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Effects of low-protein diet supplemented with exogenous protease on growth performance and intestinal health of broiler chickens

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Abstract: This experiment was conducted to study the effects of low-protein diet supplemented with exogenous protease on performance and intestinal health of broilers. A total of 560 one-day-old male Arbor Acres broiler chickens were randomly divided into 5 treatments with 8 replicates (12 birds per replicate) in a completely randomised design. The treatments were as follows: (1) maize-soybean meal basal diet (CON); (2) negative control with crude protein being 1% lower than in CON (NCON); (3) NCON + 12 000 U/kg coated alkaline protease (NCAP); (4) NCON + 16 000 U/kg alkaline protease (NAP); (5) NCON + 16 000 U/kg keratinase (NKA). The remaining 80 birds (10 replicates, 8 birds per replicate) were randomly assigned to endogenous indicator measurements. The results indicated that the NCON diet resulted in a higher feed-to-gain ratio and reduced protein digestibility, ileal amino acid digestibility, and intestinal morphological parameters ($P < 0.05$). Supplementation of different exogenous proteases significantly improved the apparent and true ileal digestibility of specific amino acids, enhanced jejunal chymotrypsin activity, and promoted intestinal morphological development, including increased villus height and villus height-to-crypt depth ratio ($P < 0.05$). Our findings suggested that the addition of exogenous protease improved the intestinal morphology of broilers.

Keywords: Arbor Acres chickens; feed efficiency; intestinal morphology; low-protein diet; nutrient digestibility; protease supplementation

With the rapid development of poultry industry, the shortage of conventional feed and the rapid rise of feed raw material prices have become the key factors restricting the sustainable development

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of poultry industry. Meanwhile, in intensive livestock production, nitrogen excretion from dietary proteins can cause high ammonia emissions, eutrophication, and soil acidification. Therefore, the substitution of maize and soybean meal reduction is an inevitable trend to lower production costs and mitigate environmental impacts. Adopting a low-protein diet can reduce feed costs and relieve environmental problems, but it could cause the reduced growth performance of broilers (Hofmann et al. 2019). Exogenous enzyme supplementation is one of the best ways to improve the bioavailability of nutrients and eliminate anti-nutritional factors, which could improve the growth performance and crude protein utilisation efficiency in broilers (Kwak et al. 2024).

At present, the exogenous compound enzyme has been widely used in broilers, which plays a great role in improving the utilisation rate of nutrient organisms and promoting growth (Leyva-Jimenez et al. 2024). Exogenous proteases are enzymes derived from microorganisms, plants, or animals that can hydrolysae complex proteins in feed into small peptides and amino acids, thus improving protein digestibility and feed utilisation efficiency (Liu et al. 2025). Despite these benefits, the use of a single protease remains under investigation for its potential broader applications.

Exogenous proteases can be categorised into acid, neutral, and alkaline proteases based on their optimal pH range. However, due to variations in physicochemical properties, feeding conditions and other factors, the effectiveness of protease in production also differs.

Research has shown that supplementation of exogenous protease (50 ppm~200 ppm) in low-protein diets significantly improved growth performance, enhanced nutrient digestibility, and positively influenced intestinal histomorphometry in broiler chickens (Duque-Ramirez et al. 2023). Fru-Nji et al. (2011) found that the addition of dietary protease (75 000 U/g, from *Bacillus licheniformis*) improved the efficiency of energy and protein utilisation in broilers. Nevertheless, the specific role of exogenous proteases in low-protein diets requires further investigation to establish their optimal use in poultry nutrition.

Therefore, the objective of this experiment was to determine the effects of diets supplemented with exogenous proteases on the performance and intestinal health of broilers.

MATERIAL AND METHODS

Experimental design

A total of 560 one-day-old male Arbor Acres broiler chickens with an initial body weight (BW) of 44.9 ± 0.18 g were purchased from a commercial chicken farm (Buen Agriculture and Animal Husbandry Technology Co., Ltd, Linyi, P.R. China), individually weighed, and then randomly distributed into 5 dietary treatments with 8 replicates (12 birds per replicate) in a completely randomised design. The treatments were as follows: (1) maize-soybean meal basal diet (CON); (2) negative control with crude protein being 1% lower than in the CON (NCON); (3) NCON + 12 000 U/kg coated alkaline protease (NCAP); (4) NCON + 16 000 U/kg alkaline protease (NAP); (5) NCON + 16 000 U/kg keratinase (NKA). The remaining 80 birds (10 replicates, 8 birds per replicate) were randomly assigned to endogenous measurements. Among them, 40 birds (8 replicates) were used for the metabolic rate of nutrients and the metabolisable energy of diet, and the other 40 birds (8 replicates) were used for the true ileal digestibility of amino acids. The basal diet was formulated according to two-phase feeding programs (0–21 d and 21–42 d) recommended by the Feeding Standard of Chickens of the People's Republic of China (NY/T 33-2004) (Table 1). The NCAP, NAP and NKA of feed grade were all provided by Shandong Longkete Enzyme Preparation Co., Ltd. (Linyi, Shandong, P.R. China).

Animals and management

The experimental broilers were raised in cages and vaccinated according to the normal immunisation program, and they were allowed to feed (except the endogenous treatment) and water *ad libitum*. The room temperature was maintained at 35 °C for the first week and then it was decreased by 1 °C every two days until 21 °C. The experiment lasted for 42 days. During the experiment, all birds were inspected at least three times per day and any mortalities were removed in accordance with the guidelines for the care and use of laboratory animals prescribed by the Animal Nutrition Research Institute of Shandong Agricultural University and the Ministry of Agriculture of China (Approval No. SDAUA-2021-0410; date of approval: 10 April

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Table 1. Ingredients and nutrient levels of basal diet (air dry basis, g/kg)

