

Responses in growth performance and nutrient digestibility to a multi-protease supplementation in amino acid-deficient broiler diets

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Abstract

The present study was conducted to investigate the effect of a multi-protease on production indicators of broiler chickens fed a crude protein and amino acid deficient-diets for 35 days immediately after hatch. A total of 448 one-day-old Ross 308 male broiler chicks were allocated in a completely randomized design into one of eight dietary treatments (positive control [PC], negative control [NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine], extreme negative control [ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine], and plus multi-protease 150 or 300 g per ton [e. g., PC-150]; PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300) to give eight replicates with seven birds in a battery cage. Body weight, average daily gain, average daily feed intake, feed conversion ratio, and mortality were measured every week. Carcass traits, proximate analysis of breast meat, and ileum digestibility were analyzed on day 21 and 35. Feeding a multi-protease (i.e., more than 150 g/ton) for 35 days immediately after hatching improved feed efficiency and ileum digestibility (i.e., dry matter, crude protein, and energy) compared to their counterparts (i.e., diets without multi-protease: PC, NC, and ENC). In conclusion, our results indicated that broiler chickens fed nutrients deficient-diet (i.e., crude protein and amino acids) supplemented a multi-protease had an ability to compensate and (or) improve their growth performance commensurate with increased ileal digestibility for 35 days immediately after hatch.

Keywords: Amino acids, Broiler chickens, Crude protein, Ileal digestibility, Multi-protease

INTRODUCTION

Exogenous enzymes have been used in the poultry industry for a long time, and in recent years, interest in feed enzyme technology to maintain or improve growth performance as a result of increased feed

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Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and material

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

Authors' contributions

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Ethics approval and consent to participate

The experimental protocol used in this study was approved by the Animal Ethics Committee of the Chungnam National University (201909A-CNU-127).

costs and environmental concerns has emerged [1–3]. It is well documented that enzymes accelerate the digestion of ingredients and improve the assimilation of less digestible feed as a result of catalysts secreted by cells in the gastrointestinal tract of broilers [4,5].

Dietary proteases have been used to improve protein availability by breaking down protein-bound starch within feed ingredients [6,7]. The proteases improve digestion by separating the protein-starch bonds effectively and this facilitates the use of extra energy by the animal [8–10]. Adeola and Cowieson [11] demonstrated that broilers fed a diet supplemented with a dietary protease had a higher protein utilization compared to those fed a diet without a dietary protease. Moreover, multiple proteases with different pH optima and substrate specificity have been proposed to improve the digestion of soybean meal, and thus increasing protein intake and utilization, improving poultry physiology, and reducing environmental impact [12,13].

The combined action of different forms (i.e., alkaline and neutral) of proteases can be synergistically used in the animal's digestive tract by increasing free amino nitrogen content compared with the supplementation of a single protease [14,15]. This suggests that supplementation with a multi-protease could be cost-effective by reducing crude protein (CP) and synthetic amino acids (AA) in the feed formulation due to the increased bioavailability of AA in supplied protein [16–18]. In this light, the present study was designed to determine the effects of a multi-protease on the growth performance, carcass traits, proximate analysis of breast meat, and ileal digestibility of broilers fed CP- and AA-deficient diets. The hypothesis conducted was that supplementation of multi-protease into AA-deficient diets would improve growth performance and nutrient digestibility in broilers for 35 days after hatching.

MATERIALS AND METHODS

Animals and managements

The experiment was conducted by using a total of 448 one-day-old Ross 308 male broiler chickens for 35 days. Birds were allocated into eight dietary treatments. Each dietary treatment contained eight replicate pens. Seven birds were housed in each raised battery cage (76 × 61 × 46 cm³), with similar body weight (BW) (36.96 ± 0.37 g; *p* > 0.05). Birds were offered the experimental diets on an *ad libitum* basis and had free access to fresh and clean drinking water via nipple drinkers throughout the experimental period.

Experimental design, diets and treatments

The experiment was conducted using a completely randomized design. The eight dietary treatments were: (1) conventional standard positive control (PC, PC_{1–3} week: 21.5% CP, 3,060 kcal/kg metabolizable energy, and PC_{4–5} week: 19.5% CP, 3,200 kcal/kg metabolizable energy), (2) PC plus multi-protease 150 g per ton (PC-150), (3) CP (minus 0.5% from PC) and AA (minus 2% of lysine, methionine, threonine and methionine plus cysteine) deficient-diet (negative control [NC, NC_{1–3} week: 21.0% CP, 3,060 kcal/kg metabolizable energy, and NC_{4–5} week: 19.0% CP, 3,200 kcal/kg metabolizable energy), (4) NC plus multi-protease 150 g per ton (NC-150), (5) NC plus a multi-protease 300 g per ton (NC-300), (6) CP (minus 1% from PC) and AA (minus 4% of lysine, methionine, threonine and methionine plus cysteine) deficient-diet (extreme negative control [ENC], ENC_{1–3} week: 20.5%, 3,060 kcal/kg metabolizable energy, and ENC_{4–5} week: 18.5%, 3,200 kcal/kg metabolizable energy), (7) ENC plus multi-protease 150 g per ton (ENC-150), (8) ENC plus multi-protease 300 g per ton (ENC-300). Corn and SBM control diets were formulated to meet the Ross 308 nutrition specification [19] (Tables 1 and 2).

In addition, Cr₂O₃ (Chromium oxide powder, > 99.9% purity, Sigma-Aldrich, St. Louis, MO,

Table 1. Ingredient composition and calculated composition of the basal diets for manufacturing the experimental diets¹⁾

