A mono-component microbial protease improves performance, net energy, and digestibility of amino acids and starch, and upregulates jejunal expression of genes responsible for peptide transport in broilers fed corn/wheat-based diets supplemented with xylanase and phytase

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ABSTRACT A total of 90 male Ross 308 broiler chicks were used in a digestibility and performance bioassay to explore the effect of reduction in dietary protein and digestible amino acids and inclusion of an exogenous mono-component protease on amino acid digestibility, net energy, jejunal gene expression, and bird performance. Four dietary treatments were created by the supplementation, or not, of 2 control diets with a mono-component exogenous protease. The control diets were corn/wheat/soybean meal-based and were formulated to be either nutritionally adequate or reduced in protein and amino acids (around 3%). The 2 control diets were supplemented with xylanase and phytase (2000 FYT). Treatments were therefore arranged as a 2 × 2 factorial design. The reduction in diet nutrient density had no significant effect on various experimental outcomes (including bird performance, amino acid digestibility, and net energy [NE]) that were measured with the exception of a reduction in the expression of aminopeptidase N and glucose transporter 2. However, the addition of exogenous protease resulted in an increase in weight gain and a reduction in feed conversion ratio (around 4%; $P < 0.05$) and an increase in the digestibility of several amino acids ($P < 0.05$) and starch ($P = 0.06$). Protease addition also resulted in an increase in both apparent metabolizable energy (AME) (+73 kcal/kg; $P < 0.05$) and NE (+107 kcal/kg; $P < 0.05$). The addition of exogenous protease to the diet also increased the jejunal expression of genes responsible for peptide transport (PepT2; $P < 0.01$) and starch digestion (sucrase isomaltase; $P = 0.06$). These results confirm the efficacy of exogenous protease in broiler diets that contain both xylanase and phytase and suggest substantial beneficial effects that extend beyond protein and amino acid nutrition. The effect of exogenous protease on energy partitioning, starch digestibility and the efficiency of nitrogen cycling is an area for further study.

Key words: broiler, protease, net energy, nutrition, gene expression

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INTRODUCTION

The efficacy of exogenous microbial protease as a mono-component feed enzyme has been unequivocally demonstrated in recent years (Angel et al., 2011; Cowieson and Roos, 2014; Olukosi et al., 2015; Cowieson and Roos, 2016; Cowieson et al., 2018). These benefits have been reported on ileal amino acid digestibility, bird performance, and various indicators of gut health e.g., mucin, tight junctions, and nutrient transporter proteins (Cowieson et al., 2016). However, the focus of much of the previous research on proteases in the diets of poultry has been on protein and amino acid digestibility and the effect on energy metabolizability has been largely ignored. Although an increase in ileal energy digestibility (or apparent metabolizable energy [**AME**]) is expected, commensurate with increases in the digestibility of protein, reported effects are substantial and extend beyond a magnitude that can be easily explained based on the arithmetic sum of the contributing amino acids. For example, Fru-Nji et al. (2011) observed an increase in AME of 51 to 212 kcal/kg in broilers (day 36) fed corn-soy-based diets. Freitas et al. (2011) observed increases in AME of up to 194 kcal/kg when exogenous protease was added to a corn-soy-meat meal-based diet (day 42). Kalmendal and Tauson (2012) supplemented a wheat/soy-based diet with an exogenous protease and

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observed an increase in AME of 114 kcal/kg. Olukosi et al. (2015) noted an increase in AME of 177 kcal/kg when an exogenous protease was added to a corn-soy-canola-distillers-based diet. Finally, Cowieson et al. (2016) noted an increase in AME of 28 to 131 kcal/kg when an exogenous protease was added to corn/soy or corn/canola-based diets for broilers.

As the focus of much of the recent research on exogenous protease has been ileal amino acid digestibility, the effect on energy digestibility has been observed but not systematically explored. Thus, though the effects on AME are substantial (>80 kcal/kg on average), they are not well understood and so are often heavily discounted in least cost formulation *in praxis*. Furthermore, the efficacy of exogenous protease has been challenged recently (Lee et al., 2018) based on the observation that some of the beneficial effects on amino acid digestibility may be vulnerable to erosion by the simultaneous addition of adjacent enzymes, e.g., phytase, to the diet. Therefore, it was the purpose of the experiment reported herein to explore the effect of exogenous protease in corn/wheat/soy-based diets that contained both exogenous phytase and xylanase and to generate more granularity on its effect on energy partitioning and the digestibility of energy-yielding macro-nutrients. The hypothesis was that exogenous protease would significantly improve the digestibility of amino acids in diets that contain xylanase and phytase, irrespective of adequate or reduced protein and amino acid levels, and that increases in metabolizable and net energy (**NE**) would also declare themselves.

METHODOLOGY

Birds and Diets

The study procedures were reviewed and approved by the University of New England Animal Ethics Committee to ensure compliance with welfare and humane practices (AEC number 17-044).

A total of 90-day-old Ross 308 male broiler chicks were obtained from a commercial hatchery. Chicks (initial BW: 46 ± 1 g) were distributed into 4 floor pens in an environmentally controlled room bedded with fresh wood shavings, and given ad libitum access to the experimental starter (1 to 10 d) and grower (10 to 24 d) feeds (Table 1) and water. The trial comprised 4 dietary treatments as follows:

1. Positive control with adequate protein and amino acid based on Ross 308 nutrient specs
2. Negative control with crude protein (**CP**) and amino acid levels lower as per protease matrix recommendation (circa 3.5% lower)
3. Positive control + 200 ppm protease
4. Negative control + 200 ppm protease

Xylanase (Ronozyme WX, DSM Nutritional Products; 200 mg/kg—no matrix value) and phytase (Ronozyme HiPhos, DSM Nutritional Products; 200 mg/kg [2000 FYT]—matrix value for Ca, P, and Na) were added into the diets. The protease (RONOZYME ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) used in the current experiment was a serine protease expressed in *Bacillus licheniformis*. One protease (PROT) unit is defined as the amount of enzyme that releases 1 mmol of

Table 1. Ingredient and as-fed calculated nutrient composition of the experimental grower diets.

