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Original Research Article

Protease supplementation reduced the heat increment of feed and improved energy and nitrogen partitioning in broilers fed maize-based diets with supplemental phytase and xylanase

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ABSTRACT

An experiment was conducted to explore the effects of digestible amino acid (dAA) concentrations and supplemental protease on live performance and energy partitioning in broilers. Ross 308 male broilers ($n = 288$) were distributed into 24 floor pens and offered 1 of 4 dietary treatments with 6 replicates from 1 to 35 d of age. Dietary treatments consisted of a 2 × 2 factorial arrangement with dAA concentrations (standard and reduced [34 g/kg below standard]) and supplemental protease (without or with) as the main factors. At 1, 15, 28, and 35 d of age, feed and broilers were weighed to determine live performance. From 20 to 23 d of age, a total of 32 birds (2 birds/chamber, 4 replicates) were placed in closed-calorimeter chambers to determine respiratory exchange (heat production, HP), apparent metabolisable energy (AME), retained energy (RE), and net energy (NE). From 29 to 35 d of age, supplemental protease in the reduced-dAA diet decreased broiler feed conversion ratio (FCR) by 5.6 points, whereas protease supplementation in the standard-dAA diet increased FCR by 5.8 points. The indirect calorimetry assay revealed that supplemental protease decreased ($P < 0.05$) the heat increment of feed (HIF) by 0.22 MJ/kg. Also, from 20 to 23 d of age, broilers offered the reduced-dAA diet with supplemental protease had a higher daily body weight gain (BWG) (+10.4%), N intake (+7.1%), and N retention (+8.2%) than those offered the standard-dAA with supplemental protease. Broilers offered the reduced-dAA without supplemental protease exhibited a 3.6% higher AME-to-crude protein (CP) ratio than those offered other treatments. Protease supplementation in the standard- and reduced-dAA diets resulted in 2.7% and 5.6% lower AME intake-to-N retention ratios, respectively, compared with the unsupplemented controls. Reduced-dAA increased ($P < 0.05$) AME intake (+4.8%), RE (+9.8%), NE intake (+5.8%), NE intake-to-CP ratio (+3.0%), and RE fat-to-RE ratio (+8.6%). Protease supplementation increased ($P < 0.05$) respiratory quotient (+1.2%) and N retention-to-N intake ratio (+2.2%), NE-to-AME ratio (+1.9%), and reduced HP (−3.6%), heat increment (−7.4%), and NE intake-to-N retention (−2.5%). In conclusion, protease positively affected FCR and energy partitioning in broilers; responses were most apparent in diets with reduced-dAA concentrations.

1. Introduction

Decreasing dietary crude protein (CP) concentrations can reduce N excretion, the inclusion of protein contributing ingredients, and excess digestible AA (dAA) (Ferguson et al., 1998), which can reduce diet cost (Zhang et al., 2020). However, reducing dietary CP and dAA concentrations can negatively affect the balance and availability of essential nutrients, which can lead to adverse effects on broiler feed

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conversion (Zhang et al., 2020). Supplementing broiler diets with a mono-component protease enzyme may help alleviate these problems by releasing peptides and amino acids (AA) that are limiting (Angel et al., 2011), or by modulating digestive physiology to improve overall energy partitioning (Cowieson et al., 2019; Mahagna et al., 1995; Yin et al., 2018). On average, supplemental protease has been observed to improve ileal AA digestibility by 3.74% (Cowieson and Roos, 2013). Moreover, a recent study evaluating the effects of supplemental protease in diets with both phytase and xylanase demonstrated that protease supplementation improved apparent metabolisable energy (AME) and net energy (NE) by 0.30 and 0.45 MJ/kg, respectively (Cowieson et al., 2019). This larger magnitude of improvement in NE compared with AME indicates that benefits of protease supplementation extend beyond direct digestibility effects, to additional net effects and reduce the heat increment of feed (HIF). Understanding these extra-proteinaceous effects with supplemental protease may improve commercial value and increase flexibility in diet formulation when applied appropriately. However, the magnitude and mode of action of these responses have not been well defined, and varying responses have been observed with different dAA concentrations (Yu et al., 2007). Therefore, the objective of this experiment was to evaluate the effects of supplemental protease (without or with) and dAA concentrations (standard or reduced) on broiler live performance and energy partitioning. The hypothesis of this study was that protease supplementation would improve energy partitioning and live performance of broilers, and these responses would be more evident in diets with reduced concentrations of dAA.

