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Evaluation of novel protease enzymes on growth performance and apparent ileal digestibility of amino acids in poultry: enzyme screening

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Running title: Novel proteases in poultry

Abstract

Three experiments were conducted to evaluate eight neutral serine and six acid aspartic proteases on growth performance and apparent ileal amino acid digestibility (**AID**) of poults (Experiment 1) or chicks (Experiments 2 and 3). Two basal diets were formulated: a nutrient adequate positive control (**PC**), formulated to meet or exceed the nutrient requirements for poults (Experiment 1) or chicks (Experiments 2 and 3) and a negative control (**NC**) diet formulated to achieve 85% (Experiments 1 and 2) or 80% (Experiments 3) of the requirement for protein and amino acids. Phytase was included in all diets to provide 500 FTU/kg and xylanase was included in all diets to provide 10,000 (Experiments 1 and 2) or 16,000 (Experiments 3) BXU/kg. Proteases were supplemented in the NC diet at an equivalent amount of enzyme protein to create 16 experimental diets. There were five birds/pen and 10 replicate pens per treatment in each experiment. In experiment 1, birds fed the PC diet gained more ($P < 0.05$) than birds fed the NC. There were no differences in growth performance in birds fed the PC or NC in experiments 2 or 3. In all three experiments, birds fed the NC supplemented with neutral protease 1 had reduced ($P < 0.05$) feed intake (**FI**) or body weight gain (**BWG**) and increased ($P < 0.05$) feed conversion ratio (**FCR**) compared with birds fed the NC. Birds fed the NC diet supplemented with neutral protease 3, 7 (Experiment 1) or acid protease 4 (Experiment 3) had increased ($P < 0.05$) FCR and birds fed neutral protease 6 (Experiment 2) had reduced ($P < 0.05$) BWG compared with birds fed the NC. Apparent ileal amino acid digestibility was improved ($P < 0.05$) with protease supplementation to the NC diets (Experiment 1 or 3), but this was dependent on the protease and the amino acid. In conclusion, novel protease supplementation improved AID of amino acids but this was not reflected in improvements in growth performance of turkey poults or broiler chicks.

Keywords: amino acids, broiler, turkey, protease, performance, apparent ileal digestibility

INTRODUCTION

The use of protease enzymes in industrial applications, such as detergents, textiles, food processing and animal feed, is a major contributor to the $5 billion market for industrial enzymes (Juntunen et al., 2015). Proteases are classified into six groups: aspartate, cysteine, glutamate, metallo, serine and threonine proteases, based on mechanistic features within each group (Li et al., 2013). Over one-third of all known proteolytic enzymes are serine proteases with an endoproteolytic catalytic activity typically dependent on a triad of aspartate, histadine and serine residues (Di Cera, 2009). The endogenous proteases, trypsin and chymotrypsin, belong to the largest family of serine proteases and cleave polypeptide chains at positively charged arginine, lysine residues or large hydrophobic phenylalanine, tryptophan, tyrosine residues, respectively (Di Cera, 2009). It has also been suggested that serine proteases are allosteric enzymes and respond to the conditions of their environment differently, which may influence biological activity and specificity (Di Cera, 2009), and impart differences in the serine proteases selected for use in industrial applications.

Aspartic proteases are commonly called acid endopeptidases with aspartate residues at their active site (Mandujan-Gonzlalez et al., 2016). In the food industry, they are predominantly used during the process of milk clotting to make cheese and to prevent formation of wine haze (Schlander et al., 2017). Pepsin is a well known aspartic protease in the A1 family with 282 other members (Dunn, 2002), and most aspartic proteases have broad peptide bond specificity (Uniacke-Lowe and Fox, 2017). Swine pepsin and chicken pepsin have similar molecular weights but contain different basic groups, and chicken pepsin has a higher stability at alkaline solutions due to its smaller over-all negative charge (Bohak, 1969). Esumi et al., (1980) reported the optimal pH values for quail and chicken pepsin were about 3.0, with quail pepsin having a higher relative activity at alkaline pH than chicken pepsin. Therefore, within the same group of neutral serine or acidic aspartic endogenous or industrial proteases, differences exist in their biological activity, substrate specificity, pH optima and relative activity at a range of pH.

In animal feed, protease supplementation is of interest to improve protein and amino acid digestibility, particularly in very young animals where the relative activity of endogenous proteases may not be optimal (Lewis et al., 1955; Mahagna et al., 1995). In addition, protease supplementation may improve ingredient quality by reducing ingredient variability and mitigating negative effects of heat-stable trypsin-inhibitors or lectins (Cowieson et al., 2016). Lewis et al. (1955) reported improvements in gain and efficiency of piglets fed diets supplemented with pepsin, pancreatin, papain or a fungal protease from *Aspergillus oryzae*. More recently, neutral serine or acid protease supplementation is gaining in popularity in animal diets with beneficial (Angel et al., 2011; Cowieson and Roos, 2016) or inconclusive (Freitas et al., 2011; Fru-Nji et al., 2011; Yuan et al., 2015; Yuan et al., 2017) effects on growth performance, nutrient digestibility and endogenous enzyme secretion. The objective of this set of trials was to evaluate the efficacy of eight neutral serine proteases and six acid aspartic proteases when supplemented in low protein and amino acid diets fed to turkey poults or broiler chicks for approximately 3 weeks from hatch. The response variables included growth performance, apparent ileal digestibility of amino acids (**AID**), and digestible amino acid intake in g/day.

MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Missouri (Experiments 1 and 2) and the National Institute of Poultry Husbandry and approved by the Ethical Committee of Harper Adams University (Experiment 3).

