Effects of dietary supplementation with Bacillus licheniformis derived-protease on growth performance, nutrient digestibility and fecal microbial shedding in post-weaned growing pigs

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ABSTRACT
An experiment was conducted to evaluate the effect of Bacillus licheniformis derived-protease supplementation on growth performance, nutrient digestibility, and fecal microbial shedding in post-weaned and growing pigs. A total of 90 crossbred (Landrace × Yorkshire × Duroc) weaning pigs with an initial average body weight of 6.61 ± 1.73 kg were randomly allotted into 2 treatments with 9 replicate pens of 5 pigs for a 4-phase feeding trial. The treatments were: CON, basal diet; PROT, basal diet + 0.5 g/kg protease of diets (contained 75000 protease units per gram). During phase 3 (wk 3–6), average daily gain, and gain: feed ratio was increased (P < 0.05) in pigs fed PROT diet compared with those fed CON diet. During phase 3 and 4 (wk 6–11, growing phase), pigs fed PROT diet had higher (P < 0.05) apparent total tract digestibility (ATTD) of dry matter and crude protein than pigs fed CON diet. No differences were observed in fecal microflora between CON and PROT groups. In conclusion, dietary supplementation of Bacillus licheniformis derived-protease increased growth performance in post-weaned pigs and increased the digestibility of dry matter and crude protein in post-weaned and growing pigs.

Introduction
Weaning stress could decrease feed intake, impair digestive and absorptive capacity of piglets (Campbell et al. 2013; Moeser et al. 2017). Meanwhile, the high protein contained in weaning diets may cause a proliferation of pathogenic bacteria in the gastrointestinal tract, causing poor growth rate, higher feed costs, low feed conversion, and high incidence of colibacillosis (Boudry et al. 2004; Wijtten et al. 2011; Jha and Berrocoso 2016). Furthermore, the growth and protein deposition rate is increased dramatically during the growing phase, which requires numerous amounts of protein rich feed ingredients. The prices of these feed grains are dramatically increasing due to the numerous demands by its inclusion in many dominant feed ingredients and human consumption industries, which reduced the profit margins of pork production (Niemi et al. 2010; Stein et al. 2016). Therefore, the use of highly digestible ingredients is an essential component in a successful weaning and growing period.

Protease, as stand-alone enzyme or as a part of enzyme cocktails, has shown positive effects on growth performance and nutrient digestibility to pigs (Jo et al. 2012; Zuo et al. 2015; Tactacan et al. 2016). However, to date, most of the studies on protease have been generated from enzyme cocktails, no enough data about the supplementation of Bacillus licheniformis derived-protease in post-weaned and growing pigs. The B. licheniformis derived-protease is produced by submerged fermentation of B. licheniformis from Nocardiopsis prasina, and is claimed to benefits the performance of animals by hydrolyzing and solubilizing dietary proteins (Fru-Nji et al. 2011). Therefore, the objective of the current study was to examine effects of supplementation with B. licheniformis derived-protease on growth performance, apparent total tract digestibility (ATTD), and fecal microbial shedding in post-weaned and growing pigs.

Materials and methods
The experimental protocol of this study was subjected to approval by the Animal Care and Use Committee of Dankook University, South Korea.

The protease was obtained from a commercial company (Ronozyme ProAct; DSM Nutritional Products, Ltd., Heerlen, The Netherlands). It is produced by submerged fermentation of B. licheniformis containing transcribed genes from N. prasina. Protease activity is 75,000 protease units (PROT)/g. One PROT is defined as the amount of enzyme that releases 1 µmol of p-nitroaniline from 1 µmol/L of substrate/min at pH 9.0 and 37°C.

Experimental design, diets and management
A total of 90 crossbred (Landrace × Yorkshire × Duroc) weaning pigs with an initial average body weight of 6.61 ± 1.73 kg were
used in an 11-week feeding trial. Pigs were randomly allotted into one of two treatment groups consisting of nine replicate pens per treatment, with five pigs (three barrows and two gilts) per pen. A 4-phase (wk 0–1, wk 1–3, wk 3–6, and wk 6–11) feeding programme was employed in the current study, which consisted of post-weaning (wk 0–6) and growing (wk 6–11) periods. The dietary treatments were: CON, basal diet; PROT, CON +0.5 g/kg protease. The composition and nutrient content of basal diets for the experimental phases are presented in Table 1. All diets used in the present study were formulated to meet or exceed the nutritional requirements of the NRC (2012). During the first 6 weeks, all pigs were housed in an environmentally controlled room with a slatted plastic floor. Temperature during wk 1 was maintained at 32°C and was reduced 1.2°C each week thereafter. After the post-weaning period (wk 6), the pigs were transferred to growing facility. The target room temperature and humidity were 25°C and 60%, respectively. Each pen was equipped with a 1-sided self-feeder and one nipple waterer that allowed for ad libitum access to the feed and water throughout the experiment.