Items	Phases			
	1–21 d		22–42 d	
Ingredients (g/kg)	CON	NCON	CON	NCON
Corn	346	374	394	422
Soybean meal	282	257	246	221
Rough rice	150	150	100	100
Wheat flour	80.0	80.0	80.0	80.0
Corn gluten meal	20.0	20.0	20.0	20.0
Cottonseed meal	40.0	40.0	40.0	40.0
Feather meal	15.0	15.0	15.0	15.0
$CaHPO_3$	9.00	9.00	8.00	8.00
Pulverised limestone	16.0	16.0	15.0	15.0
Duck oil	18.0	15.0	62.0	59.0
Premix[1]	20.0	20.0	20.0	20.0
TiO_2	4.00	4.00	4.00	4.00
Total	1 000	1 000	1 000	1 000
Nutrient levels[2]				
Metabolisable energy (J/kg)	12.3	12.3	13.6	13.6
Crude protein (g/kg)	230	220	215	205
Calcium (g/kg)	9.00	9.00	8.30	8.30
Total phosphorus (g/kg)	5.80	5.80	5.30	5.30
Lysine (g/kg)	14.6	13.9	13.2	12.5
Methionine (g/kg)	6.00	5.80	5.30	5.10
Threonine (g/kg)	10.1	9.60	9.20	8.70

[1]Supplied per kg of diet: VA 4 525 IU, VD_3 975 IU, VE 13 IU, VK_3 2.5 mg, VB_1 1.3 mg, VB_2 4.0 mg, VB_{12} 0.01 mg, pantothenic acid 7.50 mg, niacin 17.50 mg, biotin 0.1 mg, folic acid 0.6 mg, Mn (as $MnSO_4 \cdot H_2O$) 30.00 mg, Fe (as $FeSO_4 \cdot H_2O$) 40 mg, Zn (as $ZnSO_4 \cdot H_2O$) 30 mg, Cu (as $CuSO_4 \cdot 5H_2O$) 4.25 mg, I (as KIO_3) 0.135 mg, Se (as Na_2SeO_3) 0.1 mg; [2]Digestible energy is the calculated value, and the levels of other nutrients are analysed values
CON = the basal diet; NCON = a negative control with crude protein being 1% lower than in CON

2021). The daily feed intake and BW (on days 21 and 42) per replicate were recorded to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed-to-gain ratio (F/G).

Nutrient availability experiment

SAMPLE COLLECTIONS

The nutrient availability experiment was conducted according to the method of total faeces collection. All excreta from 12 birds per replicate were collected continuously on d 18 to 20. Feathers and shredded dry skin in excreta were removed carefully, and then the excreta were weighed, pooled by replicate, sampled and stored at –20 °C for analysis. Before collecting endogenous measurements, all selected birds were fasted for 24 h with access to water only, and faeces were then collected for the next 48 hours.

NUTRIENT AVAILABILITY AND DIETARY METABOLISABLE ENERGY

Firstly, crude protein (CP) was determined in fresh feed and faeces by the Kjeldahl method (CP = nitrogen × 6.25). Secondly, the faecal samples were dried at 65 °C and the dried samples were finely ground using a mortar and pestle, and then stored in sealed containers for the subsequent analysis of dry matter (DM), organic matter (OM), CP, crude ash (CA) and gross energy (GE) accord-

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ing to AOAC (2012). DM was analysed by drying at 103 ± 2 °C for 48 h, EE and CA were determined by ether extraction and ashing at 550 °C in a muffle furnace (SX2-4-10; Longkou Electric Furnace Manufacturer, Yantai, P.R. China), and GE was determined using the Parr adiabatic bomb calorimeter (Model 6200; Parr Instruments Co, Moline, IL, USA) (Zhang et al. 2009). And the apparent metabolisable energy (AME), nitrogen corrected apparent metabolisable energy (AMEn), true metabolisable energy (TME), and nitrogen corrected true metabolisable energy (TMEn) were calculated by the method described by Sibbald (1976). The formulas for calculating the metabolic rate of nutrients and the metabolisable energy of diet were as follows:

$$\text{Apparent metabolic rate} = [(\text{TNI} - \text{TNE})/\text{TNI}] \times 100 \quad (1)$$

$$\text{True metabolic rate} = [(\text{TNI} - \text{TNE} + \text{TNEE})/\text{TNI}] \times 100 \quad (2)$$

where:

TNI – total nutrient intake (g) of DM, OM, CP, CA and GE;

TNE – total nutrients in the excreta of DM, OM, CP, CA and GE;

TNEE – total nutrients in the endogenous excreta of DM, OM, CP, CA and GE.

$$\text{AME (MJ/kg)} = [(\text{TNI} - \text{TNE})/\text{FI}] \quad (3)$$

$$\text{TME (MJ/kg)} = [(\text{TNI} - \text{TNE} + \text{TNEE})/\text{FI}] \quad (4)$$

$$\text{AMEn (MJ/kg)} = \text{AME} - (34.39 \times \text{RN}) \quad (5)$$

$$\text{TMEn (MJ/kg)} = \text{TME} - (34.39 \times \text{RN}) \quad (6)$$

where:

AME – apparent metabolisable energy;

AMEn – nitrogen corrected apparent metabolisable energy;

TNI – total amount (MJ) of energy in daily intake of each bird;

TNE – total amount of energy in daily collected excreta of each bird;

TME – true metabolisable energy;

TNEE – total amount of energy in daily endogenous excreta of each bird without being fed;

FI – daily feed intake of each bird (DM basis);

RN – N retention calculated as the difference between N intake and N output;

TMEn – nitrogen corrected true metabolisable energy.

Slaughter experiment

At the end of the experiment, 2 broilers were randomly selected from each replication for morphology and chymotrypsin activity sampling, and the other 10 broilers were slaughtered for ileal chyme sampling. Under aseptic conditions, the 4-cm duodenum, jejunum and ileum samples were isolated from a uniform position, rinsed with normal saline repeatedly, and then rapidly fixed in Bouin's fluid for morphologic examination. At the same time, the jejunum chyme of the same duplicate broilers was collected into an RNase-free 5-ml frozen tube and stored at –80 °C for subsequent chymotrypsin activity analysis. Before collecting the endogenous ileal chyme, all 64 birds were fasted for 24 h with access to water only. The ileal chyme of the same duplicate broilers was collected into a culture dish, and freeze-dried at –80 °C to determine the ileal amino acid digestibility.

