5.70%), and *Lactobacillus* (5.61%) were followed in PRO the group. On day 7, dietary PRO increased ($P < 0.05$) *Pediococcus* and *Enterococcus* compared with CON (12.24% vs. 3.59%; 16.47% vs. 3.19%, respectively), but *Weissella* was lower ($p < 0.05$; 9.69% vs. 22.53%; Fig. 4A). On day 21, the PRO had higher ($p < 0.05$) *Lactobacillus* (44.52% vs. 21.09%) and *Streptococcus* (13.85% vs. 5.55%) than the CON, but *Pediococcus* (10.50% vs. 23.81%) and *Clostridium* (1.89% vs. 12.17) were lower ($p < 0.05$; Fig. 4B). In jejunal microbiota, pigs fed PRO had higher ($p < 0.05$) Firmicutes than those fed CON (95.91% vs. 71.72%) on day 35, but lower ($p < 0.05$) Proteobacteria (3.93% vs. 28.13%; Fig. 3C and Fig. 4C). At the genus level, dietary PRO increased ($p < 0.05$) *Streptococcus* (17.42% vs. 1.08%) and *Turicibacter* (3.02% vs. 0.11%) compared with CON, but *Helicobacter* (2.87% vs. 15.92%) and *Escherichia* (0.43% vs. 11.44%) was decreased ($p < 0.05$; Fig. 3D and Fig. 4C). Additionally, differences between dietary treatments at the species level identified through LEfSe analysis are shown in Fig. 4. On day 7, species *Lactiplantibacillus plantarum* (*Lactobacillus plantarum*), *Enterococcus hirae*, and *Pediococcus pentosaceus* were identified in fecal microbiota of PRO group, while species *Weissella paramesenteroides* were classified as microbial features in the CON group (Fig. 4A). On day 21, species *Lactobacillus plantarum* was identified in fecal microbiota of PRO group, while species *Pediococcus pentosaceus* were identrified as taxonomic features in the CON group (Fig. 4B). On day 35, species *Lactobacillus ultunensis*, *Lactobacillus plantarum*, *Streptococcus hyointestinalis* and *Turicibacter sanguinis* were identified in jejunal microbiota of PRO group, while species *Helicobacter apri*,

Lactobacillus agilis, and *Escherichia fergusonii* were classified as microbial features in the CON group (Fig. 4C).

Discussion

The gastrointestinal (GI) tract plays a crucial role in overall health. In the weaning period, GI health is accompanied by loss of structure, barrier, local immune function and microbial dysbiosis, resulting from changes in the GI environment [1,2]. Overall, reduced digestion and absorption of nutrients leads to PWD and poor feed efficiency, thereby impairing productivity [4]. Our findings showed that dietary PRO inclusion in diet of weaned pigs enhanced growth performance compared with CON through improved intestinal health. Previous studies are consistent with our result of growth performance [19,33–35] and have reported that improvement in intestinal environment would be supported by dietary PRO. In antibiotic-free swine production, PRO have been proposed as a potential alternative for PWD caused by *E. coli* infection [36]. PWD causes dehydration due to intestinal fluids and electrolytes secretion [36,37]. However, we found no difference on diarrhea frequency with the PRO addition. Additionally, the HCT, as an indicator that increases with diarrhea, did not differ between CON and PRO. The effects of dietary PRO on PWD are inconsistent, which could be associated with PRO dosage and healthy normal or challenge conditions [17,18,38,39]. Therefore, the present growth performance result would be supported by focusing on another potential mechanism of dietary PRO in weaned pigs.

Weaned pigs suffer from accelerated weaning stress on their immature intestine, which impaired physiological structure and function: villus atrophy and crypt hyperplasia as a structural change, and digestive enzyme activity decrease as a functional change [40,41]. Collectively, post-weaning GI disturbances affect long-term growth rate and feed efficiency of pigs. In the present study, we determined that the AID of CP was improved, which may be due to the result of probiotic *Lactobacillus* strain used in the experiment affecting the activity of digestive enzymes in the GI tract. This is because PRO not only increases the utilization of nutrients by producing nutrient-decomposing enzymes, such as proteolysis, but also stimulates digestive enzymes by fermenting digesta through

increase beneficial microbiota [15,33]. In general, the small intestinal morphological indices are useful parameters of surface area for nutrient absorption and intestinal health. After weaning, immature enterocyte differentiation leads to a decrease in villus height and an increase in crypt depth, which inhibits digestive enzyme activity and nutrient absorption [42]. Additionally, these morphological changes in the intestine were associated with the low feed intake after weaning [42]. Our experiment confirmed that dietary PRO enhanced intestinal morphology by decreasing duodenal crypt depth and tending to increase jejunal villus area. In the crypts, intestinal stem cells continuously differentiate for villus development. However, as mentioned earlier, the structural appearance of the immature intestine in post-weaning pigs is characterized by shedding of villi and increased crypt depth. Thus, the improvement of crypt depth has a positive effect on villus development for nutrient absorption. Previous studies in weaned pigs were also consistent with the PRO efficacy on intestinal morphology, with positive effects varying across the small intestine segments [17,34,39]. Our results indicate that addition of dietary PRO may have produced and/or stimulated nutrient-digesting enzymes through the growth and development of beneficial microbiota including *Lactobacillus* strains in the GI tract. In addition, increased feed intake in the early post-weaning period may have stimulated the intestinal morphological development of pigs.