**Sampling and measurements**

Individual body weight (BW) was measured at the beginning and the end of each dietary phase, and feed consumption was recorded on a pen basis during the experiment to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain per feed (G:F). Chromium oxide (Cr2O3) was added to the diet as an indigestible marker at 0.20% of the diet for 7 d prior to fecal collection at the 6th wk and 11th wk for apparent total tract digestibility (ATTD) calculation of dry matter, crude protein, and gross energy (Kong and Adeola 2014). The fecal grab samples were collected randomly from at least 2 pigs in each pen (1 barrow and 1 gilt) at the end of the experiment. Fecal samples were dried at 70°C for 72 h in the oven, after which they were pulverized to pass through a 1-mm screen. The dry matter and crude protein of feed and fecal samples were analyzed according to the procedures described by AOAC International (AOAC 2006). The crude protein was determined by Kjeltc 2300 Nitrogen Analyzer (Foss Tecator AB, Hoeganaes, Sweden). And the chromium was determined by UV absorption spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan). The gross energy was determined by measuring the heat of combustion in the samples using a Parr 6100 oxygen bomb calorimeter (Parr instrument Co., Moline, IL).

At the end of 11th wk, fecal samples were randomly collected from 2 pigs of each pen (1 barrow and 1 gilt) via massaging the rectum. The samples were placed on ice for transportation to the laboratory. Approximately one gram composite fecal sample from each pen was blended with 9 mL of 1% peptone broth (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and then homogenized. The viable counts of *Lactobacillus* and *Escherichia coli* (E. coli) were conducted by plating serial 10-fold dilutions (10⁻¹–10⁻⁸) onto lactobacilli medium III agar plates (Medium 638; DSMZ, Braunschweig, Germany) and MacConkey agar plates (Difco Laboratories, Detroit, MI, USA) to isolate *Lactobacillus* and *E. coli*, respectively. The lactobacilli medium III agar plates were then incubated for 48 h at 39°C under anaerobic conditions. The MacConkey agar plates were incubated for 24 h at 37°C. The microbial colonies were counted immediately after removal from the incubator and the results were expressed as the logarithm of colony-forming units per gram (log10 cfu/g).

**Statistical analysis**

The pen was established as the experimental unit for growth performance, nutrient digestibility, and fecal microbial shedding. All data were statistically analyzed by Student’s t-test of SAS 9.4 (SAS Institute Inc., Cary, NC). Variability in the data was expressed as mean ± standard deviation, differences with *P* < 0.05 were considered to be statistically significant.

### Table 1. Ingredient composition of experimental diets as-fed basis.

| Ingredients, % | Post-weaned pigs | Growing pigs Phase 4 (wk 7–11) |
|---------------|-----------------|-------------------------------|
|               | Phase 1 (wk 0–1) | Phase 2 (wk 2–3) | Phase 3 (wk 4–6) |               |
| Extruded corn | 47.90           | 55.93                        | 63.36            | –             |
| Yellow corn   | –               | –                            | –                | –             |
| Wheat 11%     | –               | –                            | –                | 48.29         |
| Dehulled soybean meal | 18.00 | 24.00                  | 28.40            | –             |
| Fermented soybean meal | 8.00 | 5.00                     | –                | –             |
| Soybean meal 44% (imported) | – | –                     | –                | 16.60         |
| Soy oil       | 3.20            | 3.25                        | 3.65             | –             |
| Animal fat    | –               | –                            | –                | 4.50          |
| Di-calcium phosphate (DCP) | 1.34 | 1.63                     | 1.36             | 1.25          |
| Limestone     | 0.74            | 0.82                        | –                | 0.9           |
| Sugar         | 2.00            | 2.00                        | 2.00             | –             |
| Whey protein  | 8.00            | 3.00                        | –                | –             |
| Lactose       | 6.70            | 3.00                        | –                | –             |
| Molasses      | –               | –                            | –                | 3.20          |
| Salt          | –               | –                            | –                | 0.30          |
| L-Lysine-HCL 51% | 0.46 | 0.48                     | 0.38             | 0.38          |
| DL-Melowine 50% | 0.17 | 0.19                     | 0.16             | 0.05          |
| L-Threonine 98.5% | 0.29 | 0.20                     | 0.19             | 0.14          |
| L-Tryptophan 10% | –           | –                         | –                | 0.01          |
| Choline 50%   | 0.10            | 0.10                        | 0.10             | 0.08          |
| Vitamin premix³ | –          | 0.20                      | 0.20             | 0.15          |
| Mineral premix³ | 0.20 | 0.20                     | 0.20             | 0.15          |
| Total         | 100.00          | 100.00                     | 100.00           | 100.00        |

