Effects of dietary protease supplementation on growth rate, nutrient digestibility, and intestinal morphology of weaned pigs

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Abstract
The addition of dietary proteases (PRO) to weaner diets hydrolyzes soybean-based anti-nutritive factors and improves weaned pig’s dietary digestibility and growth performance. Therefore, this study explores the effects of PRO in a lower crude protein (CP) level diet than that in a commercial diet on the growth performance, nutrient digestibility, and intestinal morphology of weaned pigs. A total of 90 weaned pigs were randomly assigned to 3 dietary treatments with 6 pigs per pen and 5 replicated pens per treatment using a randomized complete block design (block = body weight [BW]): 1) a commercial weaner diet as a positive control (PC; phase1 CP = 23.71%; phase2 CP: 22.36%), 2) lower CP diet than PC as a negative control (NC; 0.61% less CP than PC), and 3) an NC diet with 0.02% PRO. Pigs fed PC and PRO had higher (p < 0.05) final BW, average daily gain, and/or gain to feed ratio for the first three weeks and the overall experimental period than NC. The PC and PRO groups had greater (p < 0.05) apparent ileal digestibility of dry matter, CP, and energy than the NC group. Moreover, pigs fed PC and PRO increased (p < 0.05) apparent total tract digestibility of CP compared with those fed NC. In addition, the PRO group had a higher number of goblet cells than the PC and NC groups. However, pig fed PC and PRO increased (p < 0.05) villus height and height to crypt depth ratio in the ileum compared with those fed NC. In conclusion, PRO supplementation in a commercial weaner diet with low CP levels improves growth rate and nutrient digestibility by modulating the intestinal morphology of weaned pigs.

Keywords: Commercial weaner diet, Dietary protease, Growth rate, Intestinal morphology, Nutrient digestibility, weaned pigs

INTRODUCTION
The swine industry has been facing the problem of increasing the cost of feeds, especially the cost of
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Competition interests
No potential conflict of interest relevant to this article was reported.

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors’ contributions
Conceptualization: Song M, Kim B, Cho JH, Kim HB, Lee JJ.
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Ethics approval and consent to participate
The animal experiment protocol for this study was approved by the Institutional Animal Care and Use Committee of the Chungnam National University, Daejeon, Korea (approval# 201909A-CNU-00611).

MATERIALS AND METHODS

The animal experiment protocol for this study was approved by the Institutional Animal Care and Use Committee of the Chungnam National University, Daejeon, Korea (approval# 201909A-CNU-00611).

Animals, diets, and study design
Ninety weaned pigs (Duroc × [Landrace × Yorkshire]; aged 28 days) with an average body weight (BW) of 6.96 ± 0.06 kg were randomly assigned to three dietary treatments with five replicates of six pigs per pen using a randomized complete block design (block = BW). The dietary treatments were as follows: 1) a commercial weaner diet to meet or exceed the requirement of crude protein (CP) as a positive control (PC; phase1 CP = 23.71%; phase2 CP: 22.36%), 2) lower CP diet than PC as a negative control (NC; 0.61% less CP than PC), and 3) an NC diet supplemented with 0.02% dietary PRO. The PRO contained 75,000 PRO units/g, which were extracted from Nocardopsis prasina produced in Bacillus licheniformis, and was a commercial product (Ronozyme® ProAct, DSM nutrition products, Kaiseraugst, Switzerland). The formulated diets met the nutritional requirements for weaned pigs based on the National Research Council [12] (Table 1). The trial period lasted 42 days using a 2-phase feeding program with each phase of three weeks. All pigs had ad libitum access to feed and water throughout the entire period. During the final week of experiment, 0.2% chromic oxide, an indicator of indigestion, was added to all dietary treatments.
Sample collection and preparation for analysis

The weight of each pig and pen was recorded on days 1, 7, 14, 21, and 42 to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F) for the growth performance of weaned pigs. Fecal samples from one randomly selected pig per pen were collected daily for day 3 using the rectal massage method in the final week after the day 4 adaptation period. Each dietary treatment and fecal sample was stored at –80°C for analyzing apparent total tract digestibility (ATTD) of nutrients. On the last day (day 42) of the experiment, one pig randomly
selected from each pen (five pigs from each treatment) was anesthetized by an intramuscular injection of xylazine (20 mg per 20 kg of BW; ES Inc., Korea) and euthanized by CO\textsubscript{2} gas [5]. Ileal digesta were collected and stored at −20°C for analyzing apparent ileal digestibility (AID) of nutrients. A 3-cm ileal segment was collected and washed with distilled water, and then the samples were prepared for morphological analysis following the method of previous research [13].

**Chemical analysis**
Stored samples (diet, ileal digesta, and feces) were thawed and dried in a forced-air drying oven at 60°C, and then finely ground using a coffee grinder before chemical analysis. The dried samples were analyzed for dry matter (DM), gross energy (GE) by bomb calorimetry (Model C2000, IKA®, Staufen, Germany), and CP using the Kjeldahl method. The Cr concentrations of diets, ileal digesta and fecal samples were determined using graphite furnace atomic absorption spectrometry (Hitachi Z-5000 Absorption Spectrophotometer, Hitachi High-Technologies, Tokyo, Japan). The procedures for DM and CP analyses were based on the methods of AOAC International [14]. The AID and ATTD of DM, GE, and CP were calculated for each dietary treatment based on a previous report [15].

