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Effects of three kinds of protease on growth performance, apparent digestibility of nutrients and caecal microbial counts in weanling pigs

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Abstract: This experiment investigated the effects of three single kinds of protease on performance, serum parameters, apparent digestibility of nutrients and caecal microorganisms in weanling pigs. One hundred and ninety-two White × Landrace × Duroc hybrid pigs (7.51 ± 0.81 kg) were allotted to 1 of 4 treatments for 28 days. No protease was added to the control diet and the three experimental diets were supplemented with the same proportion of 10 IU/kg of alkaline, acidic and neutral protease, respectively. The supplementation of alkaline protease increased average daily gain and reduced serum concentrations of cholesterol and triglyceride compared with the control group ($P < 0.05$). The three single kinds of protease supplementation decreased the levels of serum urea nitrogen and increased the serum concentration of aspartate aminotransferase ($P < 0.05$). The apparent digestibility of crude protein was increased by the dietary supplementation of alkaline or acidic protease compared with that of pigs in the control group ($P < 0.05$). Results indicate that the alkaline protease supplementation improved the performance and apparent digestibility of crude protein in weanling pigs.

Keywords: enzyme preparation; growth; piglets

Weaning imposes large stress because pigs are facing a dramatic change in feeding switching from sow milk to plant materials-based diet (Sun et al. 2008; Kim et al. 2012). Weaning may result in a reduction in pig performance and the decrease could be restored by the dietary protease inclusion for its participation in regulating several important metabolic processes (Campbell and Bedford 1992; Guggenbuhl et al. 2012). Studies have reported that the appropriate amounts of protease supplementation could improve the utilization efficiency of nutrients (Kiarie et al. 2013; Cowieson and Roos 2016), alleviate the negative effects caused by the inadequate secretion of endogenous protease in young pigs, and thus improve the pig growth (Cowieson and Roos 2016; Norgaard et al. 2019).

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In pig production, the commonly used kinds of protease can be divided into acidic, neutral or alkaline protease (Barekatain et al. 2013; Amerah et al. 2017). The optimal pH for alkaline or neutral protease is close to the alkaline environment (Anwar and Saleemuddin 1998) and both the kinds of protease can exert the effects of enzymatic hydrolysis mainly in the small intestine because of its weak alkaline environment, while the optimal pH range for acidic protease is 3 to 5 and it mainly plays a priority role in the stomach of pigs for its acidic environment in the stomach (Gurumallesh et al. 2019). However, there is limited data on the effect of a single protease on growth performance of pigs. Therefore, this study compared the effects of three single kinds of protease on performance, apparent digestibility of nutrients, serum parameters and caecal microorganisms of piglets in order to provide a theoretical basis for the application of protease in weanling pigs.

MATERIAL AND METHODS

All animals were handled observing the rules of animal care that were approved by the Institutional Animal Care and Use Committee of China Agricultural University (Beijing, P.R. China).

Enzyme preparation

The proteases used in this study were supplied by Henan Yangshao Bio-products Co., Ltd, P.R. China and all three kinds of pure enzyme product provided 10 000 IU/g of protease. One unit of protease is defined as the amount of enzyme which liberates 1 μg of phenolic compound (tyrosine equivalents) from a casein substrate per minute.

Animal, housing, treatments, and samples collection

A total of 192 hybrid pigs (Landrace × Duroc × Yorkshire) with an average weight of 7.51 ± 0.81 kg were used in a 28-day performance trial. The experiment was conducted in the Pig Facility at Henan University of Science and Technology (Luoyang, Henan Province, P.R. China) when pigs were randomly allotted to 1 of 4 treatments with 8 pigs per pen (4 barrows and 4 gilts per pen) and 6 replications per treatment. Pigs were placed in partially steel-slatted concrete floored pens (2.5 m × 2.0 m) and each pen was equipped with a stainless steel self-feeder and a drinker. At the beginning and end of the experiment, pigs and feeders were weighed to determine average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

Before the start of the experiment, piglets were allowed a 5-day commercially prepared diet (Complete Feed; DaCheng Group, ChangChun, P.R. China) and the commercial diet was progressively replaced by experimental diets in the next four days. The basal diet was based on soybean meal and maize and formulated to meet the recommendations of NRC (2012) for piglets (Table 1). The contents of standard ileal digestible (SID) amino acids (AA) in diets were calculated by multiplying the analyzed AA contents in feed materials by the SID coefficients of the corresponding AA in those feedstuffs determined from NRC (2012). No protease was added to the control diet and the pigs in the other three groups were fed the basal diet supplemented with the acidic, neutral and alkaline protease preparations at 10 IU/g of diet, respectively. From day 21 to 28, a batch of feed was mixed with 0.25% chromium oxide as an indigestible marker and faecal samples from eight pigs in each pen were collected from day 25 to 28 and stored at –20 °C for the detection of apparent digestibility coefficients.