	PC_1-3	PC_4-5	NC_1-3	NC_4-5	ENC_1-3	ENC_4-5
Ingredients (%)						
Corn	48.51	60.96	48.96	62.93	48.64	62.84
Wheat	8.40	4.12	10.09	3.68	11.42	5.63
Wheat bran	4.10	-	3.70	-	4.30	-
SBM 48%	31.15	27.62	29.60	26.37	27.98	24.70
Vegetable oil	3.30	3.30	3.00	3.00	3.00	2.80
Limestone	1.20	0.95	1.20	0.96	1.20	0.97
Monocal P	1.65	1.39	1.70	1.39	1.70	1.39
Salt	0.30	0.35	0.35	0.35	0.35	0.35
Vitamin-mineral premix ²⁾	0.30	0.30	0.30	0.30	0.30	0.30
Lysine-HCl	0.34	0.30	0.36	0.31	0.37	0.33
DL-Methionine	0.20	0.19	0.19	0.18	0.19	0.17
L-Threonine	0.13	0.11	0.13	0.12	0.13	0.11
L-Cystine	0.12	0.11	0.12	0.11	0.12	0.11
Chromium oxide powder (Cr ₂ O ₃ , maker) ³⁾	0.30	0.30	0.30	0.30	0.30	0.30
Calculated composition ⁴⁾						
Metabolizable energy (kcal/kg)	3,060	3,200	3,060	3,200	3,060	3,200
Crude protein (%)	21.50	19.50	21.00	19.00	20.50	18.50
SID lysine (g/MJ ME)	0.94	0.80	0.92	0.79	0.90	0.77
Calculated SID amino acids (%)						
Lysine	1.20	1.08	1.18	1.05	1.15	1.03
Methionine	0.48	0.45	0.46	0.44	0.46	0.42
Cystine	0.43	0.40	0.43	0.39	0.42	0.39
Threonine	0.82	0.74	0.80	0.73	0.78	0.70
Tryptophan	0.22	0.19	0.21	0.18	0.20	0.18
Iso-leucine	0.75	0.68	0.72	0.66	0.70	0.64
Leucine	1.56	1.49	1.52	1.46	1.48	1.42
Valine	0.85	0.78	0.82	0.76	0.80	0.73
Histidine	0.54	0.49	0.52	0.48	0.51	0.46
Arginine	1.25	1.10	1.20	1.06	1.16	1.01
Methionine + cystine	0.91	0.85	0.89	0.83	0.88	0.81

¹⁾The eight dietary treatments were: positive control (PC), negative control (NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine), extreme negative control (ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine), and plus multi-protease 150 or 300 g per ton (e. g., PC-150); PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300.

²⁾Provided per kilogram of diet: vitamin A, 12,000 IU; vitamin D₃, 2,500 IU; vitamin E, 30 IU; vitamin K₃, 3 mg; D-pantothenic acid, 15 mg; nicotinic acid, 40 mg; choline, 400 mg; and vitamin B₁₂, 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.

³⁾Chromium oxide powder (Cr₂O₃; > 99.9% purity, Sigma-Aldrich, USA).

⁴⁾The values were calculated according to the values of feedstuffs [20].

SBM, soybean meal; SID, standardized ileal digestible.

USA) was added as an internal indigestible marker for digestibility analysis in a proportion of 0.3% to all experimental eight diets. This study has employed Kemzyme[®] protease (Kemin Industries Pte Limited., Singapore) as a multi-protease.

Table 2. Analyzed chemical composition of the experimental diets¹⁾

	PC_1-3	PC_4-5	NC_1-3	NC_4-5	ENC_1-3	ENC_4-5
Analyzed composition						
Gross energy (kcal/kg)	3,460	3,600	3,460	3,600	3,460	3,600
Crude protein (%)	21.52	19.52	21.03	19.02	20.55	18.52
Total amino acid (%)						
Lysine	1.34	1.20	1.32	1.17	1.29	1.15
Methionine	0.53	0.48	0.51	0.47	0.50	0.45
Cystine	0.48	0.44	0.47	0.43	0.46	0.43
Threonine	0.95	0.85	0.92	0.84	0.89	0.81
Tryptophan	0.24	0.22	0.24	0.21	0.23	0.20
Iso-Leucine	0.87	0.78	0.84	0.76	0.82	0.73
Leucine	1.02	0.93	1.00	0.90	0.97	0.88
Valine	0.98	0.89	0.95	0.86	0.93	0.84
Histidine	0.61	0.56	0.59	0.54	0.58	0.52
Arginine	1.41	1.26	1.37	1.22	1.32	1.18
Methionine + cystine	1.00	0.93	0.98	0.90	0.96	0.88

¹⁾The eight dietary treatments were: positive control (PC), negative control (NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine), extreme negative control (ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine), and plus multi-protease 150 or 300 g per ton (e. g., PC-150); PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300.

Growth performance evaluation

BW was recorded at the start and on every week of the experimental periods, and feed consumption was recorded on the same date of BW recording to calculate average daily gain, average daily feed intake, and feed conversion ratio.

Post-mortem procedure and sample collection

Eight birds per treatment (one bird per cage) were selected randomly and euthanized by cervical dislocation for sample collection on day 21 and 35. After evisceration, empty bodies were weighed. Drumsticks (skinless) and breast meat were removed from carcasses and weighed. The empty BW, drumstick, and breast meat weight were expressed as proportions relative to slaughter live BW.

Laboratory analysis

The digesta samples were pre-dried at 55 °C for 24 h, ground through a 0.75 mm sieve (ZM 200 Ultra-Centrifugal Mill, Retsch GmbH & Co. KG, Haan, Germany), and analyzed for levels of dry matter, CP (Macro-Kjeldahl, $N \times 6.25 [1/0.16 = 6.25]$ to convert nitrogen content into protein content), ether extract, and gross energy according to the methodologies of AOAC [21]. Chromium oxide concentration of the feed and digesta samples were also analyzed [22]. The digestibility coefficient of nutrients was calculated as described by [23] using the following equation:

$$\text{Digestibility coefficient} = 1 - \frac{([\text{ID} \times \text{AF}])}{([\text{IF} \times \text{AD}]})$$

Where ID is the concentration of an indigestible marker in the diet; IF is the indigestible-marker concentration in ileal digesta; AF is the nutrient concentration in ileal digesta and AD is the nutrient concentration in the diet.

The AA contents in the diets were measured according to a method described by Cohen [24]. Samples were freeze-dried and grounded through a laboratory hammermill (1 mm screen; Cyclotec

1093, Tecator AB, Höganäs, Sweden). A 200–300 mg sample was hydrolyzed with acid (6 M hydrochloric acid [HCl]) to convert protein-bound AA into free AA. The AA in the hydrolysate then underwent pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. For tryptophan analysis, a separate 50–100 mg sample was hydrolyzed in an alkaline solution (5 M sodium hydroxide [NaOH]) and neutralized before pre-column derivatization. The AA derivatives were then separated and quantified by reversed-phase high-performance liquid chromatography (HPLC; ACQUITY UPLC system with ultraviolet [UV] detector, Waters Corporation, Milford, MA, USA). For all analyses, a Waters AccQ-Tag Ultra column (BEH C18, 2.1 × 100 mm²; 1.7 μm) was used with column temperature at 55 °C, detection at 260 nm, and the flow rate 0.7 mL/min.