Ingredient %	Positive control (without protease matrix)	Negative control (with protease matrix)
Corn, 9.5% CP	18.64	18.64
Wheat, 12.5% CP	43.74	45.53
SBM, 46.5% CP	22.50	20.92
Canola Meal, 36.5% CP	7.90	7.90
Canola oil	4.118	3.919
Limestone	1.025	1.027
Dicalcium phosphate, 18% P 21% Ca	0.427	0.437
Salt	0.205	0.205
Sodium bicarbonate	0.093	0.091
Titanium dioxide	0.500	0.500
Vitamin premix[1]	0.090	0.090
Mineral premix[2]	0.100	0.100
Choline Chloride 60%	0.074	0.077
L-lysine HCl 78.4	0.264	0.262
DL-methionine	0.198	0.179
L-threonine	0.077	0.072
Phytase[3] (200 g/mt)	0.020	0.020
Xylanase[3] (WX 200 g/mt)	0.020	0.020
Calculated composition (*determined*)		
ME Poultry; kcal/kg	3120	3120
Crude Protein; %	20.91 (*21.34*)	20.38 (*20.55*)
Crude fat; %	6.08	5.91
Crude Fiber; %	2.78	2.75
d Arg; %	1.160	1.119
d Lys; %	1.110	1.071
d Met; %	0.499	0.474
d M+C; %	0.830	0.800
d Trp; %	0.235	0.227
d Ile; %	0.758	0.734
d Thr; %	0.720	0.695
d Val; %	0.848	0.825
Calcium; %	0.800 (*0.89*)	0.800 (*0.86*)
Phosphorus; %	0.400	0.400
Phosphorus; %	0.475 (*0.547*)	0.472 (*0.554*)

[1]Vitamin concentrate (DSM Nutritional Products, Wagga Wagga, NSW, Australia) 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 concentrate supplied per kilogram of diet: Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg

[3]RONOZYME HiPhos and RONOZYME WX (DSM Nutritional Products, Kaiseraugst, Switzerland) were used as sources of phytase and xylanase, respectively. In the case of phytase a matrix value of 0.15% Ca and 0.15% digestible P was used. In the case of xylanase no energy assumption was made and the product was added over the top of formulation. Enzyme recoveries were as follows: Phytase—PC 2835 FYT/kg; PC + Protease 3,327 FYT/kg; NC 2542 FYT/kg; NC + Protease 2928 FYT/kg. Protease—PC < 100 PROT/kg; PC + Protease 12,870 PROT/kg; NC < 100 PROT/kg; NC + Protease 12,320 PROT/kg.

p-nitroaniline from 1 mM substrate (Suc-Ala-Ala-Pro-Phe-pNA_ per minute at pH 9.0 and 37°C).

Prior to formulating the experimental diets, homogenous subsamples of raw materials (corn, wheat, canola meal, and soybean meal) were analyzed for CP, crude fiber, crude fat, crude ash, total phosphorus, and amino acid contents using near-infrared spectroscopy (**NIR**; AminoNIR, Evonik Industries AG, Essen, Germany). Each diet was mixed and cold pelleted (65°C) to pass through a 3 mm die. Titanium dioxide was incorporated into all grower diets as an indigestible marker at a rate of 0.5% for nutrient digestibility analysis.

The birds were divided into 4 equal groups and were fed their respective experimental diet for an acclimatization period of 24 d. Although feed intake, weight gain, and feed conversion ratio (**FCR**) were recorded during this acclimatization period, there was no replication and so statistical analysis was not possible. However, this was done in order to more accurately represent the efficacy (or otherwise) of the experimental enzyme during the experimental phase (day 24 to 30).

Indirect Calorimetric Measurement

On day 24, after 3 h fasting, chicks were weighed and (from the initial 90 birds) 32 birds within a similar body weight range were allocated to 16 calorimetric chambers (2 birds/chamber). The chambers were allocated to the 4 dietary treatments with 4 replicates chambers per treatment, employing a 2 × 2 factorial arrangement of treatments.

Closed-circuit calorimetric chambers, set up in a climate-controlled room (4 chambers per diet with 2 birds per chamber), were used to measure heat production (**HP**), AME, and **NE**) by gaseous exchange and total collection of excreta, from day 27 to 30, following a 3-d further acclimatization period from day 24 to 27 (to allow the birds to adjust to the chambers).

The O_2 consumption (L) was calculated as the difference in weight of the oxygen cylinder at the beginning and end of each run, and subsequently converted to volume using the oxygen density of 1.331 g/L. Carbon dioxide was trapped in 32% KOH and determined gravimetrically using barium precipitation as described by Annison and White (1961). The amounts of O_2 consumed and CO_2 produced were used to calculate HP, using the Brouwer (1965) equation without a correction for urinary nitrogen excretion. The respiratory quotient (**RQ**) of the 3-d run was calculated as the volume of CO_2 produced to the volume of O_2 consumed.

AME and NE Determination

During the measurement period in the chambers, feed consumption and excreta weights were recorded daily and used to calculate energy intake and excretion. A total of 4 subsamples from each replicate were collected daily and homogenized from the total amount of excreta voided at the end of the experiment for gross energy and nitrogen content analyses. The AME of the diets was determined using the following equation:

$$\text{AME (kcal/kg)} = (\text{GEI} - \text{GEE}) / \text{FI},$$

where GEI is the gross energy intake and GEE is the gross energy output of excreta (kcal/kg) and FI is the feed intake (kg).