PRO have been suggested to have beneficial effects on intestinal barrier function, such as enhance TJ integrity and defense against pathogen invasion in intestinal epithelial cell lines [43,44]. Disruption of the TJ barrier, which composed of transmembrane proteins such as the CLDN family and OCLN, and intracellular scaffold proteins such as zonula occludens, causes paracellular permeability in gut [44]. Mucin secreted by goblet cells is a major component of the intestinal mucus layer, which serves as the first line of host defense [45]. In this study, the expression of *CLDN* family (*CLDN2*, *CLDN3*, and *CLDN4*) and *OCLN* genes in the ileum were upregulated in the PRO. Moreover, the upregulation of *MUC1* gene in dietary PRO was consistent with the improvement in the number of goblet cells in the ileum. It has been reported that PRO prevents pathogen binding in the epithelial layer because it induces qualitative changes in mucin by mucin gene expression [45]. Moreover, dietary PRO can protect intestinal barrier function through maintenance of TJ-related protein genes expression from

pathogenic bacteria [46]. Thus, our findings suggest that dietary PRO enhanced intestinal integrity by stimulating mucus secretion in the ileum, thereby inhibiting pathogenic penetration and reducing intestinal permeability. In addition, the PRO addition may have improved the barrier function of the intestinal epithelium by dominating in the intestine and protecting potential opportunistic pathogens and/or microbiota from adhering to the intestinal epithelium through competitive exclusion in the intestinal microecosystem.

Weaning period elevates the systemic cortisol concentration, as a stress indicator, and induces activation of central stress pathway [47,48]. In addition, an increased WBC counts indicate systemic inflammation [49]. Since cytokines maintain local/systemic immune homeostasis by regulating inflammation in immune responses, changes in levels after weaning can support the host health condition through the immune system. In this study, we found that dietary PRO alleviated early-weaning stress of piglets via serum cortisol level. Moreover, concentration of serum TNF-α, a pro-inflammatory cytokine that is activated by macrophages and triggers an inflammatory response, was reduced by PRO addition. Under various conditions, IL-6 is classified as either pro- or anti-inflammatory responses and it was increased in the serum of PRO pigs in this study. The anti-inflammatory/regenerative properties of IL-6 are mediated by classic signaling, but the pro-inflammatory properties of IL-6 are mediated by trans-signaling in chronic inflammatory diseases [50]. In the present study, not only clinical symptoms of chronic inflammation not observed throughout the experiment, but the WBC counts in the PRO group also decreased as the experiment progressed. On this basis, we considered increased serum IL-6 in PRO to have anti-inflammatory properties. Therefore, dietary PRO appears to modulate the systemic immunity of weaned pigs through anti-inflammatory effects of circulating cytokines. In the GI immune system of pigs, weaning transition is associated with inflammation due to upregulated pro-inflammatory cytokines [51]. Pro-inflammatory cytokines are known to play a role in increasing intestinal permeability by disrupting TJ barriers [52]. Interestingly, however, dietary PRO upregulated expression of ileal pro-inflammatory cytokine and chemokine genes (*TNF-α* and *IL-1β*; *MCP1*, respectively). On the other hand, the anti-inflammatory cytokine *IL-6* has been suggested to be effective in regenerating intestinal epithelial cells and protecting the intestinal

barrier [50,52], and was also upregulated in the ileum. Through our results, dietary PRO activated the mucosal immune system as an immunostimulatory function through cytokines induction, but this did not deteriorate intestinal barrier permeability. Cytokines are mediators that regulate intestinal mucosal barrier function and have diverse effects on intestinal permeability, including nutrients and ion transport [53,54]. Additionally, because cytokine-induced barrier modification can restructure TJ protein expression [55], it is possible that upregulation of inflammatory cytokine affects upregulation of proteins composing TJ barrier. Moreover, intestinal cytokine-induced responses may be associated with feedback regulation of changes in the intestinal environment, such as microbial modifications following PRO addition. However, in order to clearly identify these effects, the relationship between intestinal cytokine profiles and TJ barrier functions needs to be supported by further studies. In addition, unlike systemic immune responses, cytokine stimulation in local immune responses may be the results of PRO-induced changes in the gut microbial environment and antimicrobial substances it can produce and secrete.

The present study showed dietary PRO did not alter fecal alpha diversity, but fecal microbial communities were distinctly separated in beta diversity by dietary PRO supplementation. Moreover, dietary PRO not only changed the jejunal alpha diversity but clustered the beta diversity on day 35 of the study. These results indicate that dietary PRO contributes to changes in alpha diversity with prolonged exposure effects to the gut. Furthermore, our results are consistent with previous report suggesting that alpha diversity was negatively correlated with average daily gain and body fat [56]. These results may be due to the potential mode of action that dietary PRO can inhibit pathogens [57]. However, in understating microbial diversities, it is important to identify whether the distribution of strains that have any effects on host health, such as potentially harmful or beneficial microbiota, has changed, rather than simply increasing or decreasing diversities. Therefore, dietary PRO supplementation may positively affect growth and intestinal health of post-weaning pigs by altering microbial abundance to utilize nutrients or energy source and produce metabolites. Overall, differences on taxonomic abundances of gut microbiota were expected due to altered microbial diversity and dissimilarity by dietary PRO addition.