Gene expression analysis

The intestinal samples (n = 6 per treatment group) were collected from broiler chickens at day-21 and day-35, respectively. Total RNA was extracted from using the HiGene™ total RNA prep kit (BIOFACT, Daejeon, Korea). Their concentration and quality of RNAs were checked by NanoDrop®ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). cDNAs were synthesized by using Quantitect® Reverse Transcription Kit (Qiagen, GmbH, Hilden, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out utilizing a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), SFCgreen®I (BIOFACT) and the following gene-specific primers and beta-actin (Bioneer, Daejeon, Korea); ACC (forward) 5'-AATGGCAGCTTTGGAGGTGT-3', (reverse) 5'-TCTGTTTGGGTGGGAGGTG-3', PEPT1 (forward) 5'-CCCCTGAGGAGGATCACTGTT-,3' (reverse) 5'-CCTTGCAGTGGGCAGGAA-3', GLUT2 (forward) 5'-CACACTATGGGCGCATGCT-3', (reverse) 5'-ATTGTCCCTGGAGGTGTTGGTG-3', beta-actin (forward) 5'-CCACCGCAAATGCTTCTAAAC-3', (reverse) 5'-AAGACTGCTGCTGACACCTTC-3'. The relative quantification of gene expression was determined by the 2^{-ΔΔCt} method and beta-actin was used as an internal control for quantification of target genes.

Calculations and statistical analysis

Data were analyzed according to a completely randomized design, using the general linear model (GLM) procedure of one-way ANOVA of SPSS software (Version 21; IBM SPSS 2012). The pen was used as the experimental unit for all growth performance measurements. Selected individual birds were considered as the experimental unit for the proportion of carcass trait weights, respectively. Mean differences were considered significant at $p < 0.05$. When treatment effects were significant ($p < 0.05$), means were separated using Turkey's multiple range test procedures of SPSS software. To determine the optimum multi-protease level in diets, linear-plateau and quadratic-plateau regression analysis were conducted using a Nutritional Response Model (Version 1.1; 16) as described previously by Wickramasuriya et al. [25].

RESULTS

Growth performance

Table 3 shows the effect of a multi-protease feeding of a crude protein and AA-deficient diet on growth performance for 35 days after hatching. Overall, the multi-protease-supplied groups had a higher BW than the non-multi-protease groups (PC, NC, and ENC); in particular, a higher BW was observed on days 7, 28, and 35 ($p < 0.05$). Average daily gain (ADG) on days 7 and 28 was higher with multi-protease ($p < 0.05$). There was no difference in average daily feed intake (ADFI)

Table 3. Effect of a multi-protease in the diet on growth performance of broiler chickens^{1,2)}

	PC	PC-150	NC	NC-150	NC-300	ENC	ENC-150	ENC-300	SEM	p-value ⁴⁾
Crude protein (%)	-	-	-0.5	-0.5	-0.5	-1	-1	-1		
Amino acid ³⁾ (%)	-	-	-2	-2	-2	-4	-4	-4		
Day 7										
BW (g)	177.26 ^{ab}	180.38 ^b	171.52 ^{ab}	174.51 ^{ab}	179.56 ^a	168.59 ^b	170.30 ^a	174.49 ^{ab}	1.073	0.038
ADFI (g/bird)	23.40	23.34	26.30	24.03	23.42	22.92	24.66	24.17	0.546	0.867
ADG (g/bird)	19.62 ^{ab}	20.08 ^a	18.81 ^{ab}	19.20 ^{ab}	19.96 ^a	18.35 ^b	18.61 ^b	19.23 ^{ab}	0.154	0.032
FCR (g feed/g gain)	1.19	1.16	1.39	1.26	1.17	1.25	1.33	1.26	0.027	0.387
Day 14										
BW (g)	440.81	447.71	431.39	434.76	446.74	428.26	429.27	433.87	2.065	0.099
ADFI (g/bird)	49.36	47.12	54.24	48.46	48.83	49.82	52.72	49.20	0.948	0.641
ADG (g/bird)	37.65	38.19	37.12	37.18	38.17	37.10	37.00	37.05	0.318	0.954
FCR (g feed/g gain)	1.32	1.24	1.46	1.31	1.29	1.34	1.43	1.34	0.018	0.061
Day 21										
BW (g)	872.90	877.47	865.42	868.59	876.84	857.43	863.96	871.57	4.266	0.950
ADFI (g/bird)	89.93	85.66	81.49	90.16	86.3	88.91	86.16	83.85	1.112	0.504
ADG (g/bird)	61.73	61.39	62.00	61.98	61.44	61.31	62.1	62.53	0.557	0.999
FCR (g feed/g gain)	1.47	1.41	1.32	1.46	1.42	1.45	1.39	1.36	0.016	0.302
Day 28										
BW (g)	1,446.29 ^{ab}	1,455.18 ^a	1,416.61 ^{ab}	1,426.38 ^a	1,448.13 ^a	1,359.90 ^b	1,410.83 ^{ab}	1,435.59 ^a	7.123	0.020
ADFI (g/bird)	122.32	115.41	125.26	116.15	115.32	122.68	118.17	120.80	1.672	0.747
ADG (g/bird)	81.91 ^a	82.53 ^a	78.74 ^a	79.68 ^a	81.61 ^a	71.78 ^b	78.13 ^{ab}	80.57 ^a	0.832	0.032
FCR (g feed/g gain)	1.52 ^{bc}	1.40 ^b	1.61 ^{ab}	1.46 ^b	1.44 ^b	1.71 ^a	1.52 ^{bc}	1.50 ^{bc}	0.018	< 0.001
Day 35										
BW (g)	2,188.27 ^a	2,222.38 ^a	2,131.93 ^{abc}	2,157.62 ^{abc}	2,188.90 ^a	2,062.95 ^c	2,083.53 ^{bc}	2,166.81 ^{ab}	12.046	0.009
ADFI (g/bird)	175.89	172.84	162.39	173.92	179.16	175.39	177.95	174.70	2.573	0.856
ADG (g/bird)	106.00	109.60	102.19	104.46	105.82	100.44	96.10	104.46	1.121	0.110
FCR (g feed/g gain)	1.68	1.58	1.58	1.71	1.72	1.77	1.89	1.69	0.029	0.203
Day 1–21										
ADFI (g/bird)	54.23	52.04	54.01	54.22	52.85	53.88	54.51	52.41	0.609	0.962
ADG (g/bird)	39.67	39.89	39.31	39.45	39.86	38.92	39.23	39.60	0.203	0.945
FCR (g feed/g gain)	1.33	1.27	1.39	1.34	1.29	1.35	1.38	1.32	0.012	0.172
Day 22–35										
ADFI (g/bird)	149.11	144.12	143.83	145.04	147.24	149.03	148.06	147.75	1.839	0.993
ADG (g/bird)	93.95 ^a	96.06 ^a	90.47 ^{abc}	92.07 ^{abc}	93.72 ^a	86.11 ^c	87.11 ^{bc}	92.52 ^{ab}	0.743	0.006
FCR (g feed/g gain)	1.60 ^{ab}	1.49 ^b	1.61 ^{ab}	1.46 ^{ab}	1.44 ^{ab}	1.71 ^a	1.52 ^a	1.50 ^{ab}	0.019	0.033
Day 1–35										
ADFI (g/bird)	92.18	88.87	89.93	90.55	90.61	91.94	91.93	90.54	0.946	0.991
ADG (g/bird)	61.38 ^a	62.36 ^a	59.77 ^{abc}	60.50 ^{abc}	61.40 ^a	57.80 ^c	58.38 ^{bc}	60.77 ^{ab}	0.344	0.009
FCR (g feed/g gain)	1.43 ^{ab}	1.36 ^b	1.48 ^a	1.44 ^{ab}	1.41 ^{ab}	1.51 ^a	1.51 ^a	1.43 ^{ab}	0.012	0.030