NE intake and NE of the diets were calculated as described by Noblet et al. (1994). Briefly, the fasting heat production (**FHP**) value plus the retained energy (**RE**) in birds gives NE intake. Therefore, RE (kcal/d) was calculated by subtracting HP (kcal/d) measured by gaseous exchange in the chambers from metabolizable energy intake (kcal/d). A FHP value of 450 kJ/$BW^{0.70}$ per day was used corresponding to the asymptotic HP (at zero activity) as estimated by Noblet et al. (2015). The NE value of the diet was calculated as kcal/kg feed as follows:

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

where NE is net energy as kcal/kg feed, RE is retained energy (kcal/d), FHP is fasting heat production (kcal/d), and FI is feed intake (kg).

Sample Collection

At the end of the NE run (day 30), both birds in each chamber were euthanized by electric stunning and decapitation. Individual blood samples from each bird were collected from the jugular vein in non-heparinized tube and centrifuged at 2000 × *g* for 10 min to obtain serum. The content of the ileum (portion of the small intestine from Meckel's diverticulum to approximately 1 cm proximal to the ileocecal junction) was gently flushed out with ultra-pure water and pooled per replicate chamber, then frozen and stored at −20 °C until processed for digestibility analyses. Individual digesta content from jejunum (beginning of jejunum to Meckel's diverticulum) was collected into Eppendorf tube, stored on ice and immediately transferred to the lab, centrifuged at 12,000 × *g* for 10 min. The supernatant was collected and stored at −20 for viscosity analyses. The jejunal mucosa (entire jejunum) of each bird was scraped off and collected into Eppendorf tube following rinsing with autoclaved PBS solution and immediately stored in liquid nitrogen, and then at −80 °C for gene expression analysis.

Chemical Analyses

The diets, excreta, and digesta DM matter were determined using methods of AOAC (2006). The gross energy contents of feed, excreta, and digesta samples was determined on a 0.5-g sample using an adiabatic bomb calorimeter (IKA Werke, C7000, GMBH and

CO., Staufen, Germany) with benzoic acid as standard. The nitrogen contents of feed, freeze-dried excreta, and digesta samples were determined on a 0.25-g sample in a combustion analyzer (Leco model FP-2000 N analyzer, Leco Corp., St. Joseph, MI) using EDTA as a calibration standard, with CP being calculated by multiplying percentage N by a correction factor (6.25). The starch content of feed and freeze-dried digesta samples was measured using the Megazyme Total Starch Assay Kit (Megazyme Int. Ireland Ltd., Wicklow, Ireland).

Individual jejunal supernatants were thawed at 4°C and 0.5 mL of thawed supernatant was used to measure viscosity with a Brookfield DVIII viscometer (Stoughton, MA, US) at 25°C with a CP 40 cone. The shear rate was from 5 to 500 s^{-1}, over which the samples did not exhibit shear thinning.

For AA analysis, samples (diets and freeze-dried digesta) were prepared by 6 N HCL hydrolysis for 24 h at 110°C followed by neutralization with 4 mL of 25% (wt/vol) NaOH, and then cooled to room temperature. Afterward, sodium citrate buffer was added and the mixture was equalized to a 50-mL volume (AOAC 1990; method 982.30). Methionine and cysteine (sulfur-containing amino acids) were analyzed by performic acid oxidation at 0°C, followed by acid hydrolysis. The amino acids in the hydrolysate were determined by an AA analyzer (Biochrom 30. 30 plus, Biochrom Ltd, Cambridge, UK).

Serum total bile acid concentration was determined using an automated clinical-chemistry analyser by adaptation of the 3α-hydroxy steroid dehydrogenase enzymatic cycling method carried out according to Qureshi et al. (1986).

Titanium dioxide concentrations were determined in triplicate and duplicate for diets and digesta samples, respectively, by the colorimetric method described by Short et al. (1996). The percentage of ileal digestibility of nutrients was calculated using the indigestible marker as follows:

$$\text{Ileal digestibility (\%)} = \{1 - -[\text{TiO}_2\text{ diet (\%)}/\text{TiO}_2\text{ digesta (\%)}] \times [\text{digetsa nutrient (\%)}/\text{diet nutrient (\%)}]\} \times 100.$$

RNA Isolation

Total RNA from each jejunal mucosa sample was extracted with TRIsureTM (Bioline, Sydney, Australia) following the manufacturer's instructions. For each sample, total RNA was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Total RNA quantity and purity were determined using a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany) was employed to measure RNA integrity (RNA Integrity Number, or RIN) using RNA 6000 Nano kit. The RIN values ranged between 7.7 and 9.5.

cDNA Synthesis

The extracted RNA of each sample was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 1 μg of extracted RNA from each sample was incubated in 2 μL of 7 × gDNA Wipeout Buffer at 42°C for 2 min in order to eliminate genomic DNA. Then, the gDNA elimination reaction was mixed with reverse-transcription reaction components contained 1 μL of Quantiscript Reverse Transcriptase, 4 μL of 7 × Quantiscript RT Buffer, and 1 μL of RT Primer Mix. The Rotorgene 6000 real-time PCR machine (Corbett, Sydney, Australia) was employed to incubate the mixture at 42°C for 15 min and at 95°C for 3 min in order to convert the RNA into cDNA. The cDNA was diluted 6 times with nuclease-free water and stored at −20°C until required.

Primer Sources. The primers were sourced from previously published studies in chickens. Table 2 shows the primers that were used in the current study. Prior to quantitative PCR analysis, the primer specificity for each pair was analyzed with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Germany) using Agilent DNA 1000 Kit (Agilent Technologies, Inc., Germany).