2. Materials and methods

2.1. Husbandry and diets

The University of New England Animal Ethics Committee, which complies with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, approved this experiment (AEC 20-104). A total of 288 Ross × Ross 308 male broiler chicks were obtained from a primary breeder (Aviagen, Goulburn, NSW) and distributed into 24 floor pens (12 chicks per pen; 0.08 m^2 per bird). Pens were equipped with fresh wood shavings (depth = 7 cm), a hanging-pan feeder, and nipple drinkers in an environmentally controlled room. Birds were provided ad libitum access to feed and water throughout the experiment. At placement, room temperature was 34 °C, and was gradually reduced as the birds advanced in age, with a final set point of 24 °C at 35 d of age. Photoperiod was set at 23L:1D and 20L:4D from 1 to 6 and 7 to 35 d of age, respectively.

Maize, soybean meal, canola meal, and meat and bone meal were analysed by near-infrared spectroscopy to predict proximates, AA concentrations and AME, using AMINONIR PROX, AMINONIR NIR, and AMINONIR NRG (Evonik Nutrition & Care, Hanua, Germany), respectively. Predicted NIR-values were used for diet formulation. Four dietary treatments were offered throughout the starter (1 to 14 d of age) and grower (15 to 35 d of age) phases (Table 1). Dietary treatments consisted of a 2 × 2 factorial arrangement with dAA concentrations (standard or reduced) and supplemental protease (without or with). All dietary treatments were formulated to be adequate in essential nutrients, except for reduced-dAA treatments which had 34 and 33 g/kg lower dAA concentrations in the starter and grower diets, respectively, than the standard-dAA treatments. All dietary treatments were formulated to contain mono-component phytase (RONOZYME HIPhos GT; 10,000 FYT/g) and xylanase (RONOZYME WX CT; 1,000 FXU/g) to achieve feed activity concentrations of 2,000 FYT/kg and 200 FXU/kg, respectively (DSM Nutritional Products, Kaiseraugst, Switzerland). Phytase was formulated to provide 1.5 g/kg of both Ca and P, but no matrix value was assigned to xylanase. A mono-component serine protease (RONOZYME ProAct CT; 75,000 PROT/g; DSM Nutritional Products, Kaiseraugst, Switzerland) was included in the supplemented treatments, to achieve feed activity concentration of 15,000 PROT/kg, but no matrix value was assigned to it. Basal diets were formulated with 0.3 g/kg of Washed Builder's Sand, and protease was included in the supplemented treatments at its expense. Diets were cold-pelleted (65 °C) and feed form consisted of crumbles during the starter phase and pellets during the grower phase.

Table 1
Ingredients and nutrient composition of basal diets fed to Ross 308 male broilers (as-fed, g/kg).

Item	1 to 14 d of age		15 to 35 d of age	
	Standard	Reduced	Standard	Reduced
Ingredients				
Maize	524.9	504.2	560.6	542.2
Soybean meal	315.7	344.1	270.9	299.7
Canola meal	60.0	60.0	70.0	70.0
Meat and bone meal	18.1	0.0	24.0	0.0
Canola oil	39.6	46.3	39.8	47.2
Bentonite	10.0	10.0	10.0	10.0
Calcium carbonate	9.8	11.4	7.9	10.1
Dicalcium phosphate	5.3	9.0	1.1	6.0
Sodium chloride	1.9	2.5	1.9	2.4
Sodium bicarbonate	2.4	2.0	2.3	2.2
Vitamin premix[1]	0.9	0.9	0.9	0.9
Mineral premix[2]	1.0	1.0	1.0	1.0
Choline chloride	3.7	3.8	3.7	3.8
L-Lys•HCl	2.1	1.2	2.0	1.3
DL-Met	3.2	2.8	2.8	2.4
L-Thr	0.6	0.1	0.4	0.1
Phytase[3]	0.2	0.2	0.2	0.2
Xylanase[3]	0.2	0.2	0.2	0.2
Sand[4]	0.3	0.3	0.3	0.3
Calculated nutrient content				
AME, MJ/kg	12.55	12.55	12.76	12.76
Starch	342.58	329.27	365.46	353.66
Crude protein	230.00	230.00	218.23	215.59
SID[5] Lys	12.69	12.26	11.86	11.47
SID Met	6.37	5.96	5.88	5.5
SID TSAA	9.26	8.95	8.66	8.37
SID Thr	8.12	7.84	7.59	7.34
SID Val	9.6	9.75	9.13	9.2
SID Arg	14.00	14.29	13.11	13.25
SID Trp	2.53	2.63	2.36	2.45
Ca	9.60	9.60	8.40	8.40
Non-phytate P	4.80	4.80	4.20	4.20
Na	1.80	1.80	1.80	1.80