Novel proteases

The type of protease, source organism, working temperature optimum, pH optimum and pH range of the evaluated proteases is listed in Table 1. There were 14 proteases evaluated in 3 poultry trials, eight neutral proteases and six acid proteases. Eleven of the proteases evaluated were novel proteases, and three proteases were obtained commercially. In Experiments 1 and 2, all 14 proteases were evaluated. However, there was not enough sample of acid protease 2 or 6 for inclusion in Experiment 3, and these proteases were replaced with 1,500 or 3,000 FTU/kg of phytase (Quantum Blue, AB Vista, UK). Due to the differences in pH optima, working temperature and substrate specificity, it was not possible to standardize the dose supplemented in the experimental diets according to a specific unit/kg or based on activity obtained from a universal assay. However, the amount of protease (mg) in each sample was analyzed by determining the protease peak area obtained from HPLC using a Superdex 75 10/300 GL column (GE Healthcare Bio-Sciences AB). Each protease was diluted with wheat flour at different concentrations to allow for inclusion at an equivalent enzyme protein concentration of 225 g in the final diet. This amount of enzyme protein was obtained from the recommended dose of a commercially available serine protease and the enzyme protein determined using the same assay as described above.

Diets and experimental design

Three separate experiments were conducted at two different Universities in the US or UK using diet and husbandry conditions specific to each location. In each experiment, two basal diets were formulated: a nutrient adequate positive control (**PC**), formulated to meet or exceed the nutrient requirements for turkey poults (Experiment 1) or broiler chicks (Experiments 2 and 3), and a negative control (**NC**) diet formulated to achieve 85% (Experiments 1 and 2) or 80% (Experiments 3) of the requirement for protein and amino acids. Phytase was included in all diets to provide 500 FTU/kg (Quantum Blue, AB Vista, UK) and xylanase was included in all diets to provide 10,000 (Experiments 1 and 2) or

16,000 (Experiments 3) BXU/kg (Econase XT, AB Vista, UK). All diets were fed in mash form, and birds were provided *ad libitum* access to feed and water throughout the duration of the studies. Chromic oxide (Experiments 1 and 2) or titanium oxide (Experiment 3) was added to the basal diets as an inert marker. The ingredient and nutrient composition of the diets for Experiment 1, 2, and 3 are provided in Tables 2, 3 and 4, respectively.

Animals and husbandry

Experiment one. Eight hundred, male, Hybrid Converter turkey poults were randomly allocated to one of 16 experimental diets from one to 18 days post-hatch. Birds were housed in Petersime battery brooder cages, with five birds/pen and 10 replicate pens/treatment. The room temperature and humidity were thermostatically controlled and temperature maintained at 29°C from d 1 to 7, 27°C from d 8 to 14, and 25°C from d 15 to 18. Light was provided to the birds 24 hours/day for the duration of the study. Feed and water were provided in troughs.

Experiment two. Eight hundred, male, Ross 308 broiler chicks were randomly allocated to one of 16 experimental diets from one to 17-days post-hatch. Similar to experiment 1, birds were housed in Petersime battery brooder cages with five birds/pen and 10 replicate pens/treatment. The room temperature and humidity were thermostatically controlled and temperature maintained at 29°C from d 1 to 7, 27°C from d 8 to 14, and 25°C from d 15 to 18. Light was provided to the birds 24 hours/day for the duration of the study. Feed and water were provided in troughs.

Experiment three. Eight hundred, male, Ross 308 broiler chicks, vaccinated for Marek's and Infectious Bursal disease at the hatchery, were randomly allocated to one of 16 experimental diets from one to 18-days post-hatch. Chicks were housed in metal battery brooders on raised wire floors with five birds/pen and 10 replicate pens/treatment. The room temperature was maintained with negative pressure ventilation, and gradually decreased from

32 to 22°C by the conclusion of the study. A standard lighting regime was used at 23:1 hours light:dark from day old to 18:6 hours light:dark at day seven and maintained until the conclusion of the trial.

Sample collection, calculations and statistical analysis

In all three experiments, feed and birds were weighed at the start and conclusion of the trial to determine feed intake (**FI**) and BW gain (**BWG**) and calculate feed conversion ratio (**FCR**). Birds were monitored daily and any culls or mortality was recorded to adjust feed intake for bird days. At the conclusion of the studies, all birds were euthanized and ileal digesta collected from the lower half of the ileum, defined as the section of intestine between the Meckel's Diverticulum and the ileo-cecal junction. Digesta was pooled/pen, homogenized, frozen and then dried for determination of amino acid and inert marker concentration.

In Experiments 1 and 2, excreta were collected on the last 3 days of the trial, pooled/pen, homogenized, frozen and dried prior to determination of starch and inert marker. Diets, ileal digesta and excreta in Experiments 1 and 2 were analyzed for chromium (method 990.08) and diets and ileal digesta were analyzed for amino acids (method 982.30) according to AOAC (2006). Starch was analyzed in the excreta samples using an enzymatic assay kit (Sigma, St Louis, MO). Diets and ileal digesta in Experiment 3 were analyzed for titanium according to methods of Short et al. (1996) and amino acids and starch according to previously mentioned methods. To calculate digestible amino acid intake in g/day, the following equation was used:

$$Digestible\ amino\ acid\ intake\ (g/d) = (analysed\ dietary\ amino\ acid\ (\%) \times \%\ AID\ amino\ acid) \times daily\ intake\ (g)$$

Data were analyzed as a two-way ANOVA using the fit model platform in JMP v. 13.0 (SAS, Cary, NC). Outliers were determined as three times the root mean square error plus or minus the mean of response. Plotting the growth performance and AID data using a normal quantile plot indicated the means were normally distributed. The model included treatment and replicate pen. When treatment was significant, means were separated using Dunnett's test for multiple comparisons. This test was used to compare each treatment to the NC. Percent mortality was analyzed as nonparametric using a one-way ANOVA using the fit Y by X platform in JMP v 13.0 (SAS, Cary, NC). If treatment was significant, differences were established using the Steel's test for multiple comparisons (the non-parametric version of the Dunnett's test). Significance was defined at $P < 0.05$, with trends discussed at $P < 0.10$.