Real-time Quantitative PCR

Quantitative PCR was performed in triplicates using a SYBR Green kit SensiFAST SYBR No-ROX (Bioline, Sydney, Australia) with Rotorgene 6000 real-time PCR machine (Corbett Research, Sydney, Australia). The PCR reaction was performed in a volume of 10 μL containing 5 μL of 2 × SensiFAST, 400 mM of each primer and 2 μL of diluted cDNA template. Post thermal cycling, amplification cycle (Cq) values for all genes were collected and imported into qBase+ version 3.0 (Biogazelle, Zwijnbeke, Belgium) software and analyzed against 2 optimized reference genes, GAPDH and HMBS, in the present study. The qBase+ applied an arithmetic mean method to transform logarithmic Cq value to linear relative quantity using exponential function for relative quantification of genes (Vandesompele et al., 2002; Hellemans et al., 2007) and the output data were exported to a statistical software for further analysis.

Statistical Analyses

All the data derived were checked for normal distribution prior to conducting statistical analyses. Data were subjected to 2-way ANOVA analysis as a 2 × 2 factorial arrangement of treatments, using General Linear Model procedure of SAS 9.3 package to assess the main effects

Table 2. Sequences of primers used for quantitative real-time polymerase chain reaction.

Gene	Gene full name	Primer sequence (5′-3′)	Size (bp)	Accession no.	Reference
ATP1A1	ATPase Na+/K+ transporting subunit alpha 1	F-GTCAACCCGAGGGATGCTAA R-ACTGCTACAATGGCACCCTG	179	NM_205521.1	Kheravii et al. (2018)
APN	Aminopeptidase N	F-AATACGCGCTCGAGAAAACC R-AGCGGGTACGCCGTGTT	70	NM_204861.1	Gilbert et al. (2007)
ASCT1	Alanine, serine, cysteine, and threonine transporter (SLC1A4)	F-TTGGCCGGGAAGGAGAAG R-AGACCATAGTTGCCTCATTGAATG	63	XM_001232899.4	Paris and Wong (2013)
PepT1	Peptide transporter-1 (SLC15A1)	F- TACGCATACTGTCACCATCA R-TCCTGAGAACGGACTGTAAT	205	AY029615.1	Guo et al. (2014)
PepT2	Peptide transporter-2 (SLC15A2)	F- TGACTGGGCATCGGAACAA R-ACCCGTGTCACCATTTTAACCT	63	NM_001319028.1	Paris and Wong (2013)
GLUT1	Glucose transporter-1 (SLC2A1)	F-TCCTCCTGATCAACCGCAAT R-TGTGCCCCGGAGCTTCT	65	NM_205209.1	Su et al. (2014)
GLUT2	Glucose transporter-2 (SLC2A2)	F-TGATCGTGGCACTGATGGTT R-CCACCAGGAAGACGGAGATA	171	NM_207178.1	Kheravii et al. (2018)
IL-18	Interleukin 18	F- TGTGTGTGCAGTACGGCTTAG R- CTTACAAAAGGCATCGCATTC	79	NM_204608.1	Forder et al. (2012)
LAT1	L type amino acid transporter-1 (SLC7A5)	F-GATTGCAACGGGTGATGTGA R-CCCCACACCCACTTTTGTTT	70	KT876067.1	Gilbert et al. (2007)
MUC-2	Mucin 2	F- CCCTGGAAGTAGAGGTGACTG R- TGACAAGCCATTGAAGGACA	143	XM_001234581.3	Fan et al. (2015)
MUC-5AC	Mucin 5	F- AAGACGGCATTTATTTCTCCAC R- TCATTACCAACAAGCCAGTGA	244	XM_003641322.2	Fan et al. (2015)
PepT1	Peptide transporter-1 (SLC15A1)	F-TACGCATACTGTCACCATCA R-TCCTGAGAACGGACTGTAAT	205	AY029615.1	Guo et al. (2014)
PepT2	Peptide transporter-2 (SLC15A2)	F-TGACTGGGCATCGGAACAA R-ACCCGTGTCACCATTTTAACCT	63	NM_001319028.1	Paris and Wong (2013)
SI	Sucrase isomaltase	F-GCTTTAAGATGGGCAAGAGGAAG R- CCACCACCAGGCAAAAGAGG	65	XM_015291762.1	Kheravii et al. (2018)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F- GAGGGTAGTGAAGGCTGCTG R-CATCAAAGGTGGAGGAATGG	113	NM_204305.1	Dalgaard et al. (2015)
HMBS	Hydroxymethylbilane synthase	F- GGCTGGGAGAATCGCATAGG R-TCCTGCAGGGCAGATACCAT	131	XM_417846.2	Yin et al. (2011)

of protease and its nutrient matrix and their interactions. For energy partitioning parameters and nutrient digestibility, each single chamber was considered as an experimental unit (n = 16); for gene expression, bile acid and viscosity analysis of each bird were considered as an experimental unit (n = 32), and the values presented in the tables are means with pooled SEM. When a significant effect of treatment was detected, Tukey's HSD test was used to make pairwise comparisons between means. Significant values are based on $P < 0.05$; P values > 0.05 and < 0.10 are reported if data suggested a trend.

To standardize traits of energy metabolism including AME, RE, and HP, body weight was raised to the power of 0.70 as metabolic body weight of the birds (Rivera-Torres et al., 2010; Noblet et al., 2015).

RESULTS

The concentration of protein, phosphorus, and calcium in the experimental diets was in line with expectations (Table 1). However, the recovery of exogenous phytase activity in the control diets was higher than expected (approximately 3,000 FYT/kg vs. a target of 2000 FYT/kg). Performance of the birds during the acclimatization period (day 1 to 24; Table 3) was as expected relative to breed guidelines (day 24 weight around 1,430 g and FCR of approximately 1.30). While not statistically confirmed, the birds that received the diets containing exogenous protease had numerically lower FCR and slightly higher body weight compared with those fed the associated control diets.