Ileal amino acid digestibility

The apparent and true ileal digestibility of amino acids was determined using the 0.4% titanium dioxide indicator method. Amino acids (AA, except tryptophan) in diet, chyme and endogenous ileal chyme were analysed in an automatic amino acid analyser (Hitachi-835; Hitachi Limited, Tokyo, Japan) by high-performance liquid chromatography (HPLC) according to the method described by Yu et al. (2019). The titanium dioxide was determined according to the method described by Short et al. (1996). Samples were ashed and dissolved in 67% sulphuric acid. Hydrogen peroxide (30% vol.) was subsequently added, resulting in the typical orange colour. Then the absorbance was determined using a UV spectrophotometer at 410 nm. The apparent and true ileal amino acids digestibility was calculated according to the following formulas:

$$\text{AID} = [1 - (\text{dietary } TiO_2/\text{ileal chyme } TiO_2) \times \times (\text{AA content of ileal chyme/AA content of diet})] \times 100 \quad (7)$$

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$$TID = AID + [(\text{dietary } TiO_2/\text{endogenous ileal chyme } TiO_2) \times (\text{AA content of endogenous ileal chyme/AA content of diet}) \times 100] \quad (8)$$

where:
AID – apparent ileal digestibility;
TiO_2 – titanium dioxide;
AA – amino acids;
TID – true ileal digestibility.

Chymotrypsin activity of jejunal chyme

The jejunal chyme samples were thawed and about 0.15 g of each sample was weighed into a 2 ml centrifuge tube, homogenised with 0.02 mmol/l Tris-HCl (pH 7.4) at 1 : 10 (mg/ml), and centrifuged (3 000 rpm) at 4 °C for 10 minutes. The supernatant was analysed for chymotrypsin activity using a commercial chymotrypsin assay kit (A080-3-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China).

Intestinal morphology

The fixed duodenum, jejunum and ileum segments were dehydrated in ethanol and xylene solutions, and then embedded according to the conventional paraffin-embedding protocol, followed by being cut into 6-μm thin slices using a Leica semi-automatic microtome (Leica Co., Wetzlar, Germany). After that, the slices were processed by haematoxylin and eosin staining to observe intestinal morphology. And both villus height (VH) and crypt depth (CD) were visualised and measured at 40× magnification using an Olympus BX51 microscope equipped with a DP70 digital camera (Olympus, Tokyo, Japan). The VH/CD ratio was calculated from VH and CD.

Statistical analysis

All data were analysed using the general linear model (GLM) in SAS v9.4 (SAS Institute Inc., Cary, NC, USA), and differences between treatments were compared by Tukey's multiple range tests. The results are presented as the mean ± standard error. When $P < 0.05$, it indicates that the data are significantly different.

RESULTS AND DISCUSSION

Growth performance

No significant differences in body weight, ADG and ADFI at 42 days of age were observed between treatments ($P > 0.05$, Table 2). Compared with CON, the F/G of NCON on 0–42 d was decreased significantly ($P < 0.05$). Compared with NCON, the F/G of NCAP on 0–42 d, as well as the F/G of NAP and NKA on 21–42 d and 0–42 d, respectively, were significantly decreased ($P < 0.05$).

Dietary protein plays a significant role in digestive system development and growth performance. Our findings showed that adding alkaline protease (16 000 U/kg), coated alkaline protease (12 000 U/kg) and keratinase (16 000 U/kg) to a diet of the negative control with a 1% reduction in the dietary crude protein level improved the F/G on 21–42 d and 0–42 d, making its growth performance comparable to that of the control diet. Similarly, a report by Mohammadigheisar and Kim (2018) indicated that supplementation of alkaline protease (37 500 U/kg) produced through submerged fermentation of *Bacillus licheniformis* improved the utilisation of crude protein in broilers, especially when dietary protein levels decreased by 1%. A 2% decrease in dietary protein levels significantly reduced the body weight of broilers, and adding HuPro protease (20 000 U/g, neutral protease) achieved a similar effect as a positive control (Wang et al. 2022a). Also, research has shown that keratinase (400 000 U/g) significantly improved ADG and F/G in broiler chickens on days 1–21 and on days 1–42 (Wang et al. 2008). However, adding serine protease (15 000 U/g) to diets with 4% lower protein content had no significant effects on body weight and F/G of broilers (Rada et al. 2013). No significant F/G was observed when adding 1.25 g/kg protease (from *Streptomyces griseus*) to a diet with a 6% reduction in crude protein and main digestible amino acids (lysine, methionine, threonine, and tryptophan) (Cardinal et al. 2019). Neither could serine protease (15 000 U/kg) eliminate the detrimental effects of a 6% reduction in the dietary crude protein level and digestible amino acids on the growth performance of broilers (Sarica et al. 2020). The results of the above different literature sources might be related to differences in dietary structure, protease source and dietary protein and limiting amino acid levels.