AME = apparent metabolisable energy; SID = standardized ileal digestible values; TSAA = total sulfur amino acid.
[1] Vitamin premix supplied per kilogram of diet: retinol 12,000 IU, cholecalciferol 5,000 IU, tocopheryl acetate 75 mg, menadione 3 mg, thiamine 3 mg, riboflavin 8 mg, niacin 55 mg, pantothenate 13 mg, pyridoxine 5 mg, folate 2 mg, cyanocobalamin 16 μg, biotin 200 μg, cereal-based carrier 149 mg, mineral oil 2.5 mg.
[2] Trace mineral premix supplied per kilogram of diet: Cu (sulphate) 16 mg, Fe (sulphate) 40 mg, I (iodide) 1.25 mg, Se (selenate) 0.3 mg, Mn (sulphate and oxide) 120 mg, Zn (sulphate and oxide) 100 mg, cereal-based carrier 128 mg, mineral oil 3.75 mg.
[3] RONOZYME HiPhos and RONOZYME WX were used as sources of phytase and xylanase, respectively. A phytase matrix value of 1.5 g/kg for both Ca and digestible P was used. No energy matrix was applied to xylanase.
[4] Sand was the variable portion in diet formulation. Protease (RONOZYME ProAct was either included at 0 or 200 mg/kg to the NC basal diets at the expense of sand to achieve the 2 dietary treatments (NC diets without or with supplemental protease).
[5] Diets were formulated on digestible AA basis using SID values predicted by AMINO NIR (Evonik Nutrition & Care, Hanua, Germany).

2.2. Growth performance

Birds and feed were weighed at d 1, 15, 29, and 35 to determine body weight (BW), BW gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Mortality was recorded daily and used to adjust FCR.

2.3. Indirect calorimetry

At 18 d of age, 32 birds (2 birds/pen from 4 replicate pens) were transferred into 16 closed-circuit calorimeter chambers. A 48-h acclimation period was provided, and birds remained on their respective dietary treatments. From 20 to 23 d of age, chambers were closed to measure respiratory exchange (heat production, HP), and AME by total collection of excreta. Oxygen (O_2) consumption (L) was determined by the difference in O_2 cylinder weights at the beginning and end of each 24 h run cycle, and was converted to volume using the density of O_2 (1.331 g/L). Carbon dioxide (CO_2) was trapped in 32% KOH solution and determined gravimetrically using barium precipitation (Annison and White, 1961). Oxygen consumption and CO_2 expiration were used to calculate HP using the equation by Brouwer (1965). No correction for uric acid nitrogen excretion was used. Respiratory quotient (RQ) of the 72-h run was calculated as the volume of CO_2 produced to the volume of O_2 consumed.

2.4. AME, NE, and HIF determination

At approximately the same time in each 24 h run cycle, chambers were opened, birds and feed were weighed, and total excreta samples were collected. Subsamples were collected from the total excreta to determine gross energy (GE) and N content, allowing for the calculation of intake and excretion. Dietary AME was calculated on a DM basis according to the following equation:

$$\text{AME (MJ/kg)} = (\text{GE}_{\text{intake}} - \text{GE}_{\text{excreta}})/\text{FI}$$

NE intake and NE of the diets were calculated as described by Noblet et al. (1994). In birds, NE intake equals fasting heat production plus the retained energy (RE). Thus, RE (MJ/d) was calculated by subtracting HP (MJ/d) from metabolisable energy intake (MEI; MJ/d). The fasting heat production (FHP) of 0.45 MJ/$BW^{0.70}$ per day was used, which corresponded to the asymptotic HP (at zero activity) (Noblet et al., 2015). Heat increment (HI) of feeding was calculated by subtracting FHP from heat production (HP). The NE value of the diet was calculated as follow:

$$\text{NE (MJ/kg)} = (\text{RE} + \text{FHP})/\text{FI}$$

Additionally, the HIF value was calculated as follows:

$$\text{HIF (MJ/kg)} = \text{AME} - \text{NE}$$

Diet and excreta samples were lyophilized using a Christ Alpha 1-2LDplus Freeze Dryer (Martin Christ Gefriertrocknungsanlagen, Osterode, Germany). Dry matter of wet samples was determined using a forced air oven at 105 °C for 12 h to a constant weight. Gross energy was determined using a 6400 automatic isoperibol oxygen bomb calorimeter (Parr Instruments, Moline, IA). Nitrogen content was determined according to the Dumas combustion method (method 990.03; AOAC, 2005) using a LECO FP-200 N analyser (Leco Corp., St. Joseph, MI), with N correction factor of 6.25 for CP determination.

2.5. Statistical analysis

This experiment was arranged in a randomized complete block design with pen and chamber location as the blocking factors. Each treatment had 6 and 4 replicate pens for growth performance and energy partitioning assays, respectively. A two-way analysis of variance in PROC GLM (Minitab, 2010) was used to evaluate the interactive and main effects of dAA concentration (standard- or reduced-dAA) and protease supplementation (without or with) on growth performance and energy partitioning. Statistical significance was established at $P < 0.05$, and a trend was considered at $P < 0.10$. Interactive and main effects were separated using Tukey's pairwise comparison test.

3. Results

3.1. Growth performance

The effects of dAA concentrations and supplemental protease on broiler growth performance are displayed in Table 2. Overall, dAA concentrations and supplemental protease only interacted to affect FCR ($P = 0.03$) from 29 to 35 d of age. Broilers receiving the standard-dAA without protease and reduced-dAA diet with protease had a 3.9% lower ($P < 0.05$) FCR than those receiving the standard-dAA diet with protease and the reduced-dAA diet without protease. No dAA or protease main effects ($P > 0.05$) on broiler growth performance were observed. However, a protease main effect trend ($P = 0.06$) on BWG from 15 to 28 d of age was observed, with broilers offered diets with supplemental protease exhibiting a 2% higher BWG than those offered unsupplemented diets.

3.2. Dietary AME, NE, and HIF

The effects of dAA concentrations and supplemental protease on AME, NE, and HIF of the diets are displayed in Table 3. Digestible AA concentration and supplemental protease did not interact ($P > 0.05$) to affect AME, NE, or HIF. However, a protease main effect trend ($P = 0.08$) on NE was observed, with supplementation increasing NE by 0.19 MJ/kg compared with those offered the unsupplemented diets. Broilers offered diets with supplemental protease had lower (0.22 MJ/kg; $P > 0.05$) HIF than those offered the unsupplemented diets.

3.3. Energy and N balance and utilisation

During the indirect calorimetry assay from 20 to 23 d of age, dietary treatments interacted to affect daily BWG ($P = 0.04$), N intake ($P = 0.02$), N retention ($P = 0.02$), AME-to-CP ratio ($P = 0.04$), and AME intake-to-N retention ratio (Table 4). Broilers offered the reduced-dAA diet with supplemental protease had a 9.4% higher ($P < 0.05$) BWG (g/bird per day) than those offered the standard-dAA diet with protease, whereas BWG of those offered the standard- and reduced-dAA diets without protease were similar ($P > 0.05$) to those offered the standard- and reduced-dAA diets with supplemental protease. Similarly, broilers offered the reduced-dAA diet with protease had a 7.1% higher ($P < 0.05$) N intake than those offered the standard-dAA with protease, but N intake of broilers offered the standard- and reduced-dAA without supplemental protease were similar ($P > 0.05$) to those offered the standard- and reduced-dAA diets with supplemental protease.

Table 2
Growth performance of Ross 308 male broilers fed maize-based diets varying in digestible AA (dAA) and supplemental protease concentrations from 1 to 35 d of age[1].