The effect of diet nutrient density and exogenous protease on performance and energy partitioning is presented in Table 4. There were no interactions ($P > 0.05$) between exogenous protease addition and diet nutrient density with the exception of a tendency ($P = 0.093$) for a slightly more robust effect of exogenous protease on retained N in the control diet with a lower CP and digestible amino acid concentration. Reducing the concentration of dietary protein and amino acids had no effect ($P > 0.05$) on broiler weight gain, FCR, feed intake, AME, retained N, RQ, or NE. There was a tendency ($P = 0.073$) for diets with lower CP and digestible amino acid concentration to result in a lower heat production (292 vs. 301 kcal/b/d) in birds fed those diets. Supplementation of the control diets with exogenous protease resulted in an increase in weight gain ($P < 0.01$), a reduction in FCR (7 points; $P < 0.001$), AMEn (49 kcal/kg; $P < 0.05$), an increase in retained N ($P < 0.001$), and an increase in diet NE (107 kcal/kg; $P < 0.05$). There was no effect ($P > 0.05$) of exogenous protease on the volumes of oxygen consumed or carbon dioxide produced, the RQ of the birds or heat production. Birds fed the protease-supplemented diets tended to have a higher AME/NE ratio (0.768 vs. 0.752; $P = 0.076$).

The effect of diet nutrient density and exogenous protease addition on ileal protein, energy, amino acid and starch digestibility and on serum bile acid concentration and digesta viscosity is presented in Table 5. Addition of exogenous protease to the diet with reduced CP and digestible amino acid concentration resulted in an increase in ileal starch digestibility, whereas there was no effect of protease on starch digestibility in the diet with higher CP and amino acid concentration (interaction $P < 0.05$). There was no effect of either diet nutrient density or exogenous protease addition (and no interactions) on ileal energy digestibility or serum bile acid concentration. There was a tendency ($P = 0.08$) for exogenous protease to increase (77.6 vs. 79.0%) ileal CP digestibility. The addition of exogenous protease resulted in an increase ($P < 0.05$) in the apparent ileal digestibility of Lys (+3.2%), Met (+3.0%), Thr (+3.9%), Cys (+3.8%), Glu (+3.0%), Ser (+4.8%), and of total amino acids (by around +2.5%). There was no effect ($P > 0.05$) of either diet nutrient density or supplemental protease on digesta viscosity. There was no effect ($P > 0.05$) of diet protein ad amino acid concentration on ileal amino acid digestibility.

The effect of exogenous protease and diet nutrient density on jejunal gene expression is presented in Table 6. There were no significant interactions between diet CP and amino acid concentration and exogenous protease addition. Reducing the concentration of dietary CP resulted in an increase in the jejunal expression of genes that encode for aminopeptidase N ($P < 0.05$), glucose transporter 2 ($P < 0.05$), and mucin 2 ($P = 0.056$). Addition of exogenous protease to the control diets resulted in an increase in the expression of peptide transporter 2 ($P < 0.01$) and tended to increase the expression of sucrase isomaltase ($P = 0.069$) and reduce the expression of interleukin 18 ($P = 0.098$).

Table 3. Performance parameters of male broiler chickens (1 to 24 d) in response to supplementation of diets of varying protein and amino acid density with a mono-component exogenous protease.

Treatments[1]	PC	NC	PC + protease	NC + protease
Body weight (g/bird)				
D 1	47.1	46.4	46.7	46.3
D 10	347	337	350	353
D 24	1432	1411	1450	1439
Feed intake (g/bird)				
Day 1 to 10	310	316	310	312
Day 10 to 24	1503	1504	1472	1490
Day 1 to 24	1814	1820	1781	1803
FCR (g:g)				
Day 1 to 10	1.036	1.086	1.022	1.020
Day 10 to 24	1.341	1.373	1.291	1.323
Day 1 to 24	1.309	1.334	1.271	1.294

Mean values are based on 20 birds per pen and one pen per treatment.
[1]PC: Positive control with adequate protein and amino acid; NC: Negative control as PC but with a crude protein and amino acid levels lower as per protease recommendations (approx. 3%).

Table 4. Energy partitioning and utilization of broiler chickens in response to supplementation of diets of varying protein and amino acid density with a mono-component exogenous protease (days 24 to 30).

	Treatment effects					Main effects				Source of variation (*P*-value)		
	PC (no matrix)		NC (with matrix)			Protease		Matrix				
Item	Protease	No Enzyme	Protease	No Enzyme	SEM	+	−	NC	PC	Enzyme	Matrix	Enzyme × Matrix
BWG (g/b/d)	116.2	112.5	114.7	109.2	1.404	115.4	110.8	112.0	114.3	0.006	0.114	0.529
Feed intake (g/b/d)	172.1	173.0	171.0	171.4	1.391	171.6	172.2	171.2	172.6	0.640	0.343	0.856
FCR (g/g)	1.481	1.538	1.491	1.570	0.016	1.486	1.554	1.531	1.511	0.001	0.226	0.531
ME diet (kcal/kg)	3238	3178	3250	3164	26.66	3244	3171	3207	3208	0.017	0.974	0.633
MEn diet (kcal/kg)	3050	3007	3058	3004	25.18	3054	3005	3031	3029	0.076	0.921	0.822
ME intake (kcal/b/d)	557	550	556	542	6.560	556	546	549	553	0.138	0.498	0.652
ME intake (kcal/kg $BW^{0.70}$)[1]	359	358	364	358	5.337	362	358	361	359	0.506	0.706	0.712
Retained N (g/b/d)	3.93	3.58	3.99	3.33	0.084	3.96	3.46	3.66	3.76	0.001	0.264	0.093
V O_2[2](L/kg of $BW^{0.70}$ per d)	38.1	39.1	37.4	38.3	0.718	37.7	38.7	37.8	38.6	0.164	0.287	0.926
V CO_2[3](L/kg of $BW^{0.70}$ per d)	38.2	39.0	37.5	38.4	0.526	37.9	38.6	38.6	37.9	0.123	0.197	0.882
RQ[4]	1.004	1.00	1.002	1.001	0.008	1.003	1.00	1.002	1.00	0.536	0.824	0.615
HP[5] (kcal/b/d)	299	303	290	294	5.260	294	299	292	301	0.362	0.073	0.937
HP (kcal/kg $BW^{0.70}$)	193	198	189	194	3.367	191	196	191	195	0.151	0.263	0.961
HI[6] (kcal/kg $BW^{0.70}$)	132	138	125	131	4.935	129	135	128	135	0.204	0.131	0.943
RE[7] (kcal/kg $BW^{0.70}$)	166	160	175	164	6.135	171	162	170	163	0.142	0.299	0.691
NE[8] diet (kcal/kg)	2468	2376	2519	2398	43.28	2494	2387	2459	2422	0.019	0.367	0.717
NE:ME %	0.762	0.747	0.774	0.758	0.008	0.768	0.752	0.766	0.754	0.076	0.171	0.872
NEn diet (kcal/kg)	2280	2205	2326	2238	41.55	2303	2222	2282	2243	0.051	0.312	0.861