In fecal taxonomic abundance, *Enterococcus*, *Streptococcus*, and *Lactobacillus* were clearly enriched in the PRO group compared with the CON group. These genera are included in LAB and are known to be beneficial in pigs. However, some species of *Enterococcus* spp. have been reported to be potential harmful to the host. In particular, *Enterococcus hirae* is known to cause enteroadherent infection and diarrhea in animals [58,59], and this species was identified in the PRO group through LEfSe analysis. This may be related to the lack of treatment group differences in clinical PWD in the early post-weaning period. However, predominance of the PRO strain may have contributed to the suppression of *E. hirae* adhesion to the GI tract of weaned pigs due to its pathogenic inhibitory properties by antimicrobial peptides secretion. This is because as dietary PRO intake continued, *Lactobacillus* (*L. plantarum*) and *Streptococcus*, were predominated in the PRO group, whereas *Clostridium* predominated in the CON group. Genus *Clostridium* includes pathogenic strains such as *C. perfrigens* and *C. difficile*, which can cause clostridial diarrhea in pigs [60]. On the other hand, it has been reported that *Streptococcus* is correlated with the BW gain of pigs [61,62], which is consistent with our experiment. Therefore, our result suggests that dietary PRO altered gut microbiota by predominating in the gut and suppressing potential pathogenic strains, resulting in positive effects on the growth of weaned pigs. In the jejunum, phylum Firmicutes was enriched in the PRO group compared with the non-PRO group, whereas the phylum Proteobacteria was less abundant. In general, Firmicutes play important roles in the breakdown of complex plant carbohydrates and are dominant under healthy conditions [63]. However, Proteobacteria include opportunistic pathogens such as *Escherichia*, *Salmonella*, and *Helicobacter*, and are associated with intestinal inflammation or dysbiosis [64,65]. In pigs fed CON, *Helicobacter apri* and *Escherichia fergusonii* were identified by LEfSe analysis, both of which are clinically pathogenic. On the other hand, *Lactobacillus plantarum*, *Lactobacillus ultunensis*, *Streptococcus hyointestinalis*, and *Turicibacter sanguinis* were characterized in the PRO group. *Streptococcus hyiointestinalis* produces bacteriocins and exhibits broad antimicrobial activity against gram-positive bacteria [66,67]. In addition, intestinal *Turicibacter sanguinis* play important roles in intestinal lipid and steroid metabolism [68,69] and is involved in serotonin production [70]. These findings revealed the lower abundances of *Escherichia* and *Helicobacter* and their belonged phylum

Proteobacteria in the jejunum of PRO pigs than CON pigs, indicating inhibitory effects of dietary PRO on these opportunistic pathogens through gut enrichment of probiotic *L*. plantarum. Moreover, dietary PRO may help gut eubiosis by interacting with beneficial microbiota. Therefore, these fecal and jejunal microbial changes suggest that dietary PRO contributed to healthy and balanced alteration in the intestinal environment of weaned pigs, which promoted intestinal integrity and affected nutrient utilization of the host. Further studies evaluating the gut metabolites and functional profiles of probiotic *L. plantarum* would be helpful to determine the potential functionality of dietary PRO in pigs.

Conclusion

This study demonstrated that probiotic *L. plantarum* CJLP 243/475 supplementation in weaner diet enhanced the growth performance of weaned pigs. The potential mechanisms of beneficial effect involve improving intestinal morphology and intestinal integrity by upregulating expression of TJ protein genes, and modifying host-microbiota interaction by altering gut microbial communities. In addition, probiotic *L. plantarum* CJLP 243/475 alleviated systemic inflammation in early weaning caused by weaning stressors and modulated local immune responses by upregulating gene expression of inflammatory cytokines in the ileum. These results support our hypothesis that probiotic *L. plantarum* CJLP 243/475 could enhance intestinal health by modulating gut microbiota, thereby improving growth performance and modulating immunity system of weaned pigs. Further studies will consider evaluating the correlation between gut microbial changes and gut-immune health indicators to establish a potential mechanism of *L. plantarum* CJLP 243/475 on the health of pigs.

Funding

This work was supported by the High Value-Added Food Technology Development Program of the Korean Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET), the Ministry for Food, Agriculture, Forestry and Fisheries of the Republic of Korea

(321037-05-3-HD030) and the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (RS-2023-00271355).

Acknowledgements

We acknowledge supports from CJBIO, CJ CheilJedang, Suwon, South Korea.

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628 **Table 1.** Composition of basal diet for weaned pigs (as-fed basis)

Item	Basal diet
Ingredient, %	
Corn	52.19
Soybean meal, 44%	18.20
Soy protein concentrate	10.28
Whey powder	13.00
Soybean oil	0.50
Fish meal, combined	3.33
Limestone	1.13
Monocalcium phosphate	0.60
Vitamin-mineral premix[1]	0.30
Lysine-HCl	0.28
DL-Methionine	0.13
L-Threonine	0.06
Total	100.00
Calculated energy and nutrient contents[2]	
Dry matter, %	88.49
Metabolizable energy, kcal/kg	3,400
Crude protein, %	22.99
SID Lysine, %	1.38
SID Methionine, %	0.46
SID Methionine + cysteine, %	0.76
SID Threonine, %	0.81
SID Tryptophan, %	0.24
Calcium, %	0.86
Phosphorous, %	0.65
STTD Phosphorous, %	0.43
ATTD Phosphorous, %	0.26
Analyzed energy and nutrient contents[3]	
Dry matter, %	88.32
Gross energy, kcal/kg	3,997
Crude protein, %	21.55
Lysine, %	1.23
Methionine, %	0.44
Methionine + cysteine, %	0.87
Threonine, %	0.84
Tryptophan, %	0.18
Calcium, %	0.84
Phosphorous, %	0.77

629 SID, standardized ileal digestible; STTD, standardized total tract digestible; ATTD, apparent
630 total tract digestible.