¹⁾The eight dietary treatments were: positive control (PC), negative control (NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine), extreme negative control (ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine), and plus multi-protease 150 or 300 g per ton (e. g., PC-150); PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300.

²⁾Values are the mean of eight replicates per treatment.

³⁾List of amino acids: lysine, methionine, threonine and methionine + cysteine.

⁴⁾There are no significance linear-plateau and quadratic-plateau regression obtain on growth performance for 5 weeks after hatching.

^{a-c)}Means with different superscripts in the same row were significantly different (Turkey's multiple range test, $p < 0.05$).

BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

among treatments during the 35 days ($p > 0.05$). Feed conversion ratio (FCR) was lower ($p < 0.05$) in the groups fed with multi-protease compared to groups without multi-protease on day 28. However, linear-plateau and quadratic-plateau regression analyses did not reflect any improvement in growth performance among treatments ($p > 0.05$). Feeding a diet with multi-proteases improves the growth performance of broilers.

Carcass traits

The effects of a multi-protease on carcass traits of 21- and 35-day-old broilers are shown in Table 4. The empty BW and weight of drumsticks were not different among feed treatments in the absence or presence of a multi-protease by days 21 and 35 ($p > 0.05$). On the other hand, the percentage of breast meat was increased in groups fed a multi-protease compared to each control group (PC, NC, and ENC; $p < 0.05$). Therefore, when comparing the results of NC and ENC in the absence or presence of a multi-protease, protein accumulation could have improved the deposition of breast meat in the broilers.

Breast meat quality

In the standard and protein and AA-deficient diets, the quality of breast meat was compared in broilers at days 21 and 35 with or without a multi-protease (Table 5). Overall, no effects of dietary treatment were found on the moisture, CP, crude fat, and crude ash ($p > 0.05$). However, a numerical tendency towards an increased percentage of CP in the breast meat was observed in broilers fed a multi-protease under ENC on day 35 (ENC, 20.78%; ENC-150, 20.94%; ENC-300, 21.21%; $p > 0.10$).

Ileal digestibility

The effect of a multi-protease in protein and AA-deficient diets on ileal digestibility in broilers on days 21 and 35 are presented in Table 6. Feeding a diet with a multi-protease (i.e., up to 150 g/ton) improved ($p < 0.05$) ileal digestibility of dry matter and CP on days 21 and 35 compared to those fed without a multi-protease (i.e., PC, NC, and ENC).

Table 4. Effect of a multi-protease in the diet on carcass trait of broiler chickens^{1,2)}

	PC	PC-150	NC	NC-150	NC-300	ENC	ENC-150	ENC-300	SEM	p-value
Crude protein (%)	-	-	-0.5	-0.5	-0.5	-1	-1	-1		
Amino acid ³⁾ (%)	-	-	-2	-2	-2	-4	-4	-4		
Day 21										
Empty BW (%)	89.63	89.75	89.47	89.35	86.86	89.33	90.01	89.51	0.300	0.409
Drumstick (%)	8.37	8.47	8.07	8.65	8.14	8.66	8.22	8.36	0.077	0.475
Breast meat (%)	21.09 ^{abc}	21.63 ^{ab}	20.69 ^{bc}	21.24 ^{ab}	21.88 ^a	20.16 ^c	21.37 ^{ab}	21.69 ^{ab}	0.115	0.006
Day 35										
Empty BW (%)	91.49	90.87	91.08	91.08	91.05	91.8	91.39	91.70	0.135	0.756
Drumstick (%)	8.49	8.13	8.44	8.53	8.09	8.30	8.40	8.56	0.098	0.945
Breast meat (%)	24.82 ^b	26.01 ^a	24.54 ^b	24.88 ^b	26.06 ^a	24.74 ^b	25.94 ^a	26.11 ^a	0.114	< 0.001

¹⁾The eight dietary treatments were: positive control (PC), negative control (NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine), extreme negative control (ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine), and plus multi-protease 150 or 300 g per ton (e. g., PC-150); PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300.

²⁾Values are the mean of eight replicates per treatment.

³⁾List of amino acids: lysine, methionine, threonine and methionine + cysteine.

^{a-c)}Means with different superscripts in the same row were significantly different (Turkey's multiple range test, $p < 0.05$).

BW, body weight.

Table 5. Effect of a multi-protease in the diet on meat quality analysis of broiler chickens^{1,2)}

	PC	PC-150	NC	NC-150	NC-300	ENC	ENC-150	ENC-300	SEM	p-value
Crude protein (%)	-	-	-0.5	-0.5	-0.5	-1.0	-1.0	-1.0		
Amino acid ³⁾ (%)	-	-	-2.0	-2.0	-2.0	-4.0	-4.0	-4.0		
Day 21										
Moisture (%)	75.37	75.48	76.16	75.71	76.01	75.53	75.55	76.01	0.141	0.833
Crude protein (%)	20.99	21.11	20.95	20.83	20.63	20.64	20.73	20.52	0.077	0.745
Crude fat (%)	2.46	1.98	1.70	2.34	1.99	2.38	2.54	2.20	0.123	0.440
Crude ash (%)	1.19	1.43	1.19	1.11	1.37	1.45	1.18	1.27	0.043	0.271
Day 35										
Moisture (%)	75.71	76.08	75.73	75.91	75.07	75.07	75.83	75.26	0.143	0.577
Crude protein (%)	21.17	20.98	21.00	20.64	21.08	20.78	20.94	21.32	0.097	0.887
Crude fat (%)	1.96	1.76	2.21	2.20	2.46	2.74	1.73	2.09	0.093	0.139
Crude ash (%)	1.16	1.18	1.07	1.25	1.40	1.41	1.50	1.33	0.057	0.112

¹⁾The eight dietary treatments were: positive control (PC), negative control (NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine), extreme negative control (ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine), and plus multi-protease 150 or 300 g per ton (e. g., PC-150); PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300.