[1]$BW^{0.70}$: metabolic BW.
[2]Volume of oxygen consumed.
[3]Volume of carbon dioxide produced.
[4]Respiratory quotient (CO_2/O_2).
[5]Heat production.
[6]Heat increment
[7]Retained energy.
[8]Metabolisable and Net energy (ME and NE) values are expressed based on as-is basis of the feed (total collection method); NE calculated as fasting heat production + RE.

Table 5. Ileal protein, energy, amino acid and starch digestibility, serum bile acids concentration and digesta viscosity of broiler chickens in response to supplementation of diets of varying protein and amino acid density with a mono-component exogenous protease.

	Treatment effects					Main effects						
	PC (no matrix)		NC (with matrix)			Protease		Matrix		Source of variation (P-value)		
Item	Protease	No Enzyme	Protease	No Enzyme	SEM	+	–	NC	PC	Enzyme	Matrix	Enzyme × Matrix
Digesta viscosity (mPa s)	2.42	2.36	2.33	2.46	0.100	2.38	2.41	2.40	2.39	0.761	0.945	0.335
Serum bile acids (μmol/L)	24.4	20.5	21.8	22.2	1.776	23.1	21.3	22.0	22.5	0.335	0.796	0.248
Energy digestibility	77.1	74.0	76.6	74.7	1.419	76.9	74.4	75.7	75.6	0.106	0.945	0.655
Starch digestibility	95.1[a,b]	94.8[a,b]	97.3[a]	93.6[b]	0.690	96.2	94.2	95.5	94.2	0.014	0.411	0.028
CP digestibility	78.9	76.4	80.0	78.1	1.176	79.4	77.2	79.0	77.6	0.080	0.265	0.835
Indispensable AA												
Arg	85.7	84.2	85.5	83.9	1.344	85.6	84.1	84.7	84.9	0.279	0.859	0.968
His	81.5	78.7	80.8	78.1	1.331	81.1	78.4	79.5	80.1	0.064	0.642	0.980
Ile	78.6	79.0	79.4	77.3	1.534	79.0	78.2	78.4	78.8	0.599	0.794	0.436
Leu	78.4	76.8	78.9	77.2	1.303	78.6	77.0	78.1	77.5	0.231	0.712	0.946
Lys	85.0	82.5	85.1	82.6	1.143	85.1	82.5	83.9	83.7	0.049	0.898	0.995
Met	88.8	85.3	87.6	85.8	1.124	88.2	85.6	86.7	87.1	0.037	0.717	0.460
Phe	81.7	79.2	80.8	79.7	1.107	81.3	79.5	80.3	80.5	0.131	0.830	0.552
Thr	77.2	73.7	76.6	74.3	1.058	76.9	74.0	75.4	75.5	0.016	0.950	0.574
Trp	79.8	77.5	77.8	78.1	0.692	78.8	77.8	77.9	78.6	0.175	0.301	0.084
Val	77.6	76.1	75.9	75.3	0.896	76.7	75.7	75.6	76.8	0.270	0.210	0.610
Dispensable AA												
Ala	79.2	77.7	79.1	78.0	0.946	79.1	77.9	78.6	78.5	0.219	0.930	0.815
Asp	76.9	74.9	75.9	75.3	1.368	76.4	75.1	75.6	75.9	0.338	0.827	0.618
Cys	74.3	71.9	74.0	71.1	0.938	74.2	71.5	72.6	73.1	0.015	0.579	0.815
Glu	90.9	87.5	89.3	87.5	0.984	90.1	87.5	88.3	89.2	0.020	0.422	0.438
Gly	78.0	74.6	77.4	74.5	1.366	77.7	74.6	75.9	76.3	0.042	0.786	0.861
Pro	84.5	83.5	84.1	83.8	1.111	84.3	83.6	83.9	83.9	0.579	0.988	0.759
Ser	81.6	77.4	80.4	77.2	1.515	81.0	77.3	78.8	79.5	0.030	0.654	0.758
TAA[1]	81.1	78.9	80.5	78.8	0.751	80.8	78.8	79.6	80.1	0.020	0.650	0.694

[a,b]Means in a row not sharing a superscript differ significantly at $P < 0.05$ for treatment effect.
[1]Total amino acids.

Table 6. Jejunal gene expression of broiler chickens in response to supplementation of diets of varying protein and amino acid density with a mono-component exogenous protease.