Item	Treatment effects[2]					Main effects				Analysis of variance (*P*-value)		
	Standard dAA		Reduced dAA			dAA		Protease[3]				
	Without	With	Without	With	SEM	Standard	Reduced	Without	With	dAA × Protease	dAA	Protease
Body weight, g												
14 d of age	466	471	471	455	3	468	463	469	462	0.09	0.38	0.25
28 d of age	1,662	1,687	1,657	1,669	8	1,675	1,663	1,660	1,678	0.72	0.45	0.27
35 d of age	2,508	2,489	2,450	2,481	11	2,498	2,465	2,485	2,479	0.26	0.14	0.79
Body weight gain, g												
1 to 14 d of age	435	439	441	424	3	437	432	438	431	0.08	0.39	0.26
15 to 28 d of age	1,197	1,217	1,185	1,214	6	1,207	1,200	1,191	1,216	0.73	0.58	0.06
29 to 35 d of age	845	802	794	823	9	824	808	819	813	0.07	0.43	0.73
1 to 35 d of age	2,476	2,458	2,419	2,461	10	2,467	2,440	2,448	2,459	0.17	0.21	0.58
Feed intake, g												
1 to 14 d of age	466	466	470	453	3	466	461	468	459	0.19	0.43	0.20
15 to 28 d of age	1,545	1,560	1,566	1,609	16	1,553	1,587	1,555	1,584	0.66	0.29	0.37
29 to 35 d of age	1,187	1,175	1,168	1,163	10	1,181	1,166	1,178	1,169	0.85	0.47	0.68
1 to 35 d of age	3,199	3,201	3,204	3,224	19	3,200	3,214	3,201	3,213	0.81	0.72	0.76
Feed conversion ratio, g:g												
1 to 14 d of age	1.072	1.062	1.066	1.069	0.003	1.067	1.067	1.069	1.065	0.34	0.95	0.57
15 to 28 d of age	1.292	1.282	1.323	1.325	0.014	1.287	1.324	1.307	1.303	0.83	0.21	0.89
29 to 35 d of age	1.408[a]	1.466[b]	1.473[b]	1.417[a]	0.012	1.437	1.445	1.440	1.441	0.03	0.74	0.98
1 to 35 d of age	1.292	1.302	1.324	1.311	0.006	1.297	1.317	1.308	1.306	0.36	0.12	0.89
Mortality, %												
1 to 35 d of age	7.0	4.2	6.9	8.3	1.5	5.6	7.6	6.9	6.2	0.56	0.56	0.84

[a, b] Means within a row for a given measurement not sharing a common superscript differ significantly ($P \leq 0.05$) and were separated using Tukey's Pairwise Comparison test.
[1] Each value represents the least-squared means of 6 replicate pens with approximately 12 chicks at placement.
[2] Dietary treatments consisted of 4 possible treatments: 1) standard dAA without protease, 2) standard dAA with protease, 3) reduced dAA (digestible amino acid density 34 g/kg below the standard dAA concentration) without protease, 4) reduced dAA with protease.
[3] Protease (RONOZYME ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) provides 75,000 PROT/g was included at 200 mg/kg in the supplemented treatments to achieve an activity of 15,000 PROT/kg.

Table 3
Gross energy (GE), apparent metabolisable energy (AME), net energy (NE), and heat increment of feed (HIF) of maize-based diets varying in digestible amino acid (dAA) and supplemental protease concentrations fed to Ross 308 male broilers from 20 to 23 d of age[1].

dAA	Protease[2]	GE, MJ/kg	AME, MJ/kg	NE[3], MJ/kg	HIF[4], MJ/kg
Standard	Without	19.49	14.88	10.77	4.108
	With	19.33	14.89	10.95	3.942
Reduced	Without	19.44	14.98	10.90	4.083
	With	19.41	14.93	11.12	3.812
SEM		0.016	0.039	0.065	0.052
dAA main effects					
Standard		19.41	14.88	10.86	4.025
Reduced		19.42	14.96	11.01	3.947
SEM					
Protease main effects					
Without		19.46	14.93	10.84	4.095
With		19.37	14.91	11.03	3.877
SEM					
Analysis of variance (*P*-value)					
dAA × Protease		–	0.27	0.32	0.59
dAA		–	0.89	0.44	0.43
Protease		–	0.30	0.08	0.04

[1] Each value represents the least-square means of 4 replicate chambers with 2 birds per chamber. Values are expressed on a DM basis using the total collection method.
[2] Protease (RONOZYME ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) provides 75,000 PROT/g was included at 200 mg/kg in the supplemented treatments to achieve an activity of 15,000 PROT/kg.
[3] NE = Fasting heat production + Retained energy.
[4] HIF = Apparent metabolisable energy − Net energy.