²⁾Values are the mean of eight replicates per treatment.

³⁾List of amino acids: lysine, methionine, threonine and methionine + cysteine.

Table 6. Effect of multi-protease in the diet on ileum digestibility co-efficient of broiler chickens^{1,2)}

	PC	PC-150	NC	NC-150	NC-300	ENC	ENC-150	ENC-300	SEM	p-value
Crude protein (%)	-	-	-0.5	-0.5	-0.5	-1.0	-1.0	-1.0		
Amino acid ³⁾ (%)	-	-	-2.0	-2.0	-2.0	-4.0	-4.0	-4.0		
Day 21										
Dry matter	0.79 ^{ab}	0.80 ^a	0.78 ^{bc}	0.80 ^{abc}	0.81 ^{ab}	0.77 ^c	0.78 ^{bc}	0.79 ^{bc}	0.002	< 0.001
Crude protein	0.70 ^{bc}	0.73 ^a	0.67 ^{de}	0.70 ^{bc}	0.71 ^{ab}	0.66 ^e	0.68 ^{cde}	0.69 ^{bcd}	0.003	< 0.001
Energy	0.79 ^{ab}	0.80 ^a	0.78 ^b	0.79 ^{ab}	0.79 ^{ab}	0.77 ^b	0.77 ^b	0.78 ^{ab}	0.003	0.030
Day 35										
Dry matter	0.81 ^{bc}	0.82 ^a	0.80 ^{cd}	0.81 ^{ab}	0.81 ^a	0.80 ^d	0.80 ^{cd}	0.81 ^{bc}	0.001	0.005
Crude protein	0.81 ^b	0.82 ^a	0.79 ^{bc}	0.80 ^{bc}	0.81 ^b	0.78 ^c	0.79 ^{bc}	0.79 ^c	0.002	< 0.001
Energy	0.80 ^{ab}	0.81 ^a	0.79 ^{bc}	0.80 ^{ab}	0.80 ^{ab}	0.78 ^c	0.78 ^c	0.79 ^{bc}	0.002	0.002

¹⁾The eight dietary treatments were: positive control (PC), negative control (NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine), extreme negative control (ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine), and plus multi-protease 150 or 300 g per ton (e. g., PC-150); PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300.

²⁾Values are the mean of eight replicates per treatment.

³⁾List of amino acids: lysine, methionine, threonine and methionine + cysteine.

^{a-e}Means with different superscripts in the same row were significantly different (Turkey's multiple range test, $p < 0.05$).

Gene expression analysis

The transcription levels of genes involved in the intestinal nutrient transporter genes, such as ACC (acetyl-CoA carboxylase), PEPT1 (peptide transporter 1), and GLUT2 (glucose transporter 2) were examined. There was no dramatic increase in expression over 2-fold, but in the multi-protease, treated groups, particularly NC-150 and ENC-150, the genes were upregulated (Fig. 1; $p > 0.10$).

DISCUSSION

A study related to enzyme ability in corn-soybean meal-based diets demonstrated that specific enzyme activities could enhance the absorption of nutrients in broiler intestines [26]. Thus, research

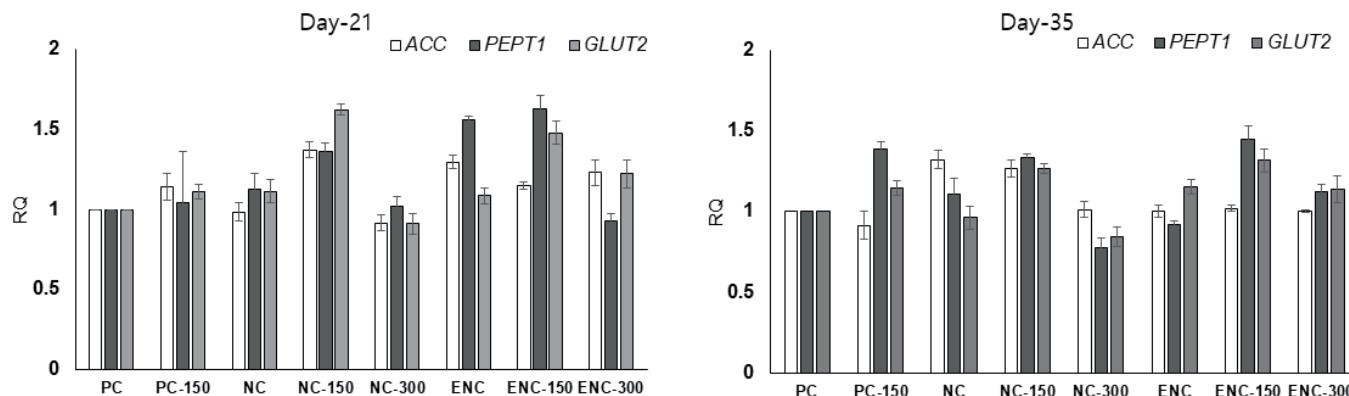


Fig. 1. RT-PCR analysis: gene expression was analyzed in intestinal samples. The eight dietary treatments were: positive control (PC), negative control (NC: minus 0.5% from PC, and minus 2% of lysine, methionine, threonine and methionine plus cysteine), extreme negative control (ENC: minus 1% from PC, minus 4% of lysine, methionine, threonine and methionine plus cysteine), and plus multi-protease 150 or 300 g per ton (e. g., PC-150); PC, PC-150, NC, NC-150, NC-300, ENC, ENC-150, ENC-300. Statistical analysis was performed using one-way ANOVA and Tukey's post hoc test for multiple comparisons. ACC, acetyl-CoA carboxylase; PEPT1, peptide transporter 1; GLUT2, glucose transporter 2; RQ, relative quantification.

on exogenous enzymes (i.e., carbohydrase, phytase, and protease) has been conducted to improve the digestibility of nutrients in animals [27,28]. The addition of exogenous proteases to poultry and swine diets has been attempted over the past decades to improve the status of deficient endogenous peptidases in the neonate.

There are inconsistent results from protease incorporation into the lower protein diets in previous studies because the accessibility of exogenous enzymes highly depends on the protein matrices and cell wall structure of the protein sources. Therefore, it is important to consider the protein structure and cell wall components present in the protein sources in low protein diets to obtain better broiler performance by adding protease [29].