RESULTS

Experiment one

Nutrients, phytase and xylanase recoveries in the experimental diets were similar to formulated values (Table 2). Overall mortality was 2.76% and not influenced by diet (Table 5). Poults fed the NC diet tended to gain less than those fed the PC ($P < 0.10$). Poults fed neutral protease 1 ($P < 0.05$) ate less and gained less than poults fed the NC. Feed conversion ratio tended to be higher in poults fed neutral protease 3 ($P < 0.10$) or neutral protease 7 ($P < 0.10$) when compared with those fed the NC. There were no other significant effects of diet on growth performance in Experiment 1.

Apparent ileal digestibility of glutamate ($P < 0.10$), methionine ($P < 0.05$), isoleucine ($P < 0.05$), leucine ($P < 0.10$), phenylalanine ($P < 0.10$) and arginine ($P < 0.10$) were greater in poults fed the PC when compared with poults fed the NC (Table 6). Apparent ileal amino acid digestibility of all measured amino acids was improved in poults fed neutral proteases 3 ($P < 0.05$) or 4 ($P < 0.10$), or acid proteases 2 ($P < 0.05$), 4 ($P < 0.05$) or 6 ($P < 0.10$) when compared with poults fed the NC. Excluding tryptophan, AID of all other measured amino acids was improved in poults fed neutral proteases 1 ($P < 0.05$), 2 ($P < 0.10$) or 6 ($P < 0.05$), or acid protease 1 ($P < 0.05$) when compared with poults fed the NC. Poults fed neutral protease 5 ($P < 0.10$) had improved AID of all amino acids measured except proline, methionine, or tryptophan, when compared with poults fed the NC. Finally, poults fed neutral protease 7 had improved glycine ($P < 0.10$), cysteine ($P < 0.10$), valine ($P < 0.05$), isoleucine ($P < 0.10$), histidine ($P < 0.10$) and arginine ($P < 0.05$) digestibility, poults fed neutral protease 8 had improved cysteine ($P < 0.05$), lysine ($P < 0.10$), and methionine ($P < 0.05$) digestibility, poults fed acid protease 3 had improved serine ($P < 0.05$) and histidine ($P < 0.05$) digestibility, and poults fed acid protease 5 had improved histidine ($P < 0.10$) digestibility when compared with poults fed the NC. Excreta starch retention was greater in poults fed neutral protease 1 ($P < 0.05$) compared with poults fed the NC. There were no other effects of treatment on excreta starch retention.

Poults fed the PC ($P < 0.05$) diet had a higher digestible amino acid intake for all amino acids when compared with poults fed the NC diet (Table 7). Poults fed neutral proteases 3 ($P < 0.05$) or 6 ($P < 0.05$) or acid proteases 1 ($P < 0.05$), 2 ($P < 0.05$), 3 ($P < 0.05$), 5 ($P < 0.05$) or 6 ($P < 0.05$) had a higher digestible amino acid intake for all amino acids, except tryptophan, when compared with poults fed the NC. Poults fed neutral proteases 2 ($P < 0.10$), 7 ($P < 0.10$) or 8 ($P < 0.05$) had increased digestible amino acid intake, except for proline and/or tryptophan, when compared with poults fed the NC. Poults fed neutral protease 5 ($P < 0.05$) or acid protease 4 ($P < 0.10$) had a higher digestible amino acid intake, except for methionine or tryptophan, compared with poults fed the NC. Finally, poults fed neutral protease 1 ($P < 0.05$) had reduced digestible amino acid intake of all amino acids, except cysteine, when compared with poults fed the NC. There was no effect of neutral protease 4 on digestible amino acid intake.

Experiment two

Phytase and xylanase recoveries in the experimental diets were in agreement with formulated values (Table 3). When analyzed in the diets, the protein and amino acid concentration in the NC diet was only reduced by 6% compared with the PC (Table 3). This was less than the expected 15% reduction from the PC, and may explain the non-significant difference in FI, BWG or FCR of chicks fed the PC when compared with those fed the NC (Table 8). Chicks fed neutral proteases 1, 4 or 6 ate significantly less and chicks fed neutral proteases 1 or 6 gained significantly less than chicks fed the NC. There was no effect of diet on FCR. Overall mortality was 4.0% and not influenced by diet.

There were very few effects of diet on AID of amino acids or excreta starch retention (Table 9). Apparent ileal serine ($P < 0.10$) and lysine ($P < 0.05$) digestibility were improved in chicks fed neutral protease 5 or acid protease 6, and methionine ($P < 0.05$) digestibility was improved in chicks fed acid protease 5 or the PC when compared with chicks fed the NC. There were no other effects of neutral protease supplementation on AID of amino acids. Chicks fed acid proteases 1 ($P < 0.10$) or 3 ($P < 0.10$) had a lower AID of cysteine or isoleucine compared with chicks fed the NC, and phenylalanine digestibility was reduced in chicks fed acid protease 1 ($P < 0.05$) compared with chicks fed the NC. The AID of tryptophan was reduced in chicks fed the PC ($P < 0.05$) compared with chicks fed the NC. There were no other effects of acid protease supplementation on the AID of amino acids. Apparent excreta starch retention was increased in chicks fed neutral proteases 1 ($P < 0.05$) or 3 ($P < 0.10$) compared with chicks fed the NC. There were no other effects of diet on starch retention.

