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# 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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**Competing interests**
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Not applicable.

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

**Authors' contributions**
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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).

ingredients used as a protein source such as soybean meal (SBM). Such increasing costs jeopardize this industry's profit [1]. Plant-based protein sources have a lower digestibility than animal-based protein sources and increase the flow of undigested proteins to the large intestine [2]. Thus, feed passes without appropriate digestion through the intestine, which is an inevitable event during the weaning period when the digestive tract has not yet matured, causing diarrhea and damaging the intestinal epithelial cells [3,4].

Several studies have reported that exogenous protease (PRO) improved the diet digestibility and growth performance of pigs during the early post-weaning period, in which there is a low ability for digestion of nutrients, and this improvement was caused because it complements their immature digestive system [5–7]. Moreover, the dietary PRO addition hydrolyzes soybean's anti-nutrient factors (ANFs) such as trypsin inhibitor, glycinin, and β-conglycinin that induce a decrease in intestinal epithelial integrity by damaging to the intestinal mucosa [4,8,9]. In other words, the attenuation of the intestinal damage can save energy and nutrients, and these surpluses may be distributed for the growth performance of weaned pigs [10]. In addition, exogenous proteolytic enzymes increase protein utilization in the small intestine [7,11]. In our previous studies using an experimental weaner diet, exogenous PRO supplementation with low protein feed showed improved immune response without compromising growth performance [5,9,11]. Many studies related to the dietary PRO based on sufficient protein formulation of the experimental diets conducted the positive effects of the results, but studies on commercial diets that use the minimum protein requirement of weaner diets are insufficient. Thus, PRO supplementation in low protein commercial weaner diets was hypothesized to alter the intestinal morphology with potential gut health mechanisms of weaned pigs. Therefore, this study validates the beneficial effects of PRO supplementation in a commercial weaner diet with a reduced protein source on intestinal morphology for improving growth performance and nutrient digestibility of weaned pigs.

# 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 *Nocardiopsis 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.

**Table 1. Composition of the commercial diets for weaned pigs (as-fed basis)[1)]**

| Items | Phase 1 | | Phase 2 | |
|---|---|---|---|---|
| | PC | NC | PC | NC |
| Ingredients (%) | 100.00 | 100.00 | 100.00 | 100.00 |
| Corn | 45.00 | 47.00 | 50.86 | 52.58 |
| Soybean meal (44%) | 18.00 | 16.00 | 27.00 | 25.28 |
| Dried whey | 15.00 | 15.00 | 10.00 | 10.00 |
| Soy protein concentrate | - | - | - | - |
| Fish meal | 11.46 | 11.46 | 8.00 | 8.00 |
| Spray-dried plasma | 4.00 | 4.00 | - | - |
| Lactose | 3.00 | 3.00 | - | - |
| Soybean oil | 3.00 | 3.00 | 3.00 | 3.00 |
| Limestone | 0.50 | 0.50 | 0.60 | 0.60 |
| Monocalcium phosphate | - | - | 0.40 | 0.40 |
| Vit-Min premix[2)] | 0.04 | 0.04 | 0.04 | 0.04 |
| L-Lysine-HCl | - | - | 0.10 | 0.10 |
| DL-Methionine | - | - | - | - |
| L-Threonine | - | - | - | - |
| Calculated nutrient compositions | | | | |
| Metabolizable energy (Mcal/kg) | 3.54 | 3.54 | 3.49 | 3.49 |
| Crude protein (%) | 23.71 | 23.10 | 22.36 | 21.75 |
| Calcium (%) | 0.85 | 0.85 | 0.82 | 0.82 |
| Phosphorus (%) | 0.72 | 0.72 | 0.68 | 0.68 |
| Lysine (%) | 1.54 | 1.50 | 1.40 | 1.36 |
| Methionine (%) | 0.44 | 0.43 | 0.41 | 0.40 |
| Threonine (%) | 1.02 | 0.97 | 0.90 | 0.85 |
| Tryptophan (%) | 0.29 | 0.29 | 0.26 | 0.26 |
| Cysteine (%) | 0.42 | - | 0.35 | - |
| Arginine (%) | 1.39 | 1.36 | 1.38 | 1.35 |
| Histidine (%) | 0.63 | 0.62 | 0.60 | 0.59 |
| Isoleucine (%) | 0.98 | 0.96 | 0.94 | 0.92 |
| Leucine (%) | 2.02 | 1.99 | 1.88 | 1.85 |
| Phenylalanine (%) | 1.09 | - | 1.04 | - |
| Valine (%) | 1.17 | 1.14 | 1.02 | 0.99 |

[1)] Phase 1: week 1 to 3 (21 days); phase 2: week 4 to 6 (21 days);

[2)] Provided per kilogram of diet: vitamin A, 12,000 IU; vitamin $D_3$, 2,500 IU; vitamin E, 30 IU; vitamin $K_3$, 3 mg; D-pantothenic acid, 15 mg; nicotinic acid, 40 mg; choline, 400 mg; and vitamin $B_{12}$, 12 μg; Fe, 90 mg from iron sulfate; Cu, 8.8 mg from copper sulfate; Zn, 100 mg from zinc oxide; Mn, 54 mg from manganese oxide; I, 0.35 mg from potassium iodide; Se, 0.30 mg from sodium selenite.

PC, positive control; NC, negative control.