After an overnight fast, 24 randomly selected pigs (one pig per pen) were bled on day 29. Pigs were bled via the jugular vein puncture and the samples were kept in uncoated vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA).

The blood samples were collected and then placed on ice for 1 h and centrifuged at 3 000 × *g* (Heraeus Biofuge 22R Centrifuge, Hanau, Germany) for 10 minutes. The serum was obtained and frozen at –80 °C until analysis. The 24 pigs were then slaughtered to collect the caecal digesta to evaluate the microbial population according to the procedures described by Zhang et al. (2003).

Chemical analyses

Faecal samples were freeze-dried using a Virtis Genesis Wizzard 2.0 Freeze Dryer (SP Industries, Warminster, PA, USA). Proximate nutrients in

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Table 1. Composition and nutrient levels of the experimental basal diet (g/kg, as-fed)

Ingredients	Basal diet
Corn	649.7
Dehulled soybean meal	150.0
Extruded full fat soybean	90.0
Concentrated soybean protein	20.0
Whey powder	50.0
Soybean oil	5.0
Limestone	8.0
Dicalcium phosphate	11.0
Salt	3.0
Vitamin-mineral premix[1]	5.0
L-Lysine HCl (78.8%)	4.4
DL-Methionine (99%)	1.2
L-Threonine (99%)	1.6
L-Tryptophan (98.5%)	0.3
L-Isoleucine (98%)	0.4
L-Valine (98%)	0.4
Chemically determined values (g/kg)	
Crude protein	179.2
Lysine	12.2
Methionine + cysteine	7.1
Threonine	8.4
Tryptophan	2.2
Calculated values	
DE (MJ/kg)	14.5
SID amino acids (g/kg)	
Lysine	11.0
Methionine + cystine	6.0
Threonine	6.8
Tryptophan	2.0

[1]Premix provided per kg of the complete diet for weanling pigs: vitamin A, 9 000 IU; vitamin D3, 2 500 IU; vitamin E, 60 IU; vitamin K3, 2.2 mg; thiamine, 1.5 mg; riboflavin, 4 mg; niacin, 30 mg; pantothenic acid, 13.8 mg; pyridoxine, 3 mg; folacin, 0.7 mg; biotin, 44 μg; cobalamin vitamin B12, 27.6 μg; choline chloride, 400 mg; Mn, 40 mg; Fe, 80 mg; Zn, 80 mg; Cu, 100 mg; I, 0.3 mg; Se, 0.3 mg

the feeds and faecal samples were analyzed according to AOAC (2003) procedure. Gross energy was detected by an automatic adiabatic oxygen bomb calorimeter (Parr 1281 Automatic Energy Analyzer, Moline, IL, USA). The concentration of chromium was determined using an atomic absorption spectrophotometer (Hitachi Z-5000 Automatic Absorption Spectrophotometer, Tokyo, Japan) according to Pan et al. (2016). The apparent digestibility coefficients of proximate nutrients were calculated using the indicator method based on the equation from Valencia and Chavez (2002). The dietary AA levels (except tryptophan, cystine and methionine) were detected by ion-exchange chromatography using a Hitachi L-8800 AA Analyzer (Tokyo, Japan) following acid hydrolysis with 6 N HCl. Dietary cysteine and methionine were measured by acid hydrolysis after an oxidation step, and tryptophan was determined using reverse-phase high-performance liquid chromatography (Waters 2690, Milford, MA, USA) after alkaline hydrolysis.

The level of serum urea nitrogen (SUN) was detected by a urea nitrogen colour test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China). Serum biochemical metabolites including albumin, total protein, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, glucose and triglyceride were detected with a CX-4 Auto Blood Biochemical Analyzer (Beckman, Inc., Fullerton, CA, USA). The counts of *Lactobacillus* and *Escherichia coli* in caecal digesta of pigs were determined within 24 h using the conventional microbiological plate count method described by Zhang et al. (2003).