Similar to the AID data, chicks fed the PC had an increase in digestible methionine ($P < 0.05$) intake and a decrease in digestible tryptophan ($P < 0.05$) intake when compared with chicks fed the NC (Table 10). Chicks fed neutral protease 1 ($P < 0.05$) had a lower digestible amino acid intake for all amino acids measured compared with chicks fed the NC. The digestible amino acid intake of all measured amino acids, except lysine or lysine and tryptophan, was lower in chicks fed neutral protease 6 ($P < 0.05$) or acid protease 3 ($P < 0.05$), respectively, compared with chicks fed the NC. Chicks fed neutral protease 4 ($P < 0.10$) had a reduced digestible intake of glutamate, proline, glycine, alanine, cysteine, tyrosine, isoleucine, leucine, phenylalanine, histadine and tryptophan, and chicks fed neutral protease 7 ($P < 0.10$) had reduced digestible amino acid intake of proline, tyrosine, methionine, leucine, phenylalanine, and lysine compared with chicks fed the NC. There were no other effects of neutral protease supplementation on digestible amino acid intake, except for chicks fed neutral protease 5 ($P < 0.10$) having a higher digestible lysine and lower digestible tryptophan intake compared with chicks fed the NC. Chicks fed acid protease 1 ($P < 0.10$) had a lower digestible intake of most measured amino acids, except proline, lysine, or tryptophan, compared with chicks fed the NC. Supplementation of the diets with acid protease 4 ($P < 0.10$) reduced digestible methionine, phenylalanine, or tryptophan intake, while acid protease 5 ($P < 0.10$) reduced digestible tyrosine or tryptophan intake and increased digestible methionine intake compared with chicks fed the NC. There were no other effects of acid protease supplementation on digestible amino acid intake.

Experiment three

Nutrient, phytase and xylanase recoveries in the experimental diets were as expected and similar to formulated values (Table 4). Overall mortality was 2.4% and not influenced by diet (Table 11). There were no differences in growth performance in chicks fed the PC when compared with chicks fed the NC. Birds fed neutral protease 1 ($P < 0.01$) gained less than birds fed the NC. Feed conversion ratio was higher in birds fed neutral protease 1 ($P < 0.05$) or acid protease 4 ($P < 0.10$) compared with birds fed the NC. There were no other effects of diet on growth performance.

There was no difference in AID of amino acids or starch in birds fed the PC compared with birds fed the NC (Table 12). There was no effect of acid protease or phytase supplementation on the AID of any amino acids measured, or the AID of starch. The AID of aspartate, serine, glutamate, glycine, alanine, threonine, valine, isoleucine, leucine, phenylalanine, histidine and arginine were reduced in chicks fed neutral protease 8 ($P < 0.10$) compared with chicks fed the NC. Apparent ileal serine or threonine digestibility were reduced in chicks fed neutral proteases 3 ($P < 0.05$) or 7 ($P < 0.10$) when compared with chicks fed the NC. Apparent ileal arginine or glycine digestibility were reduced ($P < 0.10$) in chicks fed neutral proteases 4 ($P < 0.05$) or 7 ($P < 0.10$), respectively, when compared with chicks fed the NC. Finally, the AID of serine, isoleucine, leucine, lysine, or histidine was increased in chicks fed neutral protease 1 ($P < 0.10$) when compared with chicks fed the NC. There were no other effects of diet on the AID of amino acids or starch.

Chicks fed the PC ($P < 0.05$) diet had a greater digestible amino acid intake compared with chicks fed the NC. Contradictory to the AID of amino acids, chicks fed neutral protease 1 ($P < 0.10$) had a lower digestible intake of most measured amino acids, except serine, glycine, tyrosine, methionine, isoleucine, or leucine, when compared with chicks fed the NC. Chicks fed neutral protease 3 ($P < 0.10$) or acid protease 3 ($P < 0.05$) had a lower digestible intake of all amino acids, except glutamate, tyrosine and methionine or proline and methionine, respectively, when compared with chicks fed the NC. Supplementation of the NC diet with acid protease 1 ($P < 0.10$) lowered the digestible intake of aspartate, proline, glycine, alanine, cysteine, threonine, valine, phenylalanine, lysine, histadine and arginine compared with chicks fed the NC. Finally, chicks fed the NC diet supplemented with neutral proteases 2 ($P < 0.10$) or 4 ($P < 0.10$) or acid protease 5 ($P < 0.05$) had a lower digestible intake of proline, glycine, cysteine, phenylalanine, histidine, and arginine or glycine, alanine, cysteine, lysine, and arginine or aspartate, serine and cysteine, respectively, compared with chicks fed the NC. Digestible intake of methionine was greater in chicks fed neutral proteases 6 ($P < 0.05$), 7 ($P < 0.10$) or 8 ($P < 0.10$), acid protease 4 ($P < 0.05$) or phytase at 3000 FTU/kg ($P < 0.05$) compared with chicks fed the NC.

DISCUSSION

Each protease evaluated had specific pH and temperature optima and substrate specificity. Protease recoveries in the experimental diets were not performed due to lack of an acceptable in-feed assay that is universal, optimal or specific to each protease. All diets were fed in mash form, and no denaturation of enzymatic activity would be expected in the diets due to processing of the feed. Due to the number of experimental cages and the large sample of proteases for testing, it was not possible to evaluate an optimum dose or dose response in the current set of trials. However, three of the novel proteases (neutral proteases 1 and 5 and acid protease 5) were used in two subsequent trials to evaluate a dose response on growth performance and apparent ileal amino acid digestibility in broilers (C. Walk et al., unpublished data). These trials evaluated doses that were lower, similar, and higher than the dose evaluated in the current experiments and indicated the optimal dose of each protease was similar or below (neutral protease 1) that of the dose employed in this set of trials (C. Walk et al., unpublished data). Therefore, some of the detrimental effects reported from supplementation the NC diet with neutral protease 1 may be associated with the dose selected in the current trials.