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Table 2. Effects of different proteases on the growth performance of broilers

Items	CON	NCON	NCAP	NAP	NKA	SEM	*P*-value
BW (g)							
0 d	44.6	45.4	45.3	44.2	45.0	0.177	0.114
21 d	876	859	873	908	879	7.86	0.433
42 d	2 597	2 546	2 581	2 637	2 648	13.2	0.072
0–21 d							
ADG (g)	39.6	38.8	39.4	39.7	41.1	0.375	0.404
ADFI (g)	50.7	50.8	50.3	51.2	52.9	0.427	0.344
F/G	1.28	1.31	1.28	1.29	1.29	0.007	0.813
21–42 d							
ADG (g)	81.9	80.3	81.3	84.2	82.4	0.565	0.274
ADFI (g)	126	127	125	127	124	0.801	0.717
F/G	1.54^{ab}	1.58^{a}	1.53^{ab}	1.51^{b}	1.51^{b}	0.008	0.012
0–42 d							
ADG (g)	60.8	59.5	60.4	62.0	61.7	0.316	0.070
ADFI (g)	88.2	88.9	87.4	89.3	88.7	0.388	0.646
F/G	1.45^{b}	1.49^{a}	1.45^{b}	1.44^{b}	1.44^{b}	0.006	0.004

^{a,b}Means differ significantly ($P < 0.05$)

ADFI = average daily feed intake; ADG = average daily gain; BW = body weight; CON = the basal diet; F/G = feed to gain ratio; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON

Table 3. Effects of different proteases on the metabolic rate of nutrients in broilers (g/kg)

Items	PCON	NCON	NCAP	NAP	NKA	SEM	*P*-value
Apparent metabolic rate (AMR)							
GE	764	759	762	764	762	0.258	0.984
DM	762	747	760	764	762	0.343	0.564
OM	776	772	774	776	774	0.283	0.988
CP	666^{a}	645^{b}	652^{ab}	657^{ab}	660^{ab}	0.207	0.011
EE	804	795	801	803	803	0.208	0.706
CA	642	616	632	631	636	0.428	0.419
True metabolic rate (TMR)							
GE	781^{b}	810^{a}	787^{b}	794^{ab}	811^{a}	0.354	0.010
DM	789	780	795	799	800	0.317	0.254
OM	803	804	808	810	812	0.258	0.823
CP	713	705	706	710	706	0.247	0.816
EE	820	825	830	831	832	0.234	0.475
CA	675	654	672	666	677	0.438	0.487

^{a,b}Means differ significantly ($P < 0.05$)

CA = crude ash; CON = the basal diet; CP = crude protein; DM = dry matter; EE = ether extract; GE = gross energy; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON; OM = organic matter

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Apparent and true availability of nutrients and gross energy

No significant differences in apparent availability (GE, DM, OM, EE and CA) and true availability (DM, OM, CP, EE and CA) were observed between treatments ($P > 0.05$, Table 3).

Compared with CON, the apparent availability of CP in NCON was significantly decreased ($P < 0.05$), however, the true availability of GE in NCON was significantly increased ($P < 0.05$). What's more, the true availability of GE of NCAP was decreased compared with NCON ($P < 0.05$).

Table 4. Effects of different proteases on AME, AMEn, TME, and TMEn of broilers MJ/kg DM

Items	PCON	NCON	NCAP	NAP	NKA	SEM	*P*-value
AME	12.2	12.3	12.4	11.9	12.2	0.058	0.124
AMEn	10.4	10.4	10.4	10.4	10.6	0.068	0.952
TME	12.6	12.2	12.5	12.4	12.5	0.066	0.550
TMEn	10.9	10.7	10.8	10.8	10.9	0.072	0.919

CON = the basal diet; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON

Table 5. Effects of different proteases on ileal metabolic rates of essential amino acids in broilers (g/kg)

Items	PCON	NCON	NCAP	NAP	NKA	SEM	*P*-value
Apparent ileal digestibility (AID)							
Lys	853	845	864	862	855	0.315	0.353
Met	811	804	831	820	827	0.354	0.088
Arg	837	823	855	843	850	0.399	0.092
His	810^{ab}	781^{b}	813^{a}	797^{ab}	799^{ab}	0.373	0.035
Leu	757	733	775	769	767	0.526	0.074
Ile	762	743	778	781	782	0.552	0.103
Thr	770	748	775	779	772	0.380	0.070
Val	761^{ab}	735^{b}	785^{a}	773^{a}	776^{a}	0.484	0.004
Phe	800	766	783	793	784	0.445	0.133
Trp	737	713	755	749	747	0.526	0.074
True ileal digestibility (TID)							
Lys	886	882	885	885	875	0.263	0.667
Met	851	837	859	850	834	0.329	0.080
Arg	882^{ab}	873^{ab}	887^{a}	853^{b}	880^{ab}	0.387	0.045
His	829	811	843	827	844	0.409	0.051
Leu	787	763	792	805	794	0.504	0.084
Ile	792^{ab}	773^{b}	808^{ab}	796^{ab}	811^{a}	0.453	0.048
Thr	788	778	797	807	805	0.373	0.062
Val	791^{ab}	766^{b}	801^{a}	801^{a}	793^{ab}	0.389	0.018
Phe	813	794	814	823	814	0.422	0.275
Trp	767	743	772	785	774	0.504	0.084

^{a,b}Means differ significantly ($P < 0.05$)

Arg = arginine; CON = the basal diet; His = histidine; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON; Phe = phenylalanine; Thr = threonine; Trp = tryptophan; Val = valine

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AME, AMEn, TME, TMEn of diets in broilers

No significant differences in the AME, AMEn, TME and TMEn of diets were observed between treatments ($P > 0.05$, Table 4).

Apparent and true ileal digestibility of essential amino acids (EAA) and non-essential amino acids (NEAA)

No significant differences in AID and TID of EAA were observed between CON and NCON ($P > 0.05$, Table 5). Compared with NCON, the AID of His and Val, and the TID of Val in NCAP were significantly increased ($P < 0.05$); the AID of Val, and the TID of Ile in NKA were significantly increased ($P < 0.05$); the AID and TID of Val in NAP were significantly increased ($P < 0.05$).

Compared with CON, the AID of Ala and TID of Ser in NCON significantly decreased ($P < 0.05$, Table 6). Compared with NCON, the AID and TID of Gly, Ala, Cys and Try in NCAP were significantly increased ($P < 0.05$); the AID of Gly, Ala and Try, and the TID of Ser, Gly and Ala in NAP were significantly increased ($P < 0.05$); the AID and TID of Gly, Ala, Cys and Try in NKA were significantly increased ($P < 0.05$).