631 [1]Provided per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 2,500 IU; vitamin E, 30 IU;
632 vitamin K3, 3 mg; $_D$-pantothenic acid, 15 mg; nicotinic acid, 40 mg; choline, 400 mg; and vitamin B_{12},
633 12 μg; Fe, 90 mg from iron sulfate; Cu, 8.8 mg from copper sulfate; Zn, 100 mg from zinc oxide; Mn,
634 54 mg from manganese oxide; I, 0.35 mg from potassium iodide; Se, 0.30 mg from sodium selenite.

635 [2]The values were calculated based on National Research Council [21].

636 [3]The values were analyzed according to the Association of Official Agriculture Chemists [22].

Table 2. Gene specific primer sequences for gene expression of tight junction-related proteins and inflammatory cytokines in the ileum

Item	Forward (5'-3')	Reverse (5'-3')
CLDN1	AGAAGATGCGGATGGCTGTC	CCCAGAAGGCAGAGAGAAGC
CLDN2	TCCCTCCCTGTTCTCCCTGATAG	CCTTGCAGTGGGCAGGAA
CLDN3	GATGCAGTGCAAAGTGTACGA	GTCCTGCACGCAGTTGGT
CLDN4	TATCATCCTGGCCGTGCTA	CATCATCCACGCAGTTGGT
OCLN	GGAGTGATTCGGATTCTGTCTATGCT	CGCCTGGGCTGTTGGGTTGA
MUC1	CCCTGGCCATCATCTATGTC	TGCCCACAGTTCTTTCGTC
INF-γ	GAGCCAAATTGTCTCCTTCTAC	CGAAGTCATTCAGTTTCCCAG
TNF-α	CTTGGGTTTGGATTCCTGGAT	CTTCCCTGGCAGCCACAT
IL-1β	GCCCTGTACCCCAACTGGTA	CCCAGGAAGACGGGCTTT
IL-6	GCGCAGCCTTGAGGATTTC	CCCAGCTACATTATCCGAATGG
MCP1	TCCCACACCGAAGCTTGAAT	CACAGGAGGGCTGCAGAGA
18s rRNA	GGCTACCACATCCAAGGAAG	TCCAATGGATCCTCGCGGAA

CLDN1, claudin-1; *CLDN2*, claudin-2; *CLDN3*, claudin-3; *CLDN4*, claudin-4; *OCLN*, occludin; *MUC1*, mucin-1; *INF-γ*, interferon-gamma; *TNF-α*, tumor necrosis factor-alpha; *IL-1β*, interleukin-1beta; *IL-6*, interleukin-6; *MCP1*, monocyte chemoattractant-1.

640 **Table 3.** Effects of probiotic *L. plantarum* on growth performance of weaned pigs[1]

Item	CON	PRO	SEM	*p*-value
day 1 to 7				
Initial BW, kg	6.90	6.96	0.06	0.528
Final BW, kg	7.89	8.29	0.19	0.130
ADG, g/d	141.43	190.00	25.03	0.165
ADFI, g/d	244.07	278.50	23.42	0.305
G:F, g/g	0.579	0.682	0.055	0.125
day 8 to 14				
Initial BW, kg	7.89	8.29	0.19	0.130
Final BW, kg	10.45	11.44	0.33	0.040
ADG, g/d	365.71	450.00	27.18	0.024
ADFI, g/d	475.57	563.16	22.46	0.009
G:F, g/g	0.769	0.799	0.054	0.680
day 1 to 14				
Initial BW, kg	6.90	6.96	0.06	0.528
Final BW, kg	10.45	11.44	0.33	0.040
ADG, g/d	253.57	320.00	23.05	0.051
ADFI, g/d	359.82	416.58	19.31	0.045
G:F, g/g	0.705	0.768	0.066	0.373
day 15 to 21				
Initial BW, kg	10.45	11.44	0.33	0.040
Final BW, kg	14.38	15.74	0.36	0.012
ADG, g/d	561.43	614.29	24.21	0.125
ADFI, g/d	718.29	744.21	26.51	0.494
G:F, g/g	0.782	0.825	0.029	0.324
day 1 to 21				
Initial BW, kg	6.90	6.96	0.06	0.528
Final BW, kg	14.38	15.74	0.36	0.012
ADG, g/d	356.19	418.10	16.77	0.013
ADFI, g/d	479.31	525.79	18.63	0.086
G:F, g/g	0.743	0.795	0.015	0.016
day 22 to 35				
Initial BW, kg	14.38	15.74	0.36	0.012
Final BW, kg	25.24	27.54	0.61	0.011
ADG, g/d	775.71	842.86	24.13	0.057
ADFI, g/d	1257.54	1173.68	44.63	0.192
G:F, g/g	0.617	0.718	0.018	< 0.001
day 1 to 35				
Initial BW, kg	6.90	6.96	0.06	0.528
Final BW, kg	25.24	27.54	0.61	0.011
ADG, g/d	524.00	588.00	17.30	0.013
ADFI, g/d	790.60	784.95	26.12	0.879
G:F, g/g	0.663	0.749	0.015	< 0.001
Frequency of diarrhea[2], %	22.67	18.95		0.373

641 CON, basal weaner diet; PRO, CON + 0.1% probiotic *L. plantarum*; SEM, standard error of
642 mean; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed
643 ratio.

644 [1]Each value is the mean value of 20 replicates (1 pig/pen).

645 [2]Frequency of diarrhea for the first 2 weeks after weaning (%) = (number of diarrhea score of
646 3 or higher / number of pen days) × 100.