Statistical analyses

The statistical analyses were determined by the GLM procedures of SAS (Statistical Analysis System, v6.12, 1998) using a randomized complete block design. The pen of pigs was served as the experimental unit.

Means are expressed as least squares means and an alpha level of $P < 0.05$ was considered to be significant.

RESULTS

Growth performance

Pig performance is shown in Table 2. Pigs fed the basal diet supplemented with alkaline protease had the greater ADG compared with the control group ($P < 0.05$). However, there were no significant differences in ADFI or FCR between the treatments.

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Table 2. Effect of dietary protease supplementation on the performance of weanling pigs (7–20 kg)[1]

Items	Basal diet	Protease supplementation			SEM[2]	*P*-value
		alkaline	acidic	neutral		
Average daily gain (g)	388[b]	411[a]	402[ab]	396[ab]	3.86	0.04
Average daily feed intake (g)	581	575	577	582	13.98	0.98
Feed conversion ratio	1.50	1.40	1.44	1.47	0.04	0.39

[a,b]Within the row, different lower case letter superscripts mean significant difference ($P < 0.05$)

[1]Data are means of six replications per treatment; [2]standard error of the mean

Table 3. Effect of dietary protease supplementation on the serum urea nitrogen and metabolite concentrations of weanling pigs[1]

Items	Basal diet	Protease supplementation			SEM[2]	*P*-value
		alkaline	acidic	neutral		
Serum urea nitrogen (mmol/l)	3.50[a]	2.60[b]	2.85[b]	2.55[b]	0.11	0.01
Aspartate aminotransferase (IU/l)	76.11[b]	95.97[a]	92.52[a]	91.49[a]	2.98	0.03
Alanine aminotransfease (IU/l)	143.00	139.51	163.50	159.78	28.86	0.91
Albumin (g/l)	28.98	30.02	31.11	30.51	1.52	0.81
Cholesterol (mmol/l)	1.29[a]	0.99[b]	1.31[a]	1.15[ab]	0.05	0.03
Creatinine (umol/l)	76.98	74.95	78.43	77.49	2.21	0.73
Glucose (mmol/l)	10.00	10.45	9.65	10.15	1.07	0.96
Total protein (g/l)	50.50	49.01	51.48	51.11	2.67	0.92
Triglyceride (mmol/l)	0.71[a]	0.45[b]	0.60[a]	0.59[a]	0.02	0.02

[a,b]Within the row, different lower case letter superscripts mean significant difference ($P < 0.05$)

[1]Data are means of six replications per treatment; [2]standard error of the mean

Serum metabolite concentrations and SUN

Serum metabolite concentrations and SUN of pigs are shown in Table 3. Compared with the control group, the supplementation of all proteases decreased the level of SUN and increased the serum level of AST ($P < 0.05$). The dietary supplementation of alkaline protease decreased the serum concentrations of cholesterol and triglyceride compared with those of pigs fed the basal diet ($P < 0.05$).

Apparent digestibility of nutrients

The apparent digestibility of nutrients is documented in Table 4. The apparent digestibility of crude protein was increased by the dietary supplementation of alkaline or acidic protease ($P < 0.05$).

However, the apparent digestibility of dry matter, gross energy, calcium and phosphorus was not influenced by protease supplementation.

Table 4. Effect of dietary protease supplementation on the apparent digestibility of nutrients in weanling pigs[1] (%)

Items	Basal diet	Protease supplementation			SEM[2]	*P*-value
		alkaline	acidic	neutral		
Dry matter	85.93	87.91	87.65	86.43	2.01	0.88
Gross energy	84.81	87.52	86.20	85.27	2.13	0.82
Crude protein	80.61[b]	85.93[a]	84.81[a]	82.87[ab]	0.94	0.02
Calcium	51.17	52.51	51.17	53.42	2.21	0.86
Phosphorus	41.47	42.30	42.27	39.23	0.29	0.76

[a,b]Within the row, different lower case letter superscripts mean significant difference ($P < 0.05$)

[1]Data are means of six replications per treatment; [2]standard error of the mean

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Table 5. Effect of dietary protease supplementation on the caecal microbial counts of weanling pigs[1]

Items	Basal diet	Protease supplementation			SEM[2]	*P*-value
		alkaline	acidic	neutral		
Escherichia coli, log(CFU/g)	6.19	5.83	5.94	6.02	0.23	0.74
Lactobacilli, log(CFU/g)	7.32	7.48	7.21	7.23	0.20	0.76

[a,b]Within the row, different lower case letter superscripts mean significant difference ($P < 0.05$)

[1]Data are means of six replications per treatment; [2]standard error of the mean

Caecal microbial counts

The bacterial counts in the caecum are shown in Table 5. The counts of *Escherichia coli* and *Lactobacilli* in pigs supplemented with the three kinds of protease were not significantly different from those in pigs fed the control diet.