| Calculated composition | | | | |
|-------------------------|--|--|---|---|
| Crude protein, %        | 19.0 | 18.5 | 18.0 | 17.6 |
| Ether extract, %         | 4.80 | 4.20 | 4.40 | 6.87 |
| Calcium, %               | 0.75 | 0.75 | 0.70 | 0.75 |
| Total phosphorus, %      | 0.65 | 0.65 | 0.60 | 0.65 |
| DE, kcal/kg              | 3,900 | 3,800 | 3,700 | 3563 |
| Lysine, %                | 1.50 | 1.40 | 1.30 | 1.00 |
| Methionine, %            | 0.45 | 0.42 | 0.39 | 0.28 |
| Lactose, %               | 12   | 5    | 0    | –   |

                               ²Provided per kg of complete diet (post-weaned pigs): 11,025 IU vitamin A; 1,103 IU vitamin D₂; 44 IU vitamin E; 4.4 mg vitamin K₃; 83 mg riboflavin; 50 mg niacin; 4 mg thiamine; 29 mg D-pantothenic; 166 mg choline; 33 µg vitamin B₁₂, provided per kg of complete diet (growing pigs): 4,000 IU vitamin A; 800 IU vitamin D₂; 154 mg vitamin E; 2 mg vitamin K₃; 4 mg riboflavin; 20 mg niacin; 4 mg thiamine; 11 mg D-pantothenic; 166 mg choline; 16 µg vitamin B₁₂. ³Provided per kg of complete diet (post-weaned and growing pigs): 12 mg Cu (as CuSO₄·5H₂O); 85 mg Zn (as ZnSO₄); 8 mg Mn (as MnO₂); 0.28 mg I (as KI); 0.15 mg Se (as Na₂SeO₃·5H₂O).
Results

Growth performance

Table 2 shows the effects of dietary protease supplementation on growth performance in post-weaned and growing pigs. Over the entirety of the 11-week feeding trial, there was no difference (P > 0.05) in BW between CON and PROT groups. No differences were detected between CON and PROT groups in ADG, ADFI and G:F during the phase 1, phase 2, and phase 4. During phase 3, dietary treatments showed no effect on ADFI, but pigs fed PROT diet had greater (P < 0.05) ADG and G:F than pigs fed CON diet. For the overall period, ADG, ADFI and G:F were not a

Apparent total tract digestibility and fecal microbial shedding

As shown in Table 3, the ATTD of gross energy was not different (P > 0.05) between CON and PROT groups, whereas pigs fed PROT diet had higher (P < 0.05) ATTD of dry matter and crude protein than pigs fed CON diet during phase 3 and 4. Pigs fed PROT diet had no effects (P > 0.05) on fecal Lactobacillus and E. coli concentrations in this study (Table 4).

Discussion

Protease has ability to degrade a wide range of proteins, especially well recognized for attacking recalcitrant structural protein (Gupta et al. 2013; Cowieson et al. 2017). The B. licheniformis derived-protease as an effective exogenous enzyme has been reported to eliminate antinutritional factors, increase nitrogen digestion, and improve growth performance in broilers, growing pigs and finishing pigs (Barekatain et al. 2013; Chen et al. 2017).