Proteases not only promote the digestion of proteins, but also they increase the digestibility of amino acids in broiler chickens. In the present study, keratinase (16 000 U/kg) addition to a diet of the negative control with a 1% reduction in the dietary crude protein level improved the apparent metabolic rate of GE, while alkaline protease (16 000 U/kg), coated alkaline protease (12 000 U/kg) and keratinase (16 000 U/kg) supplement to a diet of the negative control did not significantly improve the apparent metabolic rate of CP in broiler chickens, but there were varying degrees of improvement in the metabolic rate of amino acids (His, Val and Ile) in the ileum of broiler chickens. It has been reported that xylanase (300 U/kg), α-amylase

Table 6. Effects of different proteases on ileal metabolic rates of non-essential amino acids in broilers (g/kg)

Items	PCON	NCON	NCAP	NAP	NKA	SEM	*P*-value
Apparent ileal digestibility (AID)							
Asp	769	758	788	780	777	0.425	0.231
Ser	754	742	772	762	756	0.360	0.104
Glu	814	801	830	826	821	0.409	0.188
Pro	817	801	832	823	824	0.399	0.139
Gly	745^{ab}	733^{b}	775^{a}	769^{a}	768^{a}	0.445	0.003
Ala	764^{a}	726^{b}	787^{a}	763^{a}	767^{a}	0.529	0.002
Cys	656^{c}	666^{c}	814^{a}	713^{bc}	749^{ab}	1.322	<0.001
Tyr	738^{bc}	732^{c}	778^{a}	769^{ab}	770^{ab}	0.498	0.003
True ileal digestibility (TID)							
Asp	809	807	818	810	788	0.423	0.252
Ser	784^{a}	752^{b}	782^{ab}	788^{a}	772^{ab}	0.400	0.021
Glu	844	836	860	856	851	0.374	0.281
Pro	847	831	862	861	853	0.403	0.085
Gly	777^{ab}	763^{b}	805^{a}	799^{a}	798^{a}	0.438	0.003
Ala	783^{ab}	754^{b}	817^{a}	797^{a}	797^{a}	0.533	<0.001
Cys	716^{ab}	673^{b}	774^{a}	713^{ab}	749^{ab}	0.999	0.009
Tyr	748^{ab}	729^{b}	771^{a}	760^{ab}	773^{a}	0.514	0.026

^{a,b}Means differ significantly ($P < 0.05$)

Ala = alanine; Asp = aspartic acid; CON = the basal diet; Cys = cystine; Glu = glutamate; Gly = glycine; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON; Pro = proline; Ser = serine; Tyr = tyrosine

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(400 U/kg), serine protease (4 000 U/kg) and phytase (500 U/kg) can work together to provide the right conditions for the action of exogenous amylase on starch, which can lead to increased DM and GE digestibility (Cowieson et al. 2006). Research has shown that protease (8 000 U/g, heat- and acid-resistant protected protease) increased the digestibility of DM, CP and some amino acids (Asp, Ile and Leu) in broilers; reducing the crude protein in the diet by 2% before adding protease has a better effect (Wang et al. 2022b). A previous study in broilers also concluded that the digestibility of crude protein and most essential amino acids increased when proteases (7 500, 15 000, 30 000, and 60 000 U/kg) produced by submerged fermentation of *Bacillus licheniformis* of different gradients were added to a 2% protein reduction diet (Angel et al. 2011). In addition, alkaline protease (3 000 U/kg, from *Bacillus subtilis*) and protease enzyme (8 000 U/g of acidic and 12 000 U/g of neutral protease) addition to the corn distillers dried grains with solubles basal diet in which 3% protein was reduced, improved the growth performance, apparent digestibility of crude protein, nitrogen retention, and apparent ileal amino acid digestibility in broiler chickens (Shad et al. 2022). The results of the above study may be related to proteases that have the ability to hydrolyse large protein molecules into peptides and amino acids, enhancing the overall digestion and absorption of CP and amino acids. The differences in the above results may be related to the source of the enzyme, the dosage added, and the type of animal etc., and further confirmation is needed.

Chymotrypsin activity of jejunal chyme

As shown in Figure 1, compared with CON, the chymotrypsin activity of jejunal chyme in NCON decreased significantly ($P < 0.05$). The chymotrypsin activity in NAP was significantly increased compared with NCON ($P < 0.05$).

The digestive enzyme activity in digestion and mucosa is the main limiting factor affecting digestion, absorption, and growth in poultry. The digestive enzymes include amylase, lipase, and proteases, which play a very important role in the digestion of nutrients. Previous research found that the β-glucanase addition to the barley diet significantly increased the activity of lipase, amylase, and tryptase in the intestinal contents of broiler chickens (Almirall et al. 1995). Zhang et al. (2014) demonstrated that the supplementation of exogenous polyenzymes enhanced the activity of amylase, lipase, and protease in the small intestine of piglets. Engberg et al.(2004) also confirmed that xylanase (100 g/kg) supplementation significantly increased lipase and chymotrypsin activity in broilers. Our present study showed that a 1% reduction in dietary crude protein reduced the chymotrypsin activity in jejunum, however, the chymotrypsin activity improved when the alkaline protease (16 000 U/kg) was added to the 1% lower protein feed. This suggests that the addition of exogenous enzymes to poultry diets increases the nutritional value of the feed by promoting the decomposition of antinutrients and increasing the activity of digestive enzymes, thus improving the chicken performance.

Intestinal morphology

As shown in Figure 2, compared with CON, the villus height (VH) and crypt depth (CD) of duodenum in NCON significantly decreased ($P < 0.05$). The VH/CD of duodenum in NKA was significantly increased compared to NCON ($P < 0.05$).