647 **Table 4.** Effects of probiotic *L. plantarum* on nutrient digestibility of weaned pigs[1]

Item	CON	PRO	SEM	*p*-value
Apparent ileal digestibility, %				
Dry matter	70.44	77.14	2.71	0.155
Crude protein	73.05	77.90	1.00	0.026
Energy	75.09	76.36	0.52	0.163
Apparent total tract digestibility, %				
Dry matter	79.67	80.02	2.73	0.448
Crude protein	77.60	79.40	1.24	0.329
Energy	79.59	81.21	1.07	0.307

648 CON, basal weaner diet; PRO, CON + 0.1% probiotic *L. plantarum*; SEM, standard error of
649 mean.

650 [1]Each value is the mean value of 6 replicates (1 pig/pen).

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654 **Table 5.** Effects of probiotic *L. plantarum* on intestinal morphology of weaned pigs[1]

Item	CON	PRO	SEM	*p*-value
Duodenum				
Villus height, μm	269.43	300.00	23.89	0.387
Crypt depth, μm	229.00	195.81	9.83	0.038
VH:CD, μm/μm	1.18	1.56	0.13	0.074
Villus width, μm	62.45	63.83	4.75	0.842
Villus area, μm^2	13,519	14,408	1,217	0.617
Goblet cell, n	15.52	17.77	1.32	0.257
Jejunum				
Villus height, μm	235.45	264.47	17.54	0.269
Crypt depth, μm	180.52	169.40	21.07	0.717
VH:CD, μm/μm	1.36	1.68	0.18	0.249
Villus width, μm	78.22	87.85	5.08	0.210
Villus area, μm^2	14,496	18,252	1,275	0.064
Goblet cell, n	15.62	15.51	1.04	0.943
Ileum				
Villus height, μm	262.43	276.53	10.83	0.379
Crypt depth, μm	220.76	234.15	11.00	0.410
VH:CD, μm/μm	1.20	1.19	0.05	0.832
Villus width, μm	63.46	62.74	3.60	0.890
Villus area, μm^2	14,166	14,436	1,148	0.871
Goblet cell, n	10.70	14.47	0.93	0.017

655 CON, basal weaner diet; PRO, CON + 0.1% probiotic *L. plantarum*; SEM, standard error of
656 mean; VH:CD, villus height to crypt depth ratio

657 [1]Each value is the mean value of 6 replicates (1 pig/pen).

658

659 **Table 6.** Effects of probiotic *L. plantarum* on blood profiles of weaned pigs[1]

Item	CON	PRO	SEM	*p*-value
WBC, $\times 10^3/\mu L$				
day 1	8.84	9.02	0.80	0.869
day 7	19.58	18.42	1.26	0.513
day 21	22.53	18.19	1.20	0.014
day 35	21.91	19.05	0.95	0.037
RBC, $\times 10^6/\mu L$				
day 1	4.96	4.92	0.25	0.908
day 7	6.46	6.02	0.25	0.226
day 21	5.94	5.77	0.27	0.661
day 35	6.49	6.70	0.10	0.059
HCT, %				
day 1	34.66	34.72	1.78	0.982
day 7	40.28	40.09	1.25	0.918
day 21	37.37	36.80	1.76	0.820
day 35	41.32	40.68	0.99	0.652

660 CON, basal weaner diet; PRO, CON + 0.1% probiotic *L. plantarum*; SEM, standard error of
661 mean; WBC, white blood cells; RBC, red blood cells; HCT, hematocrit

662 [1]Each value is the mean value of 6 replicates (1 pig/pen).

663 **Table 7.** Effects of probiotic *L. plantarum* on immune responses of weaned pigs[1]

Item	CON	PRO	SEM	*p*-value
Cortisol, ng/mL				
day 1	8.60	7.92	0.51	0.372
day 7	9.73	7.13	0.29	< 0.001
day 21	5.72	4.69	1.04	0.498
TNF-α, pg/mL				
day 1	95.69	87.59	9.35	0.554
day 7	101.69	97.08	13.97	0.820
day 21	86.14	57.53	11.06	0.097
TGF-β1, pg/mL				
day 1	758.21	757.39	87.31	0.995
day 7	397.12	372.24	60.56	0.777
day 21	541.49	480.88	57.36	0.472
IL-1β, pg/mL				
day 1	250.20	243.83	5.63	0.443
day 7	241.69	244.57	4.13	0.633
day 21	227.51	239.98	6.81	0.225
IL-6, pg/mL				
day 1	26.30	29.69	1.69	0.186
day 7	27.38	31.83	4.15	0.465
day 21	24.18	30.07	1.39	0.013

664 CON, basal weaner diet; PRO, CON + 0.1% probiotic *L. plantarum*; SEM, standard error of
665 mean; TNF-α, tumor necrosis factor-alpha; TGF-β1, transforming growth factor-beta1; IL-1β,
666 interleukin-1beta; IL-6, interleukin-6