Production of digestive enzymes that remove intestinal endotoxins is insufficient for 21 days after hatching because pancreatic proteases predominate in the initial post-hatch period [30]. In addition, poultry less than 14 days of age has less developed viscera and there is a shortage of endogenous digestive enzyme secretions, so supplementation of an exogenous enzyme is more effective at this stage [31,32]. Lower protein diets with exogenous proteases can improve negative environmental effects by reducing nitrogen excretion (i.e., fecal and urinary nitrogen) [18,33,34]. Incorporating exogenous protein enzymes into the feed to ameliorate the anti-nutritive effects of a protein source is a common strategy for poultry [35]. Numerous dietary enzymes (i.e., protein digestive enzymes) have long been used to maximize the production of broilers by improving the digestibility of nutrients [36,37]. There are reports of positive results in the supplementation of exogenous proteases in diet [36,38,39]. In the current study, the supplementation of multi-proteases in CP and AA deficient-diets has improved growth performance and nutrient digestibility in broilers.

The utilization of multi-proteases has been proven by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (via the separation of phosphoproteins) to involve trypsin alone, improving the protein sequence coverage due to the increasing diversity of the peptide sequences, and this can facilitate the identification of protein sequences lacking tryptic cleavage sites by application of different cleavage site specificities [40,41]. Non-starch polysaccharides (NSP) in cereals and soybeans provide physical barriers to prevent the action of exogenous enzymes on starch and protein [42]. However, the relationship between the starch granules and protein structures in broilers may disrupt the absorption of starch. In the absence of exogenous enzymes, starch granules are closely associated with the protein matrix, which impedes the accessibility of the endotoxin enzymes, which may re-

duce starch digestion in the small intestine [43]. Amylolytic enzymes can act upon the starch granules when the protein matrix has been digested by exogenous proteases [9]. This means that feeding a diet with exogenous proteases can help to reduce the requirement of energy and AA by assisting peptidase production [8].

In the results of growth performance, NC-300 (BW, day 7, 28, and 35; ADG, days 7, 28, 29–35, and 1–35) showed similar data to those of the PC diet. Low protein diets with supplementation of proteases (i.e., up to 200 mg/kg) showed similar results to the growth performance of standard CP diets [36]. Several studies have reported improvements in weight gain and FCR under low CP diets with exogenous proteases compared to standard protein diets [39]. The main effect of multi-protease on the final BW explains a maximum of 5.03% of the variance (ENC vs. ENC-300). The results demonstrated that the cumulative supplementation of protein enzymes had a significant increase in the BW by improving the digestibility of CP and energy.

The study was designed to compare CP- and AA-deficient diets with a standard (control) diet. Supplementation of exogenous proteases could complement the activity of endogenous proteases, thus showing significant effects in the case of this study. It is the supplementation of proteases that directly affect the digestibility of AA.

Environment and other management factors influence the use of nutrients indirectly. These indirect external factors include disease status, nutrient balance, feed processing conditions, age, and animal species. There have been reports of changes in apparent ileal AA digestibility in a range of standard diets depending on the age of the animals [44]. AA digestibility may theoretically increase with the age of animals. However, AA digestibility on day 42 decreased on wheat and canola meal diets (day 42: 75.8% vs. day 14: 78.7%) compared to that on day 14 [44]. The above description may be beyond the scope of the present study. The changes in AA digestibility with animal age are explained in the literature [44–46]. In addition to the growth stage, various other factors modulate the digestibility of AA in the diets of pigs and broilers, and so will invariably influence protease efficacy. Other than animal age, there are also factors (e.g., dietary fat concentration, xylanase, phytase, dietary calcium or limestone inclusion, supplementary AA and AA density, ingredient type, and quality, anti-nutrients, and hydrothermal conditioning) that alter AA digestibility in poultry, which may be closely related to the efficacy of proteases [27,47,48,50]. Nonetheless, poultry fed a diet supplemented exogenous proteases are able to improve protein digestibility regardless of those factors.

Supplying proteases to maximize the utilization of protein is an opportunity to compensate for the lack of AA [50]. Although there was no significant difference in empty BW and drumstick weight, the breast meat yield was higher in diets with multi-proteases in the present study (i.e., up to 150 g/ton). The factors affecting the breast meat of broilers in this study might be attributed to the availability of AA. Supplementation of lysine, methionine, and valine in diets may have a direct effect on breast meat of broilers [51,52]. To increase breast meat in broilers, a study on the improvement of AA digestibility through exogenous protease supplementation is needed.

The improvement of nutrient digestibility due to multi-protease could influence and/or up-regulate the expression of genes related to protein digestible transporters in the intestine [53,54]. In this light, we have expected that those transporters and/or related genes would be altered by the multi-protease. However, multi-protease supplementation improved the ileum digestibility of dry matter, crude protein, and energy but did not increase the expression of ACC, PEPT1, and GLUT2 in the present study. Although the unexpected results could be due to unknown responses of gene expression or a limited number of samples or higher variation, which is an important part of data for future investigations intending to conduct advanced poultry productivity.

In conclusion, multi-proteases added (up to 150 g/ton) to CP- and AA-deficient diets for 35 days improve both ADG (PC vs. PC-150, 1.60%; NC vs. NC-150, 1.22%; NC vs. NC-300, 2.73%;

ENC vs. ENC-150, 1.00%; ENC vs. ENC-300, 5.14%) and FCR (PC vs. PC-150, 5.15%; NC vs. NC-150, 2.78%; NC vs. NC-300, 4.96%; ENC vs. ENC-300, 5.59% without ENC vs. ENC-150) in broilers compared to standard diets.