In present study, adding protease to the diet increased ADG and G:F ratio only during phase 3 of post-weaned and growing pigs. In agreement with our results, Tactacan et al. (2016) and Wang et al. (2011) reported that the ADG and G:F ratio were increased in weaned pigs fed diets containing protease. This may be attributable to the supplementation of exogenous protease enzyme could increase the hydrolysis of protein and stimulate the synthesis of digestive enzymes in post-weaned pigs, could alter intestinal morphological which was damaged by weaning, and could eliminate the antinutritional factors of feedstuffs, therefore, resulting in better digestion and improving growth performance in weaned pigs (Hedemann and Jensen 2004; Fru-Nji et al. 2011; Yang et al. 2016). Previous studies showed that supplementation of protease could eliminate the negative effects of trypsin inhibitors and kafirin (Caine et al. 1998; Torrallardona and Roura 2009; Cadogan and Finn 2010). Moreover, the current study showed that supplementation of PROT increased the ATTD of dry matter and crude protein compared with the non-PROT diet. Similarly, previous studies have demonstrated an increased ATTD dry matter and crude protein in pigs fed exogenous protease (Tactacan et al. 2016; Chen et al. 2017). Therefore, supplementation of exogenous protease could benefit the protein digestion and absorption capacity of post-weaned and growing pigs.

The increased digestibility not only can be attributed to the physiological function of protease on protein digestion but also can be attributed to the positive effects of protease on gut health. Two stages are involved in the digestion of protein: the first occurs in stomach where protein is exposed to gastric pepsin and the other occurs in small intestine where protein is digested by other endogenous proteases (trypsin, chymotrypsin and elastase). Poor protein digestibility of post-

Table 3. Effect of dietary supplementation of protease on apparent total tract nutrient digestibility in post-weaned and growing pigs1.

| Items, % | CON | PROT | P-value |
|---------|-----|------|---------|
| Dry Matter | 82.83 ± 2.01 | 85.14 ± 1.94 | 0.035 |
| Crude protein | 80.92 ± 2.11 | 83.89 ± 2.02 | 0.012 |
| Gross energy | 81.75 ± 2.34 | 83.99 ± 2.07 | 0.063 |
| Finish | 80.67 ± 1.29 | 83.03 ± 1.40 | 0.004 |
| Crude protein | 79.99 ± 0.99 | 82.02 ± 1.59 | 0.009 |
| Gross energy | 79.93 ± 1.10 | 80.87 ± 1.79 | 0.225 |

Results were presented as mean ± standard deviation.

1Abbreviation: CON, basal diet; PROT, basal diet +0.5 g/kg protease.

Table 4. Effect of dietary supplementation of protease on fecal microbial shedding in post-weaned and growing pigs1.

| Items, log10cfu/g | CON | PROT | P-value |
|------------------|-----|------|---------|
| wk6 | Lactobacillus | 7.50 ± 0.18 | 7.69 ± 0.08 | 0.109 |
| | E. coli | 5.52 ± 0.17 | 5.43 ± 0.13 | 0.430 |
| Finish | Lactobacillus | 7.51 ± 0.22 | 7.73 ± 0.10 | 0.122 |
| | E. coli | 5.68 ± 0.14 | 5.55 ± 0.17 | 0.292 |

Results were presented as mean ± standard deviation.

1Abbreviation: CON, basal diet; PROT, basal diet +0.5 g/kg protease.

Means within the same row with different superscripts differ (P < 0.05).
weaned pigs triggered the bacteria (especially *E. coli*) fermentation of undigested protein in the hindgut. The high levels of dietary protein content in post-weaned pigs have been associated with the susceptibility of colibacillosis (Williams et al. 2005). It will be noticed also, as proved by Williams et al. (2005), the digestibility of crude protein was increased by protease supplement, which helps thus to decrease *E. coli* fermentation in the gut and to some extent to lower the fecal *E. coli* concentrations. In the current study, the unaffected fecal microbial shedding was probably in relation to an inadequate supply of *B. licheniformis* derived-protease in both post-weaned and growing pigs. Further study is needed to assess the optimal range of *B. licheniformis* derived-protease level in post-weaned and growing pigs.

**Conclusions**

In conclusion, dietary supplementation of *B. licheniformis* derived-protease increased growth performance in post-weaned pigs and increased the digestibility of dry matter and crude protein in growing pigs. However, further investigations are required to assess the optimal range of *B. licheniformis* derived-protease level and clarify the underlying mechanisms of improving growth performance in post-weaned pigs by *B. licheniformis* derived-protease.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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