Previous authors have reported significant improvements in growth performance (Angel et al., 2011; Cowieson et al., 2016; Xu et al., 2017) or apparent ileal amino acid digestibility of ingredients (Adebiyi and Olukosi, 2015; Stefanello et al., 2016) or diets (Angel et al., 2011; Cowieson and Roos, 2014) supplemented with exogenous protease and fed to broilers or turkeys. Others have reported no effect of supplemental protease on performance, with a significant increase in apparent amino acid, protein or energy digestibility (Freitas et al., 2011) or a reduction in performance and endogenous enzyme activity as protease supplementation increased in the diet (Yuan et al., 2015; 2017). Inconsistency in the effect of exogenous proteases on growth performance or amino acid digestibility of poultry has been attributed to the inherent digestibility of amino acids in the diets (Cowieson and Roos, 2016). In addition, variability in the source and quality of soybean meal in the diet (Garcia-Rebollar et al., 2016) and protease enzymes that are not clearly defined (Freitas et al., 2011) or supplemented in combination with other enzymes may also contribute to the inconsistent reports surrounding protease supplementation in poultry diets. The objective of these experiments was to evaluate the efficacy of novel serine or aspartic proteases and three commercially available proteases on growth performance and AID of amino acids in turkeys or broilers.

Performance

To assess the potential efficacy of the novel proteases it was important that the NC diet, the diet to which the test proteases were supplemented, was deficient in protein and amino acids, to allow for noticeable improvements in growth performance, AID or digestible amino acid intake. The reduction in amino acids and protein by 10 to 15% was modelled after Angel et al. (2011), who reported a significant reduction in growth of chicks fed a low protein and amino acid diet. In that experiment, protease supplementation improved growth performance (Angel et al., 2011). In the current set of experiments, turkey poults fed the NC diet tended to gain less than poults fed the PC (Experiment 1) but there was no effect of the NC diet on growth performance of broiler chicks (Experiments 2 or 3). These results could be expected in Experiment 2 with only a 6% reduction in the analyzed protein content between the PC and NC diet. However, there was a 19% difference in protein and 28% difference in total lysine between the PC and NC diets in Experiment 3, which would have been expected to influence growth.

Regardless of the lack of an effect on growth performance between the PC and NC diets in two of the three experiments, a few consistent responses were observed for the different proteases evaluated. For example, in all three experiments birds fed neutral protease 1 ate less, gained less or were less efficient than birds fed the NC. Neutral protease 1 is an extracellular subtilisin-like serine protease with commercial application in detergent formulations (Juntunen et al., 2015). Juntunen et al. (2015) summarised the source organism as belonging to a species of fungi frequently described as phytopathogenic. However, the protease was expressed in Trichoderma, a commonly used organism for enzyme expression, and therefore the source organism would have no effect on the actual protease that was fed in the diet. More likely the significant reduction in growth performance of birds fed neutral protease 1 were the result of an excess dose of the protease in the diet. Previous authors have reported significant reductions in BWG as protease dose in the diet increased (Yuan et al., 2017).

In experiment 1, FCR tended to be higher in poults fed neutral proteases 3 or 7 when compared with poults fed the NC, and in experiment 3 acid protease 4 tended to increase FCR compared with chicks fed the NC. Neutral protease 3 is classified as a proline-specific endoprotease that can be used in the degradation of wheat gluten (Van Der Laan et al., 2017). Neutral protease 7 is a commercially available serine-protease which has been previously reported to improve feed efficiency of birds fed low protein and amino acid diets (Freitas et al., 2011). Acid protease 4 is described as a pepsin-like protease, and previous authors have reported significant improvements in rates of gain or feed efficiency with the supplementation pepsin into piglet diets (Lewis et al., 1955; Baker, 1959). However, in a set of subsequent experiments, Baker (1959) reported pepsin supplementation greater than 0.25% or in diets containing dried skim milk reduced gain with no beneficial effects reported on feed efficiency. Further, the author ran a series of experiments to determine factors that influence pepsin efficacy and reported a 10% reduction in gain and 7% loss in feed efficiency when pepsin was supplemented to low protein (15% CP) diets. Similarly, in the current trial the differences in FCR are associated with a numeric increase in FI with less of an effect on gain, and therefore the birds were able to eat through the protein deficiency but not utilize the nutrients at an equivalent rate of gain.

Apparent ileal digestibility and digestible amino acid intake

Baker (1959) reported 1.2 to 3.6% improvements in apparent protein digestibility in piglets fed low protein diets supplemented with pepsin. Unfortunately, these improvements in digestibility were not manifested as improvements in gain or efficiency in the low CP diet, and the authors speculated this was related to feed passage rate, which will be discussed in more detail below. The results in piglets presented by Baker (1959) and Freitas et al. (2011) are in agreement with the results of the current set of trials, in which the improvement in AID of amino acids (Experiment 1 or 3) was not associated with similar improvements in growth performance. Previous authors have predicted protease supplementation will improve AID of amino acids between 1.3 and 5.5%, with greater improvements noted when the control diet amino acid digestibility is low (Cowieson and Roos, 2014). In the current set of trials, the largest and most significant effect of protease supplementation was noted in experiment 1 in which the average AID of amino acids in the NC diet was 80%, whereas in experiment 2 or 3 the average AID of amino acids in the NC diet was 86 and 88%, respectively. Therefore, as previously reported, the AID of amino acids in the control diet will influence the magnitude of the effect of protease supplementation on the digestibility of the diet. This may have contributed to the lack of a significant effect of protease supplementation on AID of amino acids in Experiment 2 or 3 but does not reflect the lack of an effect of protease on growth performance, even with improvements in AID.