	Treatment effects					Main effects				Source of variation (*P*-value)		
	PC (no matrix)		NC (with matrix)			Protease		Matrix				
Genes[1]	Protease	No Enzyme	Protease	No Enzyme	SEM	+	–	NC	PC	Enzyme	Matrix	Enzyme × Matrix
ATP1A1	1.12	1.09	1.11	0.86	0.102	1.12	0.98	0.98	1.11	0.171	0.233	0.275
APN	1.27	1.16	1.07	0.81	0.127	1.17	0.99	0.94	1.22	0.120	0.022	0.514
ASCT1	1.19	1.19	1.21	0.99	0.137	1.20	1.09	1.10	1.19	0.398	0.503	0.419
GLUT1	1.23	0.94	0.97	0.91	0.114	1.10	0.92	0.94	1.08	0.114	0.188	0.286
GLUT2	1.33	1.29	1.05	1.02	0.123	1.19	1.16	1.03	1.31	0.765	0.032	0.989
LAT1	0.98	1.21	1.22	1.38	0.293	1.11	1.30	1.30	1.10	0.500	0.478	0.907
PepT1	1.05	1.04	1.26	0.92	0.136	1.16	0.98	1.09	1.04	0.192	0.737	0.217
PepT2	1.24	0.97	1.21	0.77	0.116	1.22	0.87	0.98	1.11	0.004	0.288	0.466
SI	1.32	1.12	1.26	0.98	0.128	1.29	1.06	1.13	1.22	0.069	0.456	0.757
IL-18	1.12	0.88	0.93	0.81	0.113	1.03	0.84	0.87	1.01	0.098	0.238	0.557
MUC2	1.28	1.42	0.99	0.76	0.242	1.14	1.09	0.88	1.35	0.227	0.056	0.436
MUC5	1.28	1.25	1.14	0.80	0.152	1.22	1.02	0.96	1.26	0.218	0.054	0.298

[1]ATP1A1: ATPase Na+/K+ transporting subunit alpha 1; APN: Aminopeptidase N; ASCT1: Alanine, serine, cysteine, and threonine transporter (SLC1A4); GLUT1: Glucose transporter-1 (SLC2A1); GLUT2: Glucose transporter-2 (SLC2A2); LAT1: L type amino acid transporter-1 (SLC7A5); PepT1: Peptide transporter-1 (SLC15A1); PepT2: Peptide transporter-2 (SLC15A2); SI: Sucrase isomaltase; IL-18: Interleukin-18; MUC2: Mucin 2; MUC5: Mucin.

DISCUSSION

It is interesting that while the majority of recently published literature on exogenous proteases in the diets of monogastric animals has been strongly oriented toward effects on amino acid and protein digestibility (Cowieson and Roos, 2014), the initial literature (1950s to the early 2000s) was largely unconcerned with this specific outcome. For example, the pioneering work of Lewis et al. (1955) and Baker et al. (1956) and subsequent research published by Castanon and Marquardt (1989), Huo et al. (1993), Guenter et al. (1995), Hessing et al. (1996), Simbaya et al. (1996), Rooke et al. (1998), Thorpe and Beal (2001), and Odetallah et al. (2003, 2005) focused on the effect of exogenous protease on performance (weight gain and FCR) and on the beneficial effect of protease on proteinaceous antinutrients e.g., trypsin inhibitors and lectins. Data generated in the present experiment are supportive of a beneficial effect of exogenous protease on performance of broiler chickens with an increase in weight gain and a reduction in FCR of around 4 to 5% compared with the birds fed the control diet. These responses in weight gain and FCR are comparable to previous observations (summarized in Cowieson and Roos, 2016) but are greater than would be expected based solely on amino acid digestibility changes alone (implying contributory effects from energy-yielding macro-nutrients or overall partitioning of nutrients in general).

In the present experiment, protease addition increased AME from 3,171 to 3,244 kcal/kg (+73 kcal/kg) which is in close agreement with responses in the literature (Freitas et al. 2011; Fru-Nji et al. 2011; Kalmendal and Tauson, 2012; Olukosi et al. 2015; Cowieson et al. 2016) where a mean increase in AME or ileal digestible energy of approximately 80 kcal/kg was reported. Interestingly, although the effect of protease on AME was +73 kcal/kg (2.3%), the effect on NE was +107 kcal/kg (+4.5%). Similar effects have been noted previously. For example, Olukosi et al. (2008) noted only small (or even negative) changes in AME when a corn-wheat-soy-based diet was supplemented with either phytase or a combination of carbohydrase and protease and fed to broilers but at the same time observed significant increases in NE. Importantly, Olukosi et al. (2008) observed a stronger correlation between body weight of the birds and NE than was the case for AME. This suggests that the effect of enzymes on NE may be more indicative of the "true" value of the enzyme as far as the bird is concerned than changes in AME.

Separation of the effect of protease (or alternative feed enzymes) on direct digestibility or metabolizability metrics from effects on "net" metrics such as the investment of energy in gut maintenance or in the synthesis of endogenous proteins is relevant when it comes to explanation of changes in animal growth rate and efficiency. Effects of exogenous protease and amylase on the size of the digestive organs, and the secretion of endogenous enzymes have been reported in the literature (Mahagna et al., 1995; Jiang et al., 2008; Yin et al., 2018), but the value of those savings in NE have not been quantified. In principle, it is likely that the effect of protease and amylase on NE savings are proportionally more important compared to that of phytase and xylanase, because the former directly complements the activity of endogenous enzymes. Effects of exogenous enzymes on maintenance requirements and heat increment of feeding have not been properly measured to date. This study provides evidence of the possible magnitude of those effects, which may help to accurately quantify the value of the different exogenous enzymes in the market. Clearly, increased RE, NE, and NE/AME levels by exogenous protease observed in present study suggest the benefit of the enzyme to growth of birds

due to the more efficient gain of energy through higher digestibility of nutrients in feed.