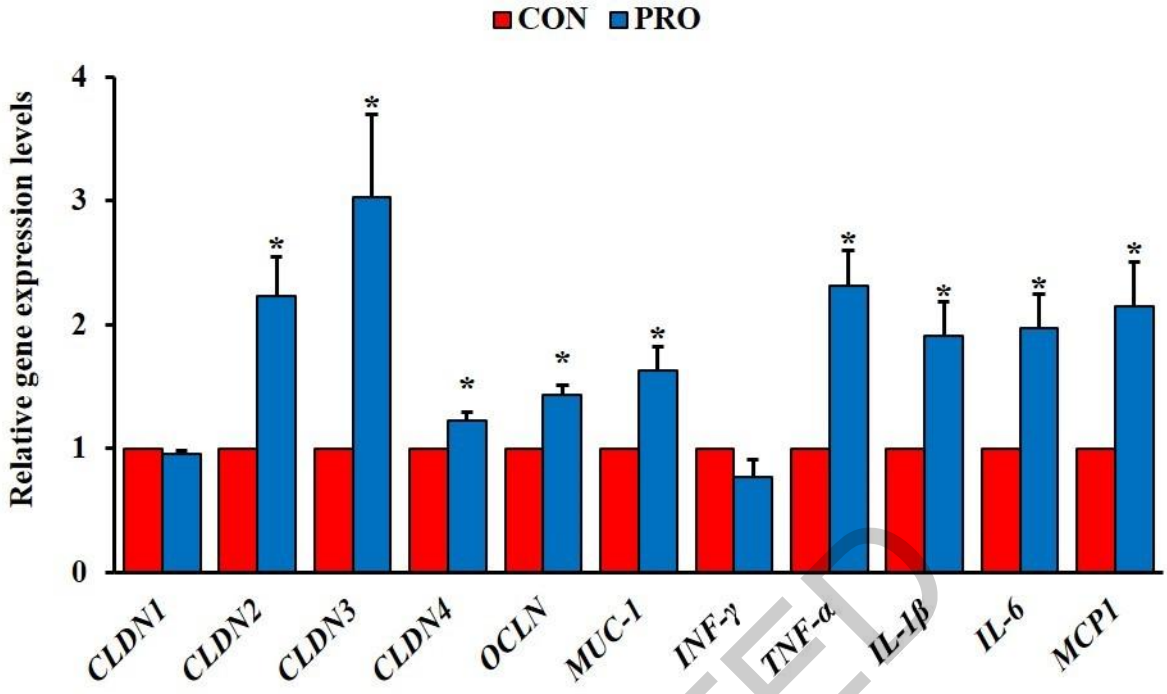
667 [1]Each value is the mean value of 6 replicates (1 pig/pen).

668 **Table 8.** Effects of probiotics *L. plantarum* on bacterial alpha diversity of weaned pigs[1]

Item	CON	PRO	*p*-value
Fecal microbiota			
day 1			
Observed OTUs	220.67 ± 46.52	194.33 ± 43.70	0.262
Chao1	259.92 ± 47.36	236.72 ± 55.17	0.423
Shannon	2.74 ± 0.54	2.49 ± 0.67	0.631
Simpson	0.852 ± 0.059	0.816 ± 0.100	0.631
day 7			
Observed OTUs	202.33 ± 69.52	207.83 ± 65.30	0.891
Chao1	230.40 ± 78.49	245.33 ± 76.23	0.749
Shannon	2.70 ± 0.30	2.74 ± 0.51	0.870
Simpson	0.869 ± 0.033	0.877 ± 0.051	0.749
day 21			
Observed OTUs	200.00 ± 30.99	214.00 ± 38.49	0.873
Chao1	236.54 ± 38.22	257.75 ± 56.08	0.522
Shannon	2.76 ± 0.24	2.69 ± 0.23	0.470
Simpson	0.880 ± 0.036	0.870 ± 0.037	0.688
Jejunal microbiota, day 35			
Observed OTUs	41.67 ± 11.72	51.67 ± 27.15	0.630
Chao1	43.67 ± 10.60	52.97 ± 27.42	0.631
Shannon	2.06 ± 0.18	1.75 ± 0.22	0.037
Simpson	0.825 ± 0.027	0.702 ± 0.083	0.010

669 CON, basal weaner diet; PRO, CON + 0.1 probiotic *L. plantarum*; OTUs, operational
670 taxonomic units

671 [1]Each value is the mean of 6 replicates (1 pig/pen) and presented as mean ± SD.



672

673 **Fig. 1.** Expression of tight junction-related protein and inflammatory cytokine genes in ileal
674 tissue of weaned pigs. Each value is the mean of 6 replicates (1 pig/pen). *Different between
675 dietary treatments ($P < 0.05$). CON, a basal weaner diet; PRO, CON + 0.1% probiotic *L.*
676 *plantarum*; *CLND1*, claudin-1; *CLDN2*, claudin-2; *CLDN3*, claudin-3; *CLDN4*, claudin-4;
677 *OCLN*, occludin; *MUC1*, mucin-1; *INF-γ*, interferon-gamma; *TNF-α*, tumor necrosis factor-α;
678 *IL-1β*, interleukin-1β; *IL-6*, interleukin-6; *MCP1*, monocyte chemoattractant protein-1.