No significant differences in VH, CD and VH/CD of jejunum in NCON were observed compared

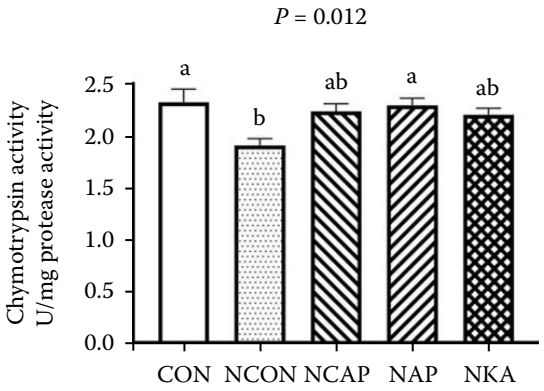

Figure 1. Effects of different proteases on chymotrypsin activity in the jejunum of broilers

[a,b]Means differ significantly ($P < 0.05$)

CON = the basal diet; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON

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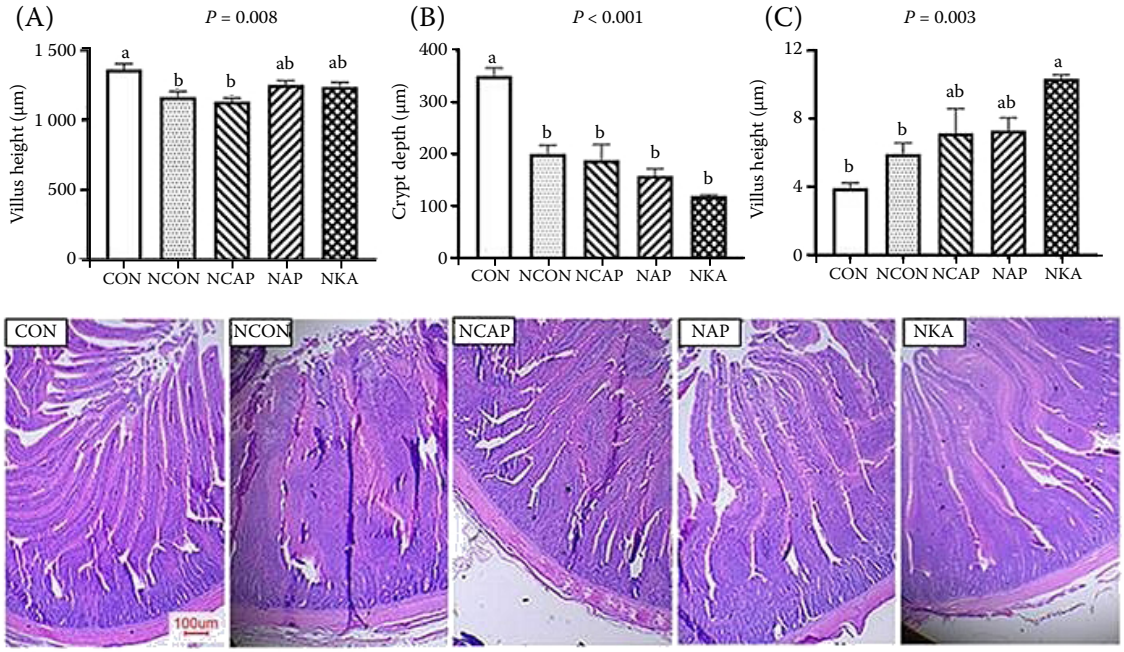

Figure 2. Effects of different proteases on duodenal morphology of broilers

Representative images of tissue samples are presented for general visualisation rather than for detailed morphological analysis

[a,b]Means differ significantly ($P < 0.05$)

CON = the basal diet; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON

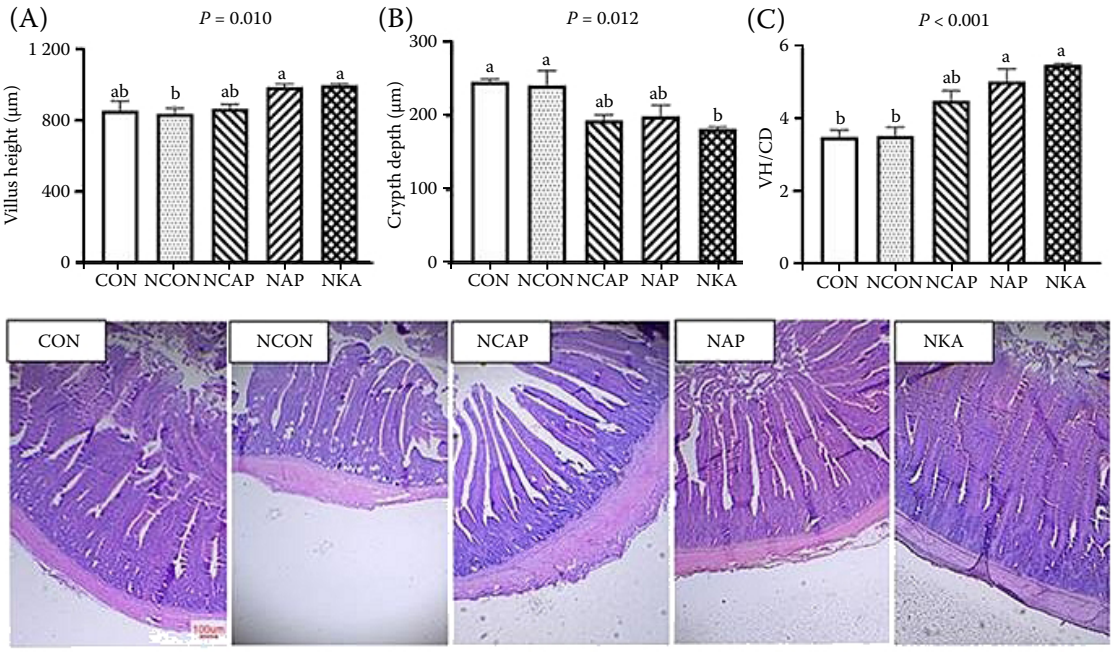

Figure 3. Effects of different proteases on jejunal morphology of broilers

Representative images of tissue samples are presented for general visualisation rather than for detailed morphological analysis

[a,b]Means differ significantly ($P < 0.05$)