To try and understand the lack of an effect on growth performance with improvements in the AID of amino acids, digestible amino acid intake in g/day was calculated from the analysed amino acid in the diet, daily intake and the AID of the amino acids. Protein deposition rate (or growth) should increase with increasing amino acid intake and variations in intake can influence AID, with birds balancing their intake to fulfil nutritional requirements (Cruz et al., 2005). However, the ability of the bird to adjust intake based on nutrient requirements depends on the quality of the ingredients (Cruz et al., 2005) or the digestible amino acids provide by the diet. Previous authors have reported pepsin supplementation may exert beneficial effects on growth performance of piglets, partially through a change in the rate of food passage with significant and positive correlations between the rate of food passage and AID of protein (Baker, 1959). While the rate of food passage was not measured in the current set of experiments, the digestible amino acid intake in g/d may provide a better explanation for the lack of correlation between performance and amino acid digestibility in the current experiments. For example, birds fed the PC diet had significantly higher digestible amino acid intake (Experiments 1 and 3) compared with birds fed the NC, while neutral protease 1 significantly improved AID of amino acids (Experiments 1 and 3) but was associated with a proportionately larger reduction in intake, resulting in reduced digestible amino acid intake of all amino acids, hence the reduction in growth and feed efficiency. Birds fed neutral protease 8 had significantly reduced AID of amino acids (Experiment 3) in the absence of an effect on growth performance, possibly due to an increase in digestible methionine intake, which was likely the most limiting amino acid in the diet.

The digestible intake of all amino acids was increased in poults fed both neutral and acid proteases, with the exception of neutral proteases 1 (as described earlier) or 4 (Experiment 1). However, in experiments 2 or 3, where there were less effects of protease supplementation on AID of amino acids, protease supplementation had no effect or decreased the digestible intake of amino acids compared with birds fed the NC, which may have resulted in the lack of an effect of protease in these diets. This is contradictory to previously published reports in similar diets (Angel et al., 2011), and may be indicative of imbalances in the digestible amino acids available in the diet or alterations in endogenous protein digestion due to an exogenous protease effect on endogenous proteolytic activity (Yuan et al. 2015; 2017).

Conclusions

In conclusion, the current set of trials evaluating the supplementation of 8 serine proteases and 6 acid proteases failed to elicit beneficial effects of protease supplementation on poultry growth performance, even in the presence of improvements in AID of amino acids. This was associated with reductions in digestible amino acid intake. Further work to evaluate a dose response of the novel proteases in the diets of poultry is ongoing.

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Table 1. Description of novel proteases evaluated

Category	#	pH (optimum)	Temperature[1], °C (optimum)	Source organism	Protease type	Experiment[2]
Neutral						
	1	6 – 11 (10)	40 – 65 (60)	*Fusarium equiseti*	Subtilisin-like serine protease	1, 2, 3
	2	6 – 11 (10)	55 – 70 (70)	*Malbranchea cinnamomea*	Subtilisin-like serine protease	1, 2, 3
	3	6 – 8.5 (7)	40 – 50 (50)	*Myricoccum thermophilum*	Proline-specific endoprotease	1, 2, 3
	4	6 – 10 (9)	30 – 55 (50)	*Trichoderma reesei*	Subtilisin-like serine protease	1, 2, 3
	5	6 – 9 (8)	ND	*Trichoderma reesei*	Serine	1, 2, 3
	6	6 – 11 (9)	40 – 65 (60)	*Verticillium dahlia*	Subtilisin-like serine protease	1, 2, 3
	7	7 – 10	60 – 80 (70)	*Nocardiopsis prasina*	Serine-specific protease	1, 2, 3
	8	7 – 10	60 – 80 (70)	*Bacillus licheniformis*	Serine	1, 2, 3
Acid						
	1	4 – 6.5 (6)	20 – 45	*Trichoderma reesei*	Subtilisin-like serine protease	1, 2, 3
	2	4 – 6 (5)	ND	*Trichoderma reesei*	Serine	1, 2
	3	3 – 8 (5 – 7)	< 50 (40)	*Trichoderma reesei*	Pepsin	1, 2, 3
	4	3 – 8 (5 – 6)	40 – 70 (60)	*Trichoderma reesei*	Pepsin	1, 2, 3
	5	2 – 5.5 (5)	(50)	*Trichoderma reesei*	Aspartyl	1, 2, 3
	6	ND[3] (acidic)	ND	*Streptomyces*		1, 2

[1] Working temperature rather than thermostable temperature.
[2] There was not enough sample of acid protease 2 or 6 to test in all 3 experiments. For consistency, the protease numbers were maintained in Experiment 3 without acid protease 2 or 6.
[3] Not determined.