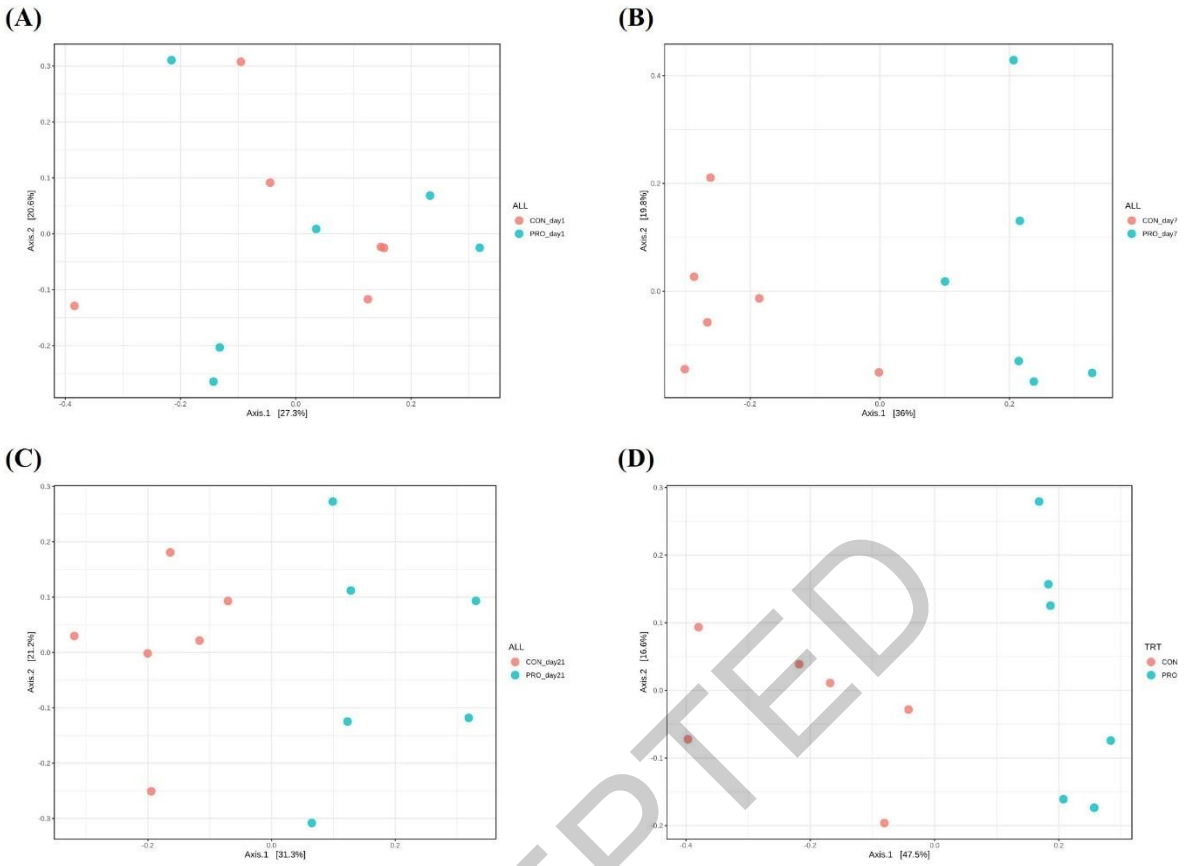


Fig. 2. Principal coordinates analysis (PCoA) based on Bray–Curtis distance of bacterial communities of weaned pigs (n = 6). Permutational multivariate analysis of variance (PERMANOVA) was used for statistical significance of clustering distances. Beta diversity analysis were represented for fecal bacteria (A) at d 1 (r^2 = 0.05; P = 0.981), (B) d 7 (r^2 = 0.32; P = 0.003), (C) d 21 (r^2 = 0.26; P = 0.003), and (D) for jejunal bacteria d 35 (r^2 = 0.41; P = 0.003) between dietary treatments. CON, basal weaner diet; PRO, CON + 0.1% probiotic *L. plantarum*.