## 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℃ for analyzing apparent total tract digestibility (ATTD) of nutrients. On the last day (day 42) of the experiment, one pig randomly

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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 $CO_2$ gas [5]. Ileal digesta were collected and stored at −20℃ 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℃, 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 $0.05 \le p < 0.10$, 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.

**Table 2. Growth performance of weaned pigs fed diets with positive control (PC), negative control (NC) and NC + 0.02% dietary protease (PRO) supplementation diets[1)]**

| Items | Dietary treatment[2)] | | | SEM | *p*-value |
|---|---|---|---|---|---|
| | PC | NC | PRO | | Diet |
| Day 1 to 21 | | | | | |
| Initial BW (kg) | 7.00 | 6.99 | 6.90 | 0.068 | 0.512 |
| Final BW (kg) | 17.75[a] | 16.00[b] | 17.88[a] | 0.282 | < 0.05 |
| ADG (g/d) | 511.90[a] | 428.94[b] | 522.83[a] | 13.66 | < 0.05 |
| ADFI (g/d) | 649.71 | 643.38 | 644.66 | 30.03 | 0.988 |
| G:F (g/g) | 0.796[a] | 0.660[b] | 0.811[a] | 0.027 | < 0.05 |
| Day 22 to 42 | | | | | |
| Initial BW (kg) | 17.75[a] | 16.00[b] | 17.88[a] | 0.282 | < 0.05 |
| Final BW (kg) | 32.50[a] | 30.26[b] | 32.95[a] | 0.504 | < 0.05 |
| ADG (g/d) | 702.40 | 679.02 | 717.84 | 23.14 | 0.510 |
| ADFI (g/d) | 1,200.68 | 1,201.81 | 1,224.98 | 55.01 | 0.941 |
| G:F (g/g) | 0.585 | 0.565 | 0.586 | 0.030 | 0.870 |
| Day 1 to 42 | | | | | |
| Initial BW (kg) | 7.00 | 6.99 | 6.90 | 0.068 | 0.512 |
| Final BW (kg) | 32.50[a] | 30.26[b] | 32.95[a] | 0.504 | < 0.05 |
| ADG (g/d) | 607.15[a] | 553.98[b] | 620.33[a] | 11.69 | < 0.05 |
| ADFI (g/d) | 921.84 | 925.62 | 934.66 | 34.08 | 0.963 |
| G:F (g/g) | 0.659 | 0.598 | 0.664 | 0.027 | 0.206 |

[1)]Each value is the mean of 5 replicates (6 pigs per pen).

[2)]PC, CP = 23.71% (phase1) and 22.36% (phase2); NC, 0.61% less CP than PC.

[a,b]Means with different letters represent statistical significance ($p < 0.05$).

BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio.

**Table 3. Apparent ileal digestibility (AID) and apparent total tract digestibility (ATTD) of weaned pigs fed diets with positive control (PC), negative control (NC), and NC + 0.02% dietary protease (PRO) supplementation diets[1)]**

| Items | Dietary treatment[2)] | | | SEM | *p*-value |
|---|---|---|---|---|---|
| | PC | NC | PRO | | Diet |
| AID | | | | | |
| Dry matter (%) | 79.64[a] | 75.82[b] | 79.18[a] | 0.45 | < 0.05 |
| Crude protein (%) | 75.17[a] | 72.37[b] | 76.28[a] | 0.36 | < 0.05 |
| Energy (%) | 80.89[a] | 76.89[b] | 79.65[a] | 0.43 | < 0.05 |
| ATTD | | | | | |
| Dry matter (%) | 86.48 | 85.89 | 86.52 | 0.46 | 0.565 |
| Crude protein (%) | 83.79[a] | 82.72[b] | 84.11[a] | 0.19 | < 0.05 |
| Energy (%) | 88.51 | 87.28 | 88.26 | 0.49 | 0.215 |

[1)]Each value is the mean of 5 replicates (6 pigs per pen).

[2)]PC, CP = 22.36% (phase2); NC, 0.61% less CP than PC.

[a,b]Means with different letters represent statistical significance ($p < 0.05$).

## 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.

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**Table 4.** Ileal morphology of weaned pigs fed diets with positive control (PC), negative control (NC), and NC + 0.02% dietary protease (PRO) supplementation diets[1)]

| Items | Dietary treatment[2)] | | | SEM | *p*-value |
|---|---|---|---|---|---|
| | PC | NC | PRO | | Diet |
| Goblet cells (n) | 11.27[a] | 10.84[a] | 15.77[b] | 1.08 | < 0.05 |
| Villus height (μm) | 294.89[a] | 233.79[b] | 322.95[a] | 19.27 | < 0.05 |
| Crypt depth (μm) | 88.25 | 93.88 | 92.40 | 6.32 | 0.811 |
| VH:CD (μm/μm) | 3.36[a] | 2.51[b] | 3.50[a] | 0.18 | < 0.05 |
| Villus width (μm) | 111.40 | 112.38 | 104.66 | 6.89 | 0.697 |
| Villus area (μm$^2$) | 23,800 | 21,977 | 22,939 | 1,872 | 0.792 |

[1)]Each value is the mean of 5 replicates (6 pigs per pen).

[2)]PC, CP = 23.71% (phase1) and 22.36% (phase2); NC, 0.61% less CP than PC.

[a,b]Means with different letters represent statistical significance ($p < 0.05$).

VH:CD, villus height to crypt depth ratio.

## 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 mucins 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 enteropahogen 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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