Broilers offered the reduced-dAA diet with supplemental protease had 6.6%, 8.3%, and 6.1% higher ($P < 0.05$) N retention than those offered the reduced-dAA without protease, the standard-dAA with protease, and the standard-dAA without protease, respectively. However, N retention values of broilers receiving the standard-dAA without and with supplemental protease and the reduced-dAA diet without protease were similar ($P > 0.05$). Broilers offered the reduced-dAA diet without protease (62.6%) had a higher ($P < 0.05$) AME-to-CP ratio than those offered standard-dAA diets without (60.5%) and with (60.1%) protease, and the reduced-dAA with protease (60.5%). Similarly, broilers offered reduced-dAA diet without protease exhibited the highest ($P < 0.05$) AME intake-to-N retention ratio, and those offered the standard-dAA diet without protease had a higher ($P < 0.05$) AME intake-to-N retention ratio those offered the standard- and reduced-dAA diets with supplemental protease.

Table 4
Performance, energy and N balance, and utilisation efficiency of Ross 308 male broilers fed maize-based diets varying in digestible AA (dAA) and supplemental protease concentrations from 20 to 23 d of age[1].

Item	Interaction effects[2]				SEM	Main effects				Analysis of variance (*P*-value)		
	Standard dAA		Reduced dAA			dAA		Protease[3]				
	Without	With	Without	With		Standard	Reduced	Without	With	dAA × Protease	dAA	Protease
BW gain, g/bird per day	99.5^{ab}	93.0^{b}	99.2^{ab}	102.7^{a}	1.3	96.2	101.0	99.3	97.8	0.04	0.05	0.51
Feed intake, g/bird per day	128.6	121.5	128.4	128.5	1.2	125.1	128.5	128.5	125.0	0.14	0.17	0.16
Feed conversion ratio, g:g	1.295	1.307	1.295	1.252	0.012	1.301	1.273	1.295	1.280	0.26	0.26	0.53
AME intake[4], MJ/kg $BW^{0.70}$	1.562	1.488	1.593	1.602	0.015	1.525	1.598	1.578	1.545	0.09	0.009	0.18
RQ	1.008	1.027	1.007	1.012	0.003	1.018	1.010	1.008	1.020	0.13	0.09	0.02
HP, MJ/kg $BW^{0.70}$	0.881	0.844	0.884	0.858	0.006	0.863	0.871	0.883	851	0.49	0.29	0.002
HI, MJ/kg $BW^{0.70}$	0.431	0.394	0.434	0.408	0.006	0.413	0.421	0.433	401	0.49	0.29	0.002
RE, MJ/kg $BW^{0.70}$	0.681	0.644	0.709	0.744	0.014	0.662	0.727	0.695	0.691	0.18	0.03	0.98
NE intake[5], MJ/kg $BW^{0.70}$	1.131	1.094	1.159	1.194	0.014	1.112	1.177	1.145	1.144	0.18	0.03	0.98
N intake, g/kg $BW^{0.70}$	4.130^{ab}	3.959^{b}	4.071^{ab}	4.239^{a}	0.037	4.045	4.155	4.101	4.099	0.02	0.10	0.98
N retention, g/kg $BW^{0.70}$	2.813^{b}	2.758^{b}	2.800^{b}	2.984^{a}	0.029	2.786	2.892	2.806	2.871	0.02	0.03	0.15
N excretion, g/bird per day	1.420	1.287	1.355	1.330	0.022	1.353	1.343	1.387	1.309	0.22	0.80	0.09
N retention-to-N intake ratio, %	68.1	69.7	68.8	70.4	0.32	68.9	69.6	68.5	70.0	0.95	0.20	0.01
AME-to-GE ratio, %	76.4	77.0	77.1	76.9	0.21	76.7	77.0	76.7	77.0	0.39	0.56	0.49
NE-to-AME ratio, %	72.4	73.5	72.8	74.5	0.35	73.0	73.6	72.6	74.0	0.66	0.34	0.05
AME-to-CP ratio, %	60.5^{b}	60.1^{b}	62.6^{a}	60.5^{b}	0.32	60.3	61.5	61.6	60.3	0.03	0.005	0.006
NE intake-to-CP ratio, %	43.8	44.2	45.6	45.0	0.30	44.0	45.3	44.7	44.6	0.39	0.03	0.91
AME intake-to-N retention ratio, %	55.5^{b}	54.0^{c}	56.9^{a}	53.7^{c}	0.37	54.7	55.3	56.2	53.8	0.004	0.03	0.001
NE intake-to-N retention ratio, %	40.2	39.7	41.4	40.0	0.24	39.9	40.7	40.8	39.8	0.24	0.06	0.02
RE fat-to-RE ratio, %	38.5	36.3	41.3	39.9	0.74	37.4	40.6	39.9	38.1	0.72	0.03	0.18