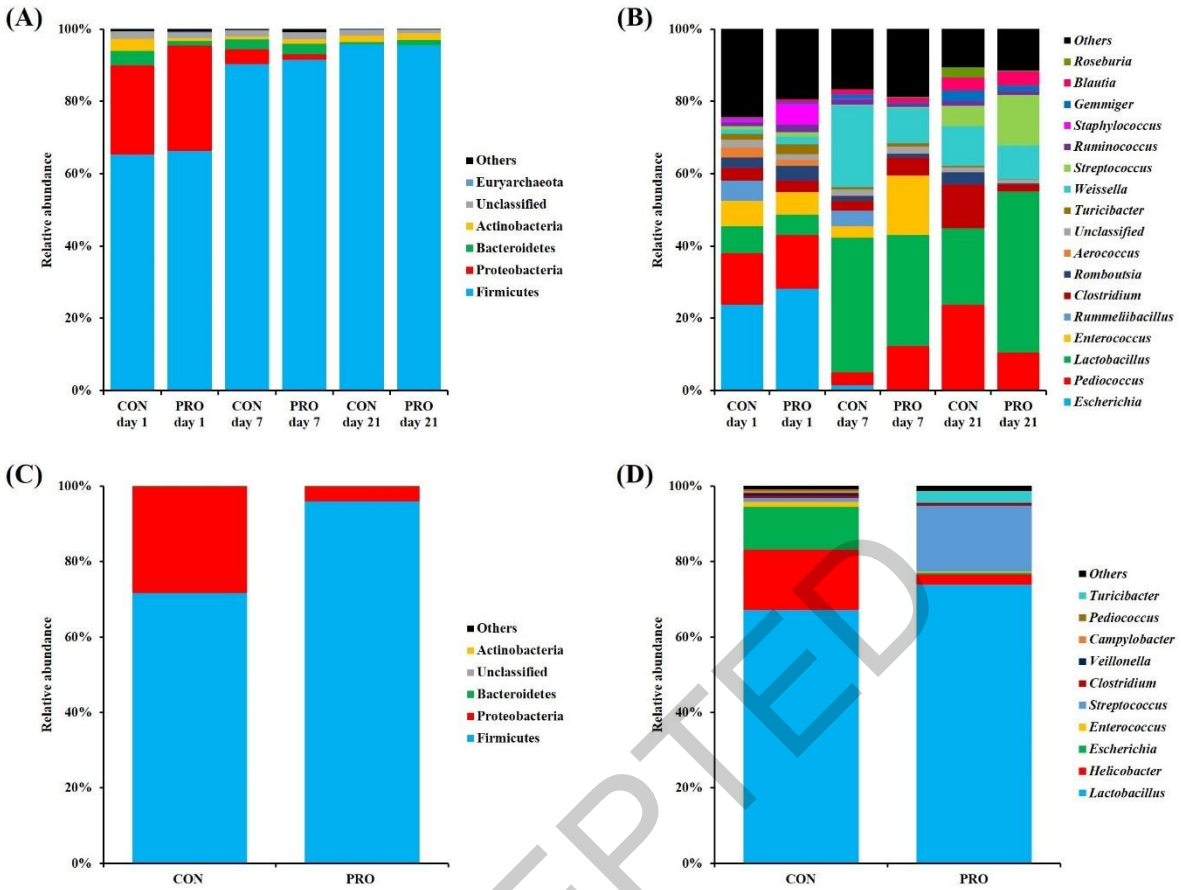
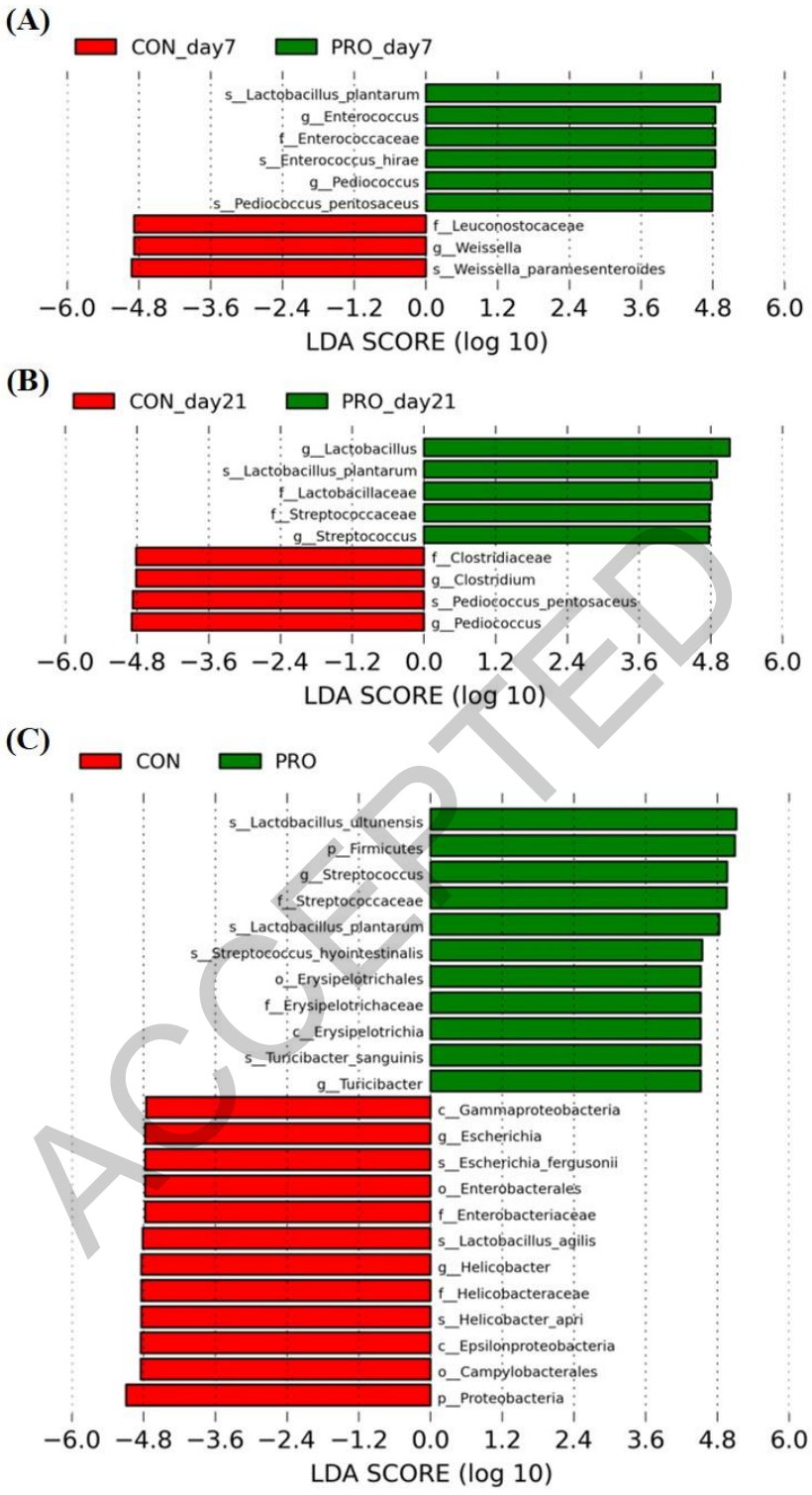


Fig. 3. Taxonomic relative abundance of the gut microbiota (A, C) at the phylum level and (B, D) genus level between dietary treatments: (A, B) fecal microbiota at d 1, 7, 21 and (C, D) jejunal microbiota at d 35. The proportions for the top five and ten bacteria are presented at the phylum level and genus level in each time period, respectively. CON, basal weaner diet; PRO, CON + 0.1% probiotic *L. plantarum*.



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694 **Fig. 4.** Taxonomic biomarker identification using linear discriminant analysis effect size (LEfSe)
695 in histogram of weaned pigs between dietary treatments: fecal microbiota (A) at d 7, (B) d 21, and
696 (C) for jejunal microbiota at d 35. CON, basal weaner diet; PRO, CON + 0.1% probiotic *L.*
697 *plantarum*.