CON = the basal diet; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON

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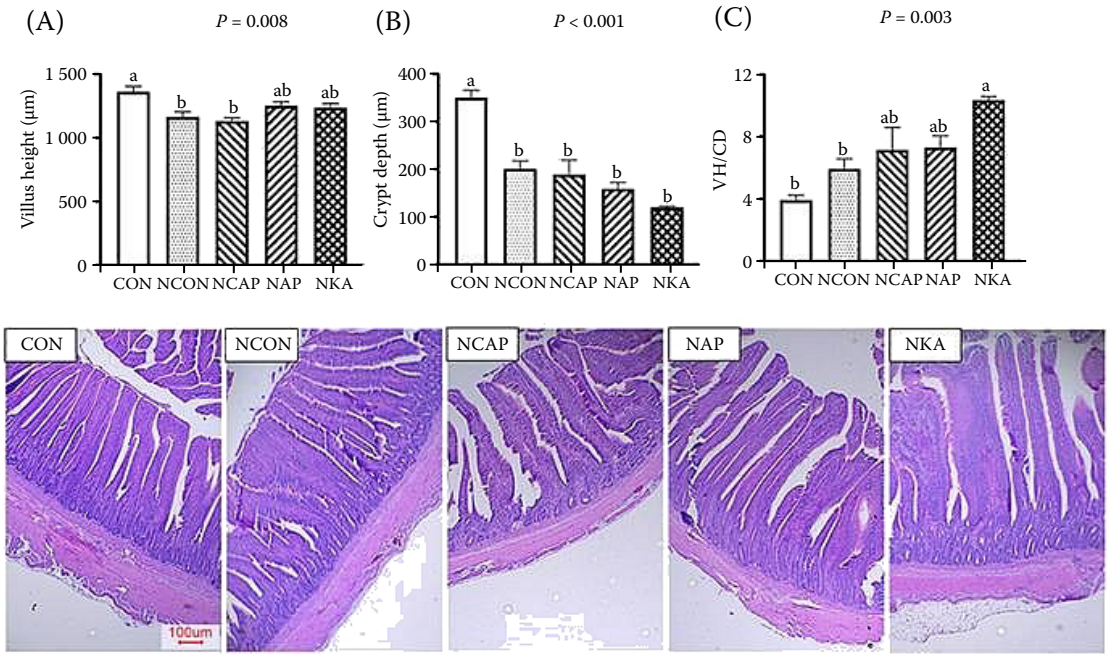

Figure 4. Effects of different proteases on ileal morphology of broilers

Representative images of tissue samples are presented for general visualisation rather than for detailed morphological analysis

[a,b]Means differ significantly ($P < 0.05$)

CON = the basal diet; NCAP, NAP and NKA = NCON diets supplemented with coated alkaline protease, alkaline protease and keratinase at the level of 12 000, 16 000 and 16 000 U/kg, respectively; NCON = a negative control with crude protein being 1% lower than in CON

with CON ($P > 0.05$, Figure 3). Compared with NCON, the CD of jejunum in NKA was significantly decreased ($P < 0.05$), while the VH and VH/CD of jejunum in NAP and NKA were significantly increased ($P < 0.05$).

No significant difference in VH, CD and VH/CD of ileum in NCON were observed compared with CON ($P > 0.05$, Figure 4). Compared with NCON, NKA showed a significant increase in ileal VH/CD and a decrease in CD ($P < 0.05$); NAP and NKA showed a significant increase in ileal VH and a decrease in CD ($P < 0.05$).

Intestinal morphology including the villus height, crypt depth and the villus height/crypt depth ratio is one of the major indicators of gut health in an animal. Lower quality and concentration of proteins can adversely impact intestinal development and function.

In our study, a 1% reduction in dietary crude protein reduced the small intestine villus height, however, adding alkaline protease (16 000 U/kg), coated alkaline protease (12 000 U/kg) and keratinase (16 000 U/kg) improved the small intestine villus height and villus height/crypt depth ratio, and reduced the crypt depth. The decreased villus height of duodenum, villus height and villus height/crypt depth ratio of jejunum, and increased crypt depth of ileum were observed in a 1% reduction in dietary crude protein of broiler chickens (Amer et al. 2021). Previous research found that the use of proteases significantly affected the size and integrity of the intestinal villi, while the protease supplementation improved the intestinal villus height and villus height/crypt depth ratio (Nabizadeh et al. 2017). This may be because the inclusion of protease increased the amount of digestible peptides available for the villus growth and the absorption capacity of the small intestine, thus improving the intestinal health of broilers.

CONCLUSION AND APPLICATIONS

1. Adding alkaline protease (16 000 U/kg), coated alkaline protease (12 000 U/kg) and keratinase (16 000 U/kg) to a diet of the negative control with a 1% reduction in the dietary crude protein level improved the F/G on 21–42 d and 0–42 d, making its growth performance comparable to that of the control diet.

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2. Adding alkaline protease (16 000 U/kg) resulted in a significant increase in ileal VH and a decrease in CD.
3. A 1% reduction in dietary crude protein decreased growth performance, nutrient digestibility and intestinal morphology in broilers; more interestingly alkaline protease (16 000 U/kg), encapsulated alkaline protease (12 000 U/kg) and keratinase (16 000 U/kg) improved the abovementioned conditions, with alkaline protease (16 000 U/kg) being more effective.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Almirall M, Francesch M, Perez-Vendrell AM, Brufau J, Esteve-Garcia E. The differences in intestinal viscosity produced by barley and beta-glucanase alter digesta enzyme activities and ileal nutrient digestibilities more in broiler chicks than in cocks. J Nutr. 1995 Apr;125(4): 947-55.

Amer SA, Beheiry RR, Abdel Fattah DM, Roushdy EM, Hassan FAM, Ismail TA, Zaitoun NMA, Abo-Elmaaty AMA, Metwally AE. Effects of different feeding regimens with protease supplementation on growth, amino acid digestibility, economic efficiency, blood biochemical parameters, and intestinal histology in broiler chickens. BMC Vet Res. 2021 Aug 25;17(1):283.