**Intestinal morphology analysis**
The measurements of intestinal morphology included villus height (VH), villus width, villus area, crypt depth (CD), VH to CD ratio (VH:CD), and the number of goblet cells, and were conducted as described previously [5]. The ileal tissue samples were immersed in paraffin, mounted on glass slides (5-μm thickness), and stained with hematoxylin and eosin. The stained samples were scanned using a light microscope (Eclipse TE2000, Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (DS-Fi1, Nikon), and all measurements were conducted using NIS-Elements BR software 3.00 (Nikon).

**Statistical analysis**
Data were analyzed using the General Linear Model Procedure of SAS (Version 9.4, 2013, SAS, Cary, NC, USA) in a randomized complete block design with the initial BW as a block. The pen was the experimental unit. The statistical model for growth performance, AID and ATTD, intestinal morphology, and number of goblet cells included the effects of dietary treatments as a fixed effect. Statistical significance and tendency were considered at $p < 0.05$ and $0.05 \leq p < 0.10$, respectively.

**RESULTS**

**Growth performance**
Pigs fed PC and PRO diets increased ($p < 0.05$) final BW, ADG, and G:F from d 1 to 21 compared with the NC diet (Table 2). Moreover, PC and PRO had higher ($p < 0.05$) final BW and ADG during the overall experimental period than NC. However, no differences in the growth performance of weaned pigs were found over the overall experimental period between PC and PRO treatments.

**Nutrient digestibility**
The AID of DM, CP, and energy was greater ($p < 0.05$) in the PC and PRO groups than in the NC group (Table 3). Moreover, pigs fed PC and PRO increased ($p < 0.05$) ATTD of CP compared with those fed NC. However, the PRO diet did not differ in nutrient digestibility from the PC diet.
Goblet cell number and intestinal morphology

The number of goblet cells in the pigs fed PRO significantly exceeded ($p < 0.05$) that of those fed PC and NC (Table 4). Furthermore, pigs fed with PC and PRO increased ($p < 0.05$) VH and VH:CD in the ileum compared with those fed with NC. In contrast, no difference was observed in ileal morphology between the PC and PRO treatments.
DISCUSSION

After weaning, piglets suffer from several stresses due to physiological, environmental, and immunological changes [16,17]. In particular, the immediate transition of feed from liquid milk to solid diet decreases feed intake, and nutrient digestibility and thus compromises growth performance [18]. This occurs because during this period, the activity of endogenous enzymes is not yet established to digest plant nutrients (i.e., solid diet) [19]. Furthermore, the solid diet may cause cell loss by friction, and feed antigen can induce the inflammation and alteration of VH, which is highly associated with nutrient digestibility [18–20].

Weaning pigs may not well digest the protein from SBM for various reasons. The most common reason is that the digestive system is not completely developed and the activity of digestive enzymes is low during the weaning period [7,21,22]. The exogenous PRO has been investigated for its positive effect on the digestibility of dietary protein in a corn-SBM based diet in the weaning but not in the growing-finishing period [5,7,23]. This study showed that PRO supplementation improved nutrient digestibility and the growth performance of weaned pigs. This result agrees with previous research that adding exogenous enzymes is more effective in piglets weighing < 20 kg [23], and previous research has also reported an improvement in nutrient utilization efficiency using PRO as a stand-alone enzyme [5,7]. Another problem during the weaning period is the increased resistance of the disulfide linkage of soy protein to digestion [6, 24]. Intestinal maturity is closely related to nutrient digestibility and the growth performance of piglets [3], and among other parameters, well-developed VH and CD can contribute to high feed intake of weaned pigs, which can have positive effects on growth performance [18]. Studies have also reported that plant protein sources impair intestinal morphology and PRO supplementation attenuates the morphological damage, due to increased degradation of ANFs [5,7,8]. In this study, increased digestibility of nutrients by PRO supplementation induced increased growth performance, which is believed to be closely associated with intestinal development and improvement of diarrhea. Moreover, improved protein digestion and absorption, especially AID of CP, reduces the flow of undigested proteins into the large intestine, thereby preventing the proliferation of pathogenic microbes and their harmful metabolites [25]. This study’s results agree with those of some previous studies conducted using proteolytic enzymes as an exogenous enzyme [5,23,26].

In the intestine, goblet cells secrete mucus that form a mucus layer, which serves as a barrier function to prevent the antigens from attachment to the intestinal epithelium [19]. Therefore, the
thickness of this mucus layer and the number of goblet cells are essential for preventing pathogen invasion. In this study, the number of goblet cells was increased by PRO supplementation, which might be due to an improved intestinal morphology. In the intestine, metabolites or toxins from bacteria, as well as the feed antigen in the SBM, can cause inflammation [27]; this inflammation is also accompanied by damage to epithelial cells and a decrease in growth efficiency [18,28]. However, PRO addition prevented the inflammation of epithelial cells by degrading the feed antigen in SBM and preventing enteropathogen proliferation, which may be the reason for the increased number of goblet cells [5,9,19].

CONCLUSION

This study suggests that the addition of dietary PRO in a lower CP diet improves growth performance and nutrient digestibility of weaned pigs as much as a commercial weaner diet by modulating the intestinal morphology.

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