REFERENCES

1. Amerah AM, Romero LF, Awati A, Ravindran V. Effect of exogenous xylanase, amylase, and protease as single or combined activities on nutrient digestibility and growth performance of broilers fed corn/soy diets. *Poult Sci.* 2017;96:807-16. <https://doi.org/10.3382/ps/pew297>
2. Cowieson AJ, Lu H, Ajuwon KM, Knap I, Adeola O. Interactive effects of dietary protein source and exogenous protease on growth performance, immune competence and jejunal health of broiler chickens. *Anim Prod Sci.* 2017;57:252-61. <https://doi.org/10.1071/AN15523>
3. Yegani M, Korver DR. Effects of corn source and exogenous enzymes on growth performance and nutrient digestibility in broiler chickens. *Poult Sci.* 2013;92:1208-20. <https://doi.org/10.3382/ps.2012-02390>
4. Lu H, Adedokun SA, Preynat A, Legrand-Defretin V, Geraert PA, Adeola O, et al. Impact of exogenous carbohydrases and phytase on growth performance and nutrient digestibility in broilers. *Can J Anim Sci.* 2013;93:243-9. <https://doi.org/10.4141/cjas2012-138>
5. Amerah AM. Interactions between wheat characteristics and feed enzyme supplementation in broiler diets. *Anim Feed Sci Technol.* 2015;199:1-9. <https://doi.org/10.1016/j.anifeeds-ci.2014.09.012>
6. Iji PA, Hughes RJ, Choct M, Tivey DR. Intestinal structure and function of broiler chickens on wheat-based diets supplemented with a microbial enzyme. *Asian-Australas J Anim Sci.* 2001;14:54-60. <https://doi.org/10.5713/ajas.2001.54>
7. Selle PH, Liu SY, Cai J, Cowieson AJ. Steam-pelleting temperatures, grain variety, feed form and protease supplementation of mediumly ground, sorghum-based broiler diets: influences on growth performance, relative gizzard weights, nutrient utilisation, starch and nitrogen digestibility. *Anim Prod Sci.* 2013;53:378-87. <https://doi.org/10.1071/AN12363>
8. Cowieson AJ. Factors that affect the nutritional value of maize for broilers. *Anim Feed Sci Technol.* 2005;119:293-305. <https://doi.org/10.1016/j.anifeeds-ci.2004.12.017>
9. Han JA, BeMiller JN. Effects of protein on crosslinking of normal maize, waxy maize, and potato starches. *Carbohydr Polym.* 2008;73:532-40. <https://doi.org/10.1016/j.carbpol.2007.12.022>
10. du Plessis RE, van Rensburg CJ. Carbohydrase and protease supplementation increased performance of broilers fed maize-soybean-based diets with restricted metabolizable energy content. *S Afr J Anim Sci.* 2014;44:262-70. <https://doi.org/10.4314/sajas.v44i3.8>
11. Adeola O, Cowieson AJ. Board-invited review: opportunities and challenges in using exogenous enzymes to improve non-ruminant animal production. *J Anim Sci.* 2011;89:3189-218. <https://doi.org/10.2527/jas.2010-3715>
12. Kilic U, Saricicek BZ, Garipoglu AV. Effect of enzyme supplementation to the rations in which soybean meal replaced by Canola meal on performances of broilers. *Asian J Anim Vet Adv.* 2006;1:76-81. <https://doi.org/10.3923/ajava.2006.76.81>
13. Lim A, Yong FF, Tan HM, inventors. Kemin Industries, assignee. Use of a multi-protease system to improve the protein digestibility of animal feeds containing vegetable meals. United States patent US 8815315B2. 2014 Aug 26.
14. Michalski A, Cox J, Mann M. More than 100,000 detectable peptide species elute in single shotgun proteomics runs but the majority is inaccessible to data-dependent LC-MS/MS. *J*

- Proteome Res. 2011;10:1785-93. <https://doi.org/10.1021/pr101060v>
15. Sugumar C, Bindhu LV. Kemzyme map dry improves nutrient utilization of broiler diets: a challenge study [Internet]. Kemin Industries. 2013 [cited 2020 Sep 14]. https://www.kemin.com/content/dam/tech-lit/16_july_tl-13-00041_kemzyme_map_dry_improves_nutrient_utilization_of_broiler_diets_a_challenge_study.pdf
 16. He LS, Liu HL, Xi B, Zhu Y. Enhancing treatment efficiency of swine wastewater by effluent recirculation in vertical-flow constructed wetland. *J Environ Sci*. 2006;18:221-6.
 17. Shen Y, Yuan W, Pei Z, Mao E. Culture of microalga *Botryococcus* in livestock wastewater. *Trans ASABE*. 2008;51:1395-400. <https://doi.org/10.13031/2013.25223>
 18. Chen J, Nie Q, Zhang Y, Hu J, Qing L. Eco-physiological characteristics of *pistia stratiotes* and its removal of pollutants from livestock wastewater. *Water Sci Technol*. 2014;69:2510-8. <https://doi.org/10.2166/wst.2014.176>
 19. Aviagen W. Ross 308 broiler nutrition specifications. Huntsville, AL: Aviagen Group. 2014. <https://www.winmixsoft.com/files/info/Ross-308-Broiler-PO-2014-EN.pdf>
 20. NRC [National Research Council]. Nutrient requirements of poultry. Washington, DC: National Academies Press; 1994.
 21. AOAC [Association of Official Analytical Chemists] International. Official methods of analysis of AOAC International. 18th ed. Gaithersburg, MD: AOAC International; 2005.
 22. Fenton TW, Fenton M. An improved procedure for the determination of chromic oxide in feed and feces. *Can J Anim Sci*. 1979;59:631-4. <https://doi.org/10.4141/cjas79-081>
 23. Huang RL, Yin YL, Wu GY, Zhang YG, Li TJ, Li LL, et al. Effect of dietary oligochitosan supplementation on ileal digestibility of nutrients and performance in broilers. *Poult Sci*. 2005;84:1383-8. <https://doi.org/10.1093/ps/84.9.1383>
 24. Cohen SA. Amino acid analysis using precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. In: Cooper C, Packer N, Williams K, editors. Amino acid analysis protocols. Totowa, NJ: Humana Press; 2001. p. 39-47.
 25. Wickramasuriya SS, Yoo J, Kim JC, Heo JM. The apparent metabolizable energy requirement of male Korean native ducklings from hatch to 21 days of age. *Poult Sci*. 2016;95:77-83. <https://doi.org/10.3382/ps/pev321>
 26. Kocher A, Choct M, Porter MD, Broz J. The effects of enzyme addition to broiler diets containing high concentrations of canola or sunflower meal. *Poult Sci*. 2000;79:1767-74. <https://doi.org/10.1093/ps/79.12.1767>
 27. Cowieson AJ, Bedford MR. The effect of phytase and carbohydrase on ileal amino acid digestibility in monogastric diets: complementary mode of action? *World's Poult Sci J*. 2009;65:609-24. <https://doi.org/10.1017/S0043933909000427>
 28. Cowieson AJ. Strategic selection of exogenous enzymes for corn/soy-based poultry diets. *J Poult Sci*. 2010;47:1-7. <https://doi.org/10.2141/jpsa.009045>
 29. Leinonen I, Williams AG. Effects of dietary protease on nitrogen emissions from broiler production: a holistic comparison using Life Cycle Assessment. *J Sci Food Agric*. 2015;95:3041-6. <https://doi.org/10.1002/jsfa.7202>
 30. Krogdahl Å, Sell JL. Influence of age on lipase, amylase, and protease activities in the pancreatic tissue and intestinal contents of young turkeys. *Poult Sci*. 1989;68:1561-8. <https://doi.org/10.3382/ps.0681561>
 31. Meng X, Slominski BA. Nutritive values of corn, soybean meal, canola meal, and peas for broiler chickens as affected by a multicarbohydrase preparation of cell wall degrading enzymes. *Poult Sci*. 2005;84:1242-51. <https://doi.org/10.1093/ps/84.8.1242>
 32. Asmare B. Effect of common feed enzymes on nutrient utilization of monogastric animals. *Int*