Table 2. Ingredient and nutrient composition of the starter diets, as-fed basis (Experiment 1)

Ingredient, %	Positive control	Negative control
Corn	43.59	55.59
Soybean meal	48.54	37.95
Corn oil	1.67	0.21
Salt	0.26	0.26
Sodium bicarbonate	0.17	0.17
DL methionine	0.33	0.25
Lysine HCl	0.30	0.32
Threonine	0.04	0.05
Limestone	1.25	1.27
Dicalcium phosphate	2.89	2.95
Phytase[1]	0.01	0.01
Vitamin premix[2]	0.12	0.12
Trace mineral premix[3]	0.13	0.13
Space (enzyme)	0.00	0.60
Chromic oxide	0.10	0.10
Xylanase[4]	0.006	0.006
Nutrient composition, %		
Crude protein	27.50	23.38
ME, kcal/kg	2865	2865
Calcium	1.29	1.29
Phosphorus	1.03	1.00
Available phosphorus	0.65	0.65
Crude fat	3.99	2.89
Crude fiber	2.70	2.62
Methionine	0.74	0.61
Cysteine	0.43	0.38
TSAA	1.17	0.99
Lysine	1.80	1.53
Tryptophan	0.33	0.27
Threonine	1.10	0.94
Arginine	1.90	1.57
Phytate phosphorus	0.25	0.23
Sodium	0.17	0.17
Chloride	0.25	0.25
Analysed nutrients, %		
Crude protein	27.45	23.07
Total lysine	1.76	1.56
Total threonine	1.01	0.89
Total methionine	0.71	0.52
Phytase, FTU/kg	710	574
Xylanase, BXU/kg	17,600	13,413

[1] Quantum Blue 5G (AB Vista) included to provide 500 FTU/kg.
[2] Supplied per kilogram of diet: vitamin A, 7700 IU; vitamin D3, 2750 IU; vitamin E, IU

16.5; vitamin B_{12}, 11 ug; vitamin K, 0.83 mg; riboflavin, 6.6 mg; thiamin, 1.1 mg; pantothenic acid, 6.6 mg; niacin, 27.5 mg; pyridoxine, 1.37 mg; folic acid, 0.69 mg; biotin, 33 mg; choline, 385 mg.

[3] Supplied per kilogram of diet: manganese (manganese sulfate), 100 mg; zinc (zinc oxide), 100 mg; iron (ferrous sulfate), 50 mg; cupper (copper sulfate), 11.25 mg; iodine (calcium iodate), 1.5 mg; selenium (sodium selenite), 0.15 mg.

[4] Econase XT 25G (AB Vista) included to provide 9,600 BXU/kg.

Table 3. Ingredient and nutrient composition of the starter diets, as-fed basis (Experiment 2)

Ingredient, %	Positive control	Negative control
Corn	57.18	67.25
Soybean meal	36.85	27.95
Corn oil	1.40	0.17
Salt	0.29	0.29
Sodium bicarbonate	0.17	0.17
DL methionine	0.35	0.29
Lysine HCl	0.25	0.28
Threonine	0.09	0.08
Tryptophan	0.00	0.01
Limestone	1.14	1.16
Dicalcium phosphate	1.32	1.37
Phytase[1]	0.01	0.01
Vitamin premix[2]	0.12	0.12
Trace mineral premix[3]	0.13	0.13
Space (enzyme)	0.00	0.60
Chromic oxide	0.10	0.10
Xylanase[4]	0.006	0.006
Nutrient composition, %		
Crude protein	23.00	19.55
ME, kcal/kg	3000	3000
Calcium	0.85	0.85
Phosphorus	0.66	0.64
Available phosphorus	0.35	0.35
Crude fat	4.12	3.19
Crude fiber	2.63	2.56
Methionine	0.71	0.60
Cysteine	0.37	0.33
TSAA	1.08	0.93
Lysine	1.44	1.22
Tryptophan	0.27	0.23
Threonine	0.97	0.82
Arginine	1.54	1.26
Phytate phosphorus	0.23	0.22
Sodium	0.18	0.18
Chloride	0.26	0.26
Analysed nutrients, %		
Crude protein	20.40	19.13
Total lysine	1.30	1.31
Total threonine	0.84	0.79
Total methionine	0.58	0.53
Phytase, FTU/kg	652	575
Xylanase, BXU/kg	14,700	13,893

[1] Quantum Blue 5G (AB Vista) included to provide 500 FTU/kg.

[2] Supplied per kilogram of diet: vitamin A, 7700 IU; vitamin D3, 2750 IU; vitamin E, IU 16.5; vitamin B_{12}, 11 ug; vitamin K, 0.83 mg; riboflavin, 6.6 mg; thiamin, 1.1 mg; pantothenic acid, 6.6 mg; niacin, 27.5 mg; pyridoxine, 1.37 mg; folic acid, 0.69 mg; biotin, 33 mg; choline, 385 mg.

[3] Supplied per kilogram of diet: manganese (manganese sulfate), 100 mg; zinc (zinc oxide), 100 mg; iron (ferrous sulfate), 50 mg; cupper (copper sulfate), 11.25 mg; iodine (calcium iodate), 1.5 mg; selenium (sodium selenite), 0.15 mg.

[4] Econase XT 25G (AB Vista) included to provide 9,600 BXU/kg.

Table 4. Ingredient and nutrient composition of the starter diets, as-fed basis (Experiment 3)

Ingredient, %	Positive control	Negative control
Wheat	59.20	70.67
Soybean meal	32.36	20.74
Soy oil	4.10	3.43
Salt	0.25	0.25
Sodium bicarbonate	0.19	0.18
DL methionine	0.36	0.32
Lysine HCl	0.36	0.49
Threonine	0.13	0.18
Tryptophan	0.00	0.02
Limestone	1.21	1.27
Dicalcium phosphate	1.02	1.03
Phytase[1]	0.01	0.01
Vitamin mineral premix[2]	0.50	0.50
Space (enzyme)	0.00	0.60
Titanium oxide	0.30	0.30
Xylanase[4]	0.01	0.01
Nutrient composition, %		
Crude protein	22.50	18.25
ME, kcal/kg	3025	3025
Calcium	0.85	0.85
Phosphorus	0.63	0.59
Available phosphorus	0.35	0.35
Crude fat	5.39	4.83
Crude fiber	2.55	2.44
Methionine	0.68	0.58
Cysteine	0.38	0.32
TSAA	1.06	0.90
Lysine	1.43	1.22
Tryptophan	0.28	0.24
Threonine	0.93	0.79
Arginine	1.44	1.08
Phytate phosphorus	0.23	0.20
Sodium	0.18	0.18
Chloride	0.27	0.29
Analysed nutrients, %		
Crude protein	22.10	17.95
Total lysine	1.69	1.22
Total threonine	1.04	0.79
Total methionine	0.77	0.55
Phytase, FTU/kg	511	549
Xylanase, BXU/kg	17,600	19,600

[1] Quantum Blue 5G (AB Vista) included to provide 500 FTU/kg.