In the present work, exogenous protease addition resulted in an increase in ileal amino acid digestibility of just over 2.5% (mean of all amino acids) but this was more substantial for Lys, Met+Cys, Thr, His, Gly, and Ser (being between +3.0% and +4.8%). Cowieson and Roos (2014) presented a meta-analysis of 25 independently conducted experiments that reported the effect of 1 mono-component microbial protease on apparent ileal amino acid digestibility in pigs and poultry, observing a mean response of +3.74% (ranging from +2.7% for Glu to +5.6% for Thr). These values are comparable to those reported herein for the most nutritionally relevant amino acids e.g., Met+Cys, Lys, and Thr but for some other amino acids the effect of protease in the current experiment was slightly lower than the mean values reported by Cowieson and Roos (2014). This is most likely explained by natural variance in experimental conditions, the fact that the meta-analysis of Cowieson and Roos (2014) was multiple-species and also included single ingredients as well as complete diets, i.e., the use of synthetic amino acids varied from trial to trial. Nonetheless, the present data suggest that exogenous protease is capable of significantly increasing the apparent ileal digestibility of several important amino acids even in a diet that contains both carbohydrase and a high concentration of phytase. Furthermore, exogenous protease addition increased the ileal digestibility of starch, particularly in the diet with a lower concentration of protein and digestible amino acids (+3.9%). An increase in ileal starch digestibility with exogenous protease addition had also been reported by Amerah et al. (2017) in corn and soybean meal based diets. The mechanism of this effect is not clear but may be related to disruption of the protein/starch matrix in the cereal fraction of the diet with exogenous protease, as the work from McAllister et al. (1993) with in vitro fermentation of cereals plus protease addition suggested. Irrespective of the mode of action, the present results confirm a beneficial effect of exogenous protease on the digestibility of amino acids, energy, and starch in practical broiler diets that contain phytase and xylanase.

The effect of exogenous protease and the protein and amino acid concentration in the diet on jejunal gene expression is not an area that has achieved much attention in recent years. Cowieson et al. (2017) offered broiler chickens corn-based diets where the major protein source was either soybean meal or a mixture of alternative protein meals (canola, corn gluten meal, and distillers grains with solubles) and fed each without or with an exogenous mono-component protease. In this previous experiment, exogenous protease reduced the expression of MUC-2 in the jejunum of birds on both control diets but had no effect on alternative genes (IL-8, IL-10, Claudin 1, Occludin, ASCT2, and SLC7A2; Cowieson et al. 2017). In the present experiment, there was no effect of exogenous protease on the expression of MUC-2 but the expression of PepT2 and SI was increased. PepT2 (also referred to as SLC15A2) encodes for a high affinity-low capacity peptide transporter for di- and tri-peptides (Zwarycz and Wong, 2013). The addition of exogenous protease to the diet resulted in an increase in the expression of this particular peptide transporter is suggestive of adaptation by the bird to the activity of protease in the intestine and the resulting oligopeptide generation. However, it is not clear why PepT1, which appears to play a more active role in the absorption of peptides in the intestine than PepT2 (Zwarycz and Wong, 2013), was not affected by the addition of protease. The expression of sucrase isomaltase in the jejunum of the chicks that received the diets containing exogenous protease was also increased (1.06 vs. 1.29; $P = 0.069$) which may be associated with the substantial increase in ileal starch digestibility that was associated with exogenous protease addition. As exogenous protease does not act on starch per se but rather may increase starch digestibility indirectly by improving the solubility of starch/protein matrices, it is logical that an increase in soluble starch in the intestine, mediated via exogenous protease activity, would necessitate an upregulation of genes responsible for further digestion of dextrin. Indeed, it is possible that the availability of amino acids in the appropriate sections of the gut may have stimulated the dynamics for starch digestion (Liu and Selle, 2015).

The current results are in contrast with the findings of Peek et al. (2009), who measured sucrase-isomaltase activity in the intestinal mucosa of chickens challenged with *Eimeria* and supplemented or not with a bacterial protease. They reported a reduction of sucrase-isomaltase activity that accompanied an increased thickness of the mucosa in the jejunum of chickens with protease supplementation, which they attributed to a possible increase in the turnover of enterocytes. Evidently, there may be interactions between the effects of protease on starch digestion and intestinal health dependent effects on intestinal cell turnover, which may explain these observations.

Finally, the reduction in dietary CP and digestible amino acid concentration resulted in a reduction in the expression of aminopeptidase N and glucose transporter-1. The latter may explain why exogenous protease increased starch digestibility more in the low protein diet than in the conventional diet. The influence of diet protein and amino acid content on starch digestibility in broilers is an area for future study as this may be one of the reasons why poultry do not react favorably to low protein diets.

CONCLUSIONS

It can be concluded that this mono-component exogenous protease is capable of significantly improving the performance of broiler chickens fed on both a standard corn/wheat/soy-based diet and one that has been reduced in protein and digestible amino acids.

Furthermore, these beneficial effects were evident despite the control diets being formulated with both exogenous xylanase and a high concentration of exogenous phytase. Additionally, the beneficial effects of exogenous protease extend beyond improvements in ileal amino acid digestibility to substantial effects on the ileal digestibility of starch, AME and also NE. Further work is required to explore the range of effects of exogenous protease and the opportunities for adjacencies with alternative zootechnical feed additives. Equally, additional research is required to understand and quantify the source of these NE savings, which may be created by compensatory responses of the animal to exogenous enzymes, and may represent a significant value to producers beyond the known effects on digestibility. In the meantime, exogenous protease offers considerable value in feed cost reduction, environmental sustainability and for animal performance enhancement, and this value is likely to increase in the future as the full potential of this feed enzyme is realized.

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