- J Biotechnol Mol Biol Res. 2014;5:27-34. <https://doi.org/10.5897/IJBMBR2014.0191>
33. O'Connell JM, Callan JJ, O'Doherty JV. The effect of dietary crude protein level, cereal type and exogenous enzyme supplementation on nutrient digestibility, nitrogen excretion, faecal volatile fatty acid concentrations and ammonia emissions from pigs. *Anim Feed Sci Technol.* 2006;127:73-88. <https://doi.org/10.1016/j.anifeedsci.2005.09.002>
 34. Dong RL, Zhao GY, Chai LL, Beauchemin KA. Prediction of urinary and fecal nitrogen excretion by beef cattle. *J Anim Sci.* 2014;92:4669-81. <https://doi.org/10.2527/jas.2014-8000>
 35. Kong C, Park CS, Kim BG. Effects of an enzyme complex on in vitro dry matter digestibility of feed ingredients for pigs. *SpringerPlus.* 2015;4:261. <https://doi.org/10.1186/s40064-015-1060-1>
 36. Angel CR, Saylor W, Vieira SL, Ward N. Effects of a monocomponent protease on performance and protein utilization in 7- to 22-day-old broiler chickens. *Poult Sci.* 2011;90:2281-6. <https://doi.org/10.3382/ps.2011-01482>
 37. Oxenboll KM, Pontoppidan K, Fru-Nji F. Use of a protease in poultry feed offers promising environmental benefits. *Int J Poult Sci.* 2011;10:842-8. <https://doi.org/10.3923/ijps.2011.842.848>
 38. Freitas DM, Vieira SL, Angel CR, Favero A, Maiorka A. Performance and nutrient utilization of broilers fed diets supplemented with a novel mono-component protease. *J Appl Poult Res.* 2011;20:322-34. <https://doi.org/10.3382/japr.2010-00295>
 39. Fru-Nji F, Kluentner AM, Fischer M, Pontoppidan K. A feed serine protease improves broiler performance and increases protein and energy digestibility. *J Poult Sci.* 2011;48:239-46. <https://doi.org/10.2141/jpsa.011035>
 40. Schlosser A, Vanselow JT, Kramer A. Mapping of phosphorylation sites by a multi-protease approach with specific phosphopeptide enrichment and nanoLC-MS/MS analysis. *Anal Chem.* 2005;77:5243-50. <https://doi.org/10.1021/ac050232m>
 41. Linke D, Koudelka T, Becker A, Tholey A. Identification and relative quantification of phosphopeptides by a combination of multi-protease digestion and isobaric labeling. *Rapid Commun Mass Spectrom.* 2015;29:919-26. <https://doi.org/10.1002/rcm.7185>
 42. Abdollahi MR, Ravindran V, Svihus B. Pelleting of broiler diets: an overview with emphasis on pellet quality and nutritional value. *Anim Feed Sci Technol.* 2013;179:1-23. <https://doi.org/10.1016/j.anifeedsci.2012.10.011>
 43. Abdollahi MR, Ravindran V, Svihus B. Influence of grain type and feed form on performance, apparent metabolisable energy and ileal digestibility of nitrogen, starch, fat, calcium and phosphorus in broiler starters. *Anim Feed Sci Technol.* 2013;186:193-203. <https://doi.org/10.1016/j.anifeedsci.2013.10.015>
 44. Huang KH, Ravindran V, Li X, Bryden WL. Influence of age on the apparent ileal amino acid digestibility of feed ingredients for broiler chickens. *Br Poult Sci.* 2005;46:236-45. <https://doi.org/10.1080/00071660500066084>
 45. Wallis IR, Balnave D. The influence of environmental temperature, age and sex on the digestibility of amino acids in growing broiler chickens. *Br Poult Sci.* 1984;25:401-7. <https://doi.org/10.1080/00071668408454880>
 46. Ten Doeschate RAHM, Scheele CW, Schreurs VVAM, Van Der Klis JD. Digestibility studies in broiler chickens: influence of genotype, age, sex and method of determination. *Br Poult Sci.* 1993;34:131-46. <https://doi.org/10.1080/00071669308417569>
 47. Liu SY, Selle PH, Cowieson AJ. Influence of conditioning temperatures on amino acid digestibility at four small intestinal sites and their dynamics with starch and nitrogen digestion in sorghum-based broiler diets. *Anim Feed Sci Technol.* 2013;185:85-93. <https://doi.org/10.1016/>

- j.anifeedsci.2013.07.008
48. Wilkinson SJ, Selle PH, Bedford MR, Cowieson AJ. Separate feeding of calcium improves performance and ileal nutrient digestibility in broiler chicks. *Anim Prod Sci.* 2013;54:172-8. <https://doi.org/10.1071/AN12432>
 49. Wu G. Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. *J Anim Sci Biotechnol.* 2014;5:34. <https://doi.org/10.1186/2049-1891-5-34>
 50. Liu SY, Selle PH, Cowieson AJ. Influence of white- and red-sorghum varieties and hydrothermal component of steam pelleting on digestibility coefficients of amino acids and kinetics of amino acids, nitrogen and starch digestion in diets for broiler chickens. *Anim Feed Sci Technol.* 2013;186:53-63. <https://doi.org/10.1016/j.anifeedsci.2013.08.006>
 51. Rezaei M, Moghaddam HN, Reza JP, Kermanshahi H. The effects of dietary protein and lysine levels on broiler performance, carcass characteristics and N excretion. *Int J Poult Sci.* 2004;3:148-52. <https://doi.org/10.3923/ijps.2004.148.152>
 52. Corzo A, Dozier WA, Kidd MT. Valine nutrient recommendations for Ross × Ross 308 broilers. *Poult Sci.* 2008;87:335-8. <https://doi.org/10.3382/ps.2007-00307>
 53. Luo XG, Li SF, Lu L, Liu B, Kuang X, Shao GZ, et al. Gene expression of manganese-containing superoxide dismutase as a biomarker of manganese bioavailability for manganese sources in broilers. *Poult Sci.* 2007;86:888-94. <https://doi.org/10.1093/ps/86.5.888>
 54. Yuan J, Xu Z, Huang C, Zhou S, Guo Y. Effect of dietary Mintrex-Zn/Mn on performance, gene expression of Zn transfer proteins, activities of Zn/Mn related enzymes and fecal mineral excretion in broiler chickens. *Anim Feed Sci Technol.* 2011;168:72-9. <https://doi.org/10.1016/j.anifeedsci.2011.03.011>