AME = apparent metabolisable energy; $BW^{0.70}$ = metabolic BW; RQ = respiratory quotient (CO_2/O_2); HP = heat production; HI = heat increment; RE = retained energy; NE = net energy; GE = gross energy; RE fat-to-RE ratio = the ratio of energy retained as fat to RE.
[a-c] Means within a row for a given measurement not sharing a common superscript differ significantly ($P \leq 0.05$) and were separated using Tukey's Pairwise Comparisons test.
[1] Each value represents the least-squared means of 4 replicate chambers with 2 birds per chamber on a DM basis using indirect calorimetry.
[2] Dietary treatments consisted of 4 possible treatments: 1) standard dAA without protease, 2) standard dAA with protease, 3) reduced dAA (digestible amino acid density 34 g/kg below the standard dAA concentration) without protease, 4) reduced dAA with protease.
[3] Protease (RONOZYME ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) provides 75,000 PROT/g was included at 200 mg/kg in the supplemented treatments to achieve an activity of 15,000 PROT/kg.
[4] AME was measured using the total collection method.
[5] NE = fasting heat production + retained energy.

A main effect of dAA concentration affected AME intake ($P = 0.009$), RE ($P = 0.02$), NE intake ($P = 0.02$), NE intake-to-CP ratio ($P = 0.02$), and RE fat-to-RE ratio ($P = 0.03$). Broilers offered reduced-dAA diets had a higher ($P < 0.05$) AME intake (+4.8%), RE (+9.8%), NE intake (+5.8%), NE intake-to-CP ratio (+3.0%), and RE fat-to-RE ratio (+8.6%). Moreover, a main effect of protease influenced RQ ($P = 0.02$), HP ($P = 0.002$), HI ($P = 0.002$), N retention-to-N intake ratio ($P = 0.01$), NE-to-AME ratio ($P = 0.02$), and NE intake-to-N retention ratio ($P = 0.02$). Broilers offered diets with supplemental protease had a higher ($P < 0.05$) RQ (+1.2%), N retention-to-N intake ratio (+2.2%), and NE-to-AME ratio (+1.9%); but a lower HP (−3.6%), HI (−7.4%), and NE intake-to-N retention ratio (−2.5%) compared with those offered unsupplemented diets.

4. Discussion

The effects of protease supplementation and varying concentrations of dAA on broiler live performance and energy partitioning were evaluated in this study. Overall, dietary treatments only interacted to affect FCR from 29 to 35 d of age, with protease increasing FCR by 5.8 points in the standard-dAA diet but decreasing FCR by 5.6 points in the reduced-dAA diet in the performance trial. This magnitude of alteration in FCR is consistent with other experiments that evaluated supplemental protease in maize-based diets (Amerah et al., 2017; Cowieson and Ravindran, 2008). The contrasting FCR responses with varying dAA concentration are consistent with our hypothesis and demonstrated that the benefits of supplemental protease on broiler performance are realised when diets are formulated with lower dAA concentrations. It appears that there is little benefit to adding protease to a diet that already has a sufficient level of all the essential AA in ratio to the dietary energy; instead, it may alter the dAA to energy ratio, negatively affecting bird performance. Moreover, enzyme efficacy and magnitude of response appears to be dependent on the most limiting nutrient, the most abundant anti-nutrient, or the model used (Cowieson and Bedford, 2009). It is plausible that AA digestibility was not limiting in these diets. Therefore, the varying FCR responses with protease may have been due to alterations in net metrics such as digestive physiology (Jiang et al., 2008; Mahagna et al., 1995; Yin et al., 2018), which altered intestinal energy requirements (Cowieson et al., 2019).