DISCUSSION

A protease preparation has been accepted by the feed industry because of its outstanding role in improving the utilization of nutrients and growth performance of animals (Kiarie et al. 2016; Chen et al. 2017; Cowieson et al. 2017). In recent years, the kinds of protease frequently used in pig production could be divided into acidic, alkaline and neutral protease according to the suitable pH ranges to optimize the enzymatic hydrolysis reaction (Anwar and Saleemuddin 1998; Gurumallesh et al. 2019). However, limited research has investigated the effect of a single type of protease on the performance of weanling pigs and these data can provide the theoretical basis for further understanding of the application of protease in piglets. Therefore, this study compared the effects of three single proteases on growth performance, serum biochemical index and nutrient apparent digestibility of weanling pigs.

In this experiment, the ADG of pigs fed the alkaline protease was higher than that of pigs fed the acid or neutral protease. This might be because of the fact that nutrients were mainly demonstrated to be digested in the small intestine where the internal pH environment is more suitable for alkaline protease to perform the effect of enzymatic hydrolysis and then to improve the nutrient digestibility of weanling pigs (O'Shea et al. 2014; Pan et al. 2016).

The effects on apparent digestibility of nutrients by the three kinds of protease supplementation were also investigated in this study. In the present study, the inclusion of alkaline, acidic and neutral protease increased the apparent digestibility of crude protein by 6.2%, 5.0% and 2.7%, respectively. In addition, the apparent digestibility of crude protein in pigs fed alkaline protease was significantly higher than that of pigs fed the control diet, which might partly be the explanation for the higher performance of pigs in the alkaline protease group. However, the reason for the limited improvement in the apparent digestibility of crude protein by neutral protease supplementation remains to be studied further.

An increase in serum AST activity in pigs within the normal range may indicate that the addition of protease affects the metabolic capacity of liver in pigs (Ma et al. 2020), which was consistent with an earlier study of Zuo et al. (2015). The decreased SUN level reflected an improvement in the efficiency of crude protein utilization in pigs (Ma et al. 2020), which was consistent with the improvement in the apparent digestibility of crude protein in this study. In addition, the serum cholesterol and triglyceride contents of pigs in the alkaline protease group were significantly lower than those of pigs fed the control diet, which may reflect that the addition of alkaline protease could also participate in the regulation of lipid metabolism in weanling pigs. This result may be due to the fact that the dietary supplementation of alkaline protease can accelerate the metabolic rate of liver, thus indirectly affecting the lipid metabolism in weanling pigs.

The improvement in pig performance may be related to the decrease of pathogenic bacteria and the proliferation of probiotics in the intestine (Zhang et al. 2003; Sauer et al. 2012). Some studies suggested that the addition of protease can reduce the amount of undigested proteins into caecum and increase the *Lactobacillus* counts to reduce the pH of the intestine to suppress the colonization of pathogenic bacteria such as *Escherichia coli* (Kiarie et al. 2013; Cowieson et al. 2017). Another potential explanation for the changes of

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the microbe proportion in the intestine may be due to a certain amount of antinutritional factors in soybean meal such as trypsin inhibitor and lectin to disturb the digestive process in the intestine and then influence the microbial colonization (Sun et al. 2008; Kim et al. 2012). Some studies demonstrated that the antinutritional factors could partly be degraded by the dietary supplementation of alkaline protease and then lead to the reduction of the diarrhoea probability in weanling pigs (Anwar and Saleemuddin 1998; Torres-Pitarch et al. 2017).

However, the supplementation of alkaline protease resulted in a decrease in *Escherichia coli* and an increase in *Lactobacillus* in caecum in this study, but there were no significant differences from the counts of pigs fed the control diet.

Conflict of interest

The authors declare no conflict of interest.

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