Angel CR, Saylor W, Vieira SL, Ward N. Effects of a monocomponent protease on performance and protein utilisation in 7- to 22-day-old broiler chickens. Poult Sci. 2011 Oct;90(10):2281-6.

AOAC – Association of Official Analytical Chemists. Official methods of chemical analysis, 19th ed. Gaithersburg,USA: Association of Official Analytical Chemists; 2012.

Cardinal KM, Moraes MLD, Andretta I, Schirmann GD, Belote BL, Barrios MA, Ribeiro AML. Growth performance and intestinal health of broilers fed a standard or low-protein diet with the addition of a protease. Rev Bras Zootec. 2019;48:e20180232.

Cowieson AJ, Singh DN, Adeola O. Prediction of ingredient quality and the effect of a combination of xylanase, amylase, protease and phytase in the diets of broiler chicks. 1. Growth performance and digestible nutrient intake. Br Poult Sci. 2006 Aug;47(4):477-89.

Duque-Ramirez CF, Javierre JA, Penuela-Sierra LM, Diaz-Vargas M. Effect of exogenous protease on performance, nutrient digestibility, intestinal histomorphometric, meat quality characteristics, carcass yield in broilers fed low protein diets. Trop Anim Health Prod. 2023 May 4; 55(3):190.

Engberg RM, Hedemann MS, Steenfeldt S, Jensen BB. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult Sci. 2004 Jun;83(6):925-38.

Fru-Nji F, Kluenter AM, Fischer M, Pontoppidan K. A feed serine protease improves broiler performance and increases protein and energy digestibility. J Poult Sci. 2011; 48(4):239-46.

Hofmann P, Siegert W, Kenez A, Naranjo VD, Rodehutscord M. Very low crude protein and varying glycine concentrations in the diet affect growth performance, characteristics of nitrogen excretion, and the blood metabolome of broiler chickens. J Nutr. 2019 Jul 1;149(7): 1122-32.

Kwak MJ, Ha DJ, Park MY, Eor JY, Whang KY, Kim Y. Comparison study between single enzyme and multienzyme complex in distiller's dried grains with soluble supplemented diet in broiler chicken. J Anim Sci Technol. 2024 Mar;66(2):398-411.

Leyva-Jimenez H, Burden Y, Soto C, McCormick K, Woodward A, Dirks B. Effect of supplementing a multi-carbohydrase enzyme complex to corn–soy-based diets on growth performance, intestinal digesta viscosity, and carcass traits of broiler chickens. J Appl Poult Res. 2024; 33(1):100387.

Liu Y, Liu S, Chen F, Liu S, Lin Q, Dai Q. Optimizing the zymogram of exogenous proteases in broiler diets by in vitro simulated gastrointestinal digestion and response surface methodology. Sci Rep. 2025 Feb 24;15(1): 6605.

Mohammadigheisar M, Kim IH. Addition of a protease to low crude protein density diets of broiler chickens. J Appl Anim Res. 2018 Jan 1;46(1):1377-81.

Nabizadeh A, Golian A, Hassanabadi A, Zerehdaran S. Effects of nutrient density and exogenous enzymes in starter diet on performance, intestinal microflora, gut morphology and immune response of broiler chickens. Rev Bras Cienc Avic. 2017;19(3):509-18.

Rada V, Foltyn M, Lichovnikova M, Musilova A. Effects of protease supplementation of low protein broiler diets on growth parameters and carcass characteristics. Mendelnet. 2013;2013:268-72.

Sarica S, Yurtseven S, Polat I. Supplementing diets for broilers that are low in crude protein and amino acids with protease. S Afr J Anim Sci. 2020;50(4):521-8.

https://doi.org/10.17221/18/2025-CJAS

Shad AA, Ahmad T, Iqbal MF, Asad MJ. Effects of a novel protease from Bacillus subtilis K-5 in low protein corn distiller dried grains with solubles (cDDGS) based diets on performance and nutrients digestibility in broiler chickens. Braz J Poult Sci. 2022;24(2):eRBCA-2021.

Short FJ, Gorton P, Wiseman J, Boorman KN. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim Feed Sci Technol. 1996;59(4): 215-21.

Sibbald IR. A bioassay for true metabolizable energy in feedingstuffs. Poult Sci. 1976;55(1):303-8.

Wang H, Guo Y, Shih JC. Effects of dietary supplementation of keratinase on growth performance, nitrogen retention and intestinal morphology of broiler chickens fed diets with soybean and cottonseed meals. Anim Feed Sci Technol. 2008 Jan 15;140(3-4):376-84.

Wang C, Yuan T, Yang J, Zheng W, Wu Q, Zhu K, Mou X, Wang L, Nie K, Li X, Zhu Y. Responses of combined non-starch polysaccharide enzymes and protease on growth performance, meat quality, and nutrient digestibility of yellow-feathered broilers fed with diets with different crude protein levels. Front Vet Sci. 2022a Jul 18;9:946204.

Wang T, Ling H, Zhang W, Zhou Y, Li Y, Hu Y, Peng N, Zhao S. Protease or Clostridium butyricum addition to a low-protein diet improves broiler growth performance. Appl Microbiol Biotechnol. 2022b Dec;106(23): 7917-31.

Yu CY, Guo YX, Yang ZB, Yang WR, Jiang SZ. Effects of star anise (Illicium verum Hook.f.) essential oil on nutrient and energy utilisation of laying hens. Anim Sci J. 2019;90: 880-6.

Zhang GF, Yang ZB, Wang Y, Yang WR, Jiang SZ, Gai GS. Effects of ginger root (Zingiber officinale) processed to different particle sizes on growth performance, antioxidant status, and serum metabolites of broiler chickens. Poult Sci. 2009;88(10):2159-66.

Zhang GG, Yang ZB, Wang Y, Yang WR, Zhou HJ. Effects of dietary supplementation of multi-enzyme on growth performance, nutrient digestibility, small intestinal digestive enzyme activities, and large intestinal selected microbiota in weanling pigs. J Anim Sci. 2014 May;92(5): 2063-9.

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