In the current experiment, dietary treatments did not interact to affect dietary AME, NE, or HIF. However, protease supplementation numerically increased NE (+0.19 MJ/kg) and significantly decreased HIF (−0.22 MJ/kg). Cowieson et al. (2019) observed an increase in NE (+0.45 MJ/kg) and a reduction in HIF (−0.15 MJ/kg; calculated from data) with supplemental protease in maize/wheat-based diets. In addition, these authors also observed improvements in AME (+0.31 MJ/kg). This larger magnitude of responses on NE and lower HIF, in conjunction with increase in AME, observed by these authors indicates that the benefits of supplemental protease were due both to improvements in direct metabolisability and net metrics. In the current experiment however it appears that the benefits of protease supplementation were only due to an improvement in net metrics, such as improvements in energy utilization in gut maintenance or in the synthesis of endogenous proteins (Cowieson et al., 2019). Exogenous enzymes such as protease and amylase have been observed to alter digestive physiology, such as the secretion of endogenous enzymes, organ size, and jejunal gene expression. These alterations in net metrics

are likely important for understanding and explaining differences in broiler performance and efficiency with enzyme supplementation (Cowieson et al., 2019; Jiang et al., 2008; Mahagna et al., 1995; Yin et al., 2018). Consequently, these results further demonstrate that protease supplementation can lower the HIF and improve energy partitioning in growing broilers. Moreover, these beneficial responses with protease may be more pronounced when AA digestibility is limiting or dAA concentrations are reduced.

Measuring heat production through indirect calorimetry and calculating NE provides a more accurate measure of energy partitioning, because it allows for the separation of energy used for production and energy lost as heat (Council, 1981). In the present work, broilers offered the reduced-dAA diet with supplemental protease had a higher daily BWG, N intake, and N retention compared with those offered the standard-dAA with supplemental protease. These responses demonstrate protease supplementation was more efficacious in diets formulated with reduced-dAA concentrations. Reducing dAA concentrations resulted in a higher AME intake, RE, NE intake, NE intake-to-CP ratio, and RE fat-to-RE ratio. The lower concentrations of dAA likely altered protein turnover rate, which increased the amount of energy available and proportion of energy retained as fat. A higher ratio of protein synthesis to protein degradation is necessary to increase protein accretion, and may be achieved either by increasing synthesis rate or by decreasing rate of degradation, with the former being more energetically expensive (Klasing et al., 1987). Moreover, recent research evaluating effects of genetic lines, ambient temperature, and dAA densities on protein synthesis and degradation rates of *Pectoralis major* muscles in broilers at 36 and 42 d of age demonstrated that fractional degradation rates can be decreased with reduced dAA concentrations (Maharjan et al., 2020). Therefore, if the energy value observed with supplemental protease in this study was applied to diet formulation, it would allow for a reduction in dietary AME. This would reduce diet costs and possibly offset the increase in fat retention that is often observed with broilers fed diets with reduced-dAA concentrations.

In the current experiment, protease supplementation increased RQ, N retention-to-N intake ratio, and NE-to-AME ratio; but decreased HP, HI, and NE intake-to-N retention ratio. These results further confirm that protease supplementation can improve energy utilisation by reducing HP and HI, which likely improved N retention-to-N intake ratio. Again, these improvements are not likely attributable to improvements in nutrient digestibility per se, but rather to alterations in net metrics with endogenous enzyme secretions, which likely lowered heat production from N excretion, endogenous losses, maintenance energy cost due to reduced burden of protein digestion, and protein accretion energy requirements (Cowieson et al., 2019). Additional research evaluating these factors is warranted, but these results demonstrate that protease supplementation can significantly improve energy partitioning in broilers when applied appropriately.

5. Conclusions

The hypothesis of this experiment was accepted, because protease supplementation improved FCR and energy and N partitioning in broilers, and these responses were more pronounced in broilers fed diets with reduced-dAA concentrations. Moreover, the lack of responses on AME, in conjunction with the numerical increases in NE and the significant reduction in HIF, clearly demonstrates that the benefits of exogenous protease supplementation extend beyond direct digestibility to additional net effects. Further research quantifying these net effects of protease and its effects on protein turnover rate is warranted to improve understanding and application to fully capitalize on the energy-sparing effects associated with supplemental protease.

Author contributions

K.W. McCafferty: Visualization, Writing - Original Draft. **M. Choct:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **S. Musigwa:** Investigation, Formal analysis, Data Curation; Writing - Review & Editing. **N.K. Morgan:** Methodology, Writing - Review & Editing, Supervision. **A. J. Cowieson:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **A. F. Moss:** Investigation, Methodology, Writing - Review & Editing, Supervision, Project administration.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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