[2] Supplied the following per kilogram of diet: vitamin A, 5,484 IU; vitamin D_3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite, 4.38 mg; riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 µg; biotin, 55.2 µg; thiamine mononitrate,2.2 mg; folic acid, 990 µg; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 250 µg.
[3] Econase XT 25G (AB Vista) included to provide 16,000 BXU/kg.

Table 5. Growth performance of turkey poults fed reduced nutrient density diets and novel proteases from hatch to 18 days post-hatch (Experiment 1)

Diet	Protease	Feed intake, g	BW gain, g	FCR, g:g	Mortality, %
Negative control		708.7	494.3	1.434	12.5
	Neutral				
	1	512.5*	344.7*	1.499	5.0
	2	719.7	473.5	1.523	0.0
	3	749.6	480.8	1.559†	0.0
	4	685.3	463.6	1.524	4.0
	5	730.3	507.5	1.439	0.0
	6	726.1	490.2	1.486	6.0
	7	747.9	483.7	1.551†	0.0
	8	746.8	491.8	1.520	4.0
	Acid				
	1	745.9	487.9	1.529	0.0
	2	727.5	487.7	1.498	5.0
	3	748.5	486.0	1.502	0.0
	4	694.6	469.2	1.490	4.4
	5	730.8	496.3	1.475	2.2
	6	724.1	480.7	1.513	2.0
Positive control		774.1	546.3†	1.417	0.0
	SE	18.3	12.9	0.03	0.03
	Diet P-value	< 0.0001	< 0.0001	0.0316	0.12
	Replicate P-value	0.13	0.0003	0.0081	0.31

Means in the same column are significantly different from the negative control, $^{*}P < 0.05$, $^{\dagger}P < 0.10$.

Table 6. Apparent ileal amino acid digestibility and apparent excreta starch retention of turkey poults fed reduced crude protein and amino acid diets and novel proteases from hatch to 18 days post-hatch (Experiment 1)

		Neutral protease								Acid protease							
Nutrient	NC[1]	1	2	3	4	5	6	7	8	1	2	3	4	5	6	PC[2]	SE
NEAA[3]																	
Asp	80.6	84.9*	85.0*	86.9*	85.0*	85.3*	87.1*	83.5	82.7	85.6*	85.8*	83.4	86.8*	83.1	86.1*	83.6	0.9[5,6]
Ser	80.5	84.6*	84.2†	86.4*	84.6*	84.9*	87.6*	83.4	81.9	85.1*	85.3*	85.1*	87.0*	82.3	85.5*	83.5	0.9[5,6]
Glu	83.5	88.5*	87.0*	89.2*	87.5*	87.2*	89.6*	86.3	85.6	88.0*	88.9*	86.2	89.4*	85.5	88.7*	86.7†	0.9[5,6]
Pro	78.7	83.8*	82.6†	85.8*	82.9†	82.4	85.5*	81.1	80.5	83.4*	84.7*	81.0	85.6*	80.5	84.4*	81.9	1.1[5]
Gly	75.8	81.5*	81.7*	84.3*	81.4*	81.8*	83.9*	79.7†	79.3	82.3*	82.7*	79.4	83.6*	78.6	82.5*	79.7	1.0[5,6]
Ala	77.0	83.2*	81.4†	85.2*	82.5*	81.5†	84.2*	79.7	79.7	82.5*	83.6*	80.1	84.2*	78.0	82.8*	80.4	1.2[5]
Cys	63.9	73.2*	74.7*	77.0*	71.9*	72.7*	76.5*	69.9†	70.5*	75.9*	74.4*	68.7	74.5*	69.0	74.6*	69.9	1.6[5]
Tyr	78.6	85.2*	83.7*	86.8*	84.3*	83.5*	86.3*	81.5	81.1	84.3*	85.6*	81.5	85.8*	80.7	84.6*	82.0	1.0[5]
EAA[4]																	
Thr	75.9	80.8*	81.2*	83.3*	80.6*	81.2*	83.0*	79.2	78.2	81.1*	81.3*	79.4	82.9*	77.5	82.0*	79.3	1.0[5]
Val	76.6	84.6*	82.7*	84.7*	81.8*	81.4*	83.8*	81.0*	80.0	82.6*	82.6*	79.2	84.2*	79.2	83.0*	80.4	1.0[5]
Met	88.0	92.5*	91.6*	93.0*	91.3*	90.1	92.9*	90.0	91.1*	91.4*	92.2*	90.0	92.1*	89.5	92.1*	92.6*	0.7[5]
Iso	80.2	86.1*	84.9*	87.4*	84.9*	84.7*	86.4*	83.6†	83.1	85.5*	86.3*	82.3	86.6*	82.2	86.0*	84.1*	0.9[5]
Leu	79.6	86.3*	83.7†	86.9*	84.5*	83.5†	86.3*	82.6	82.2	84.6*	86.2*	82.4	86.4*	81.2	85.6*	83.5†	1.0[5]
Phe	81.7	87.6*	85.8*	88.2*	86.2*	85.8*	88.0*	84.8	84.5	86.4*	87.7*	84.3	87.8*	83.8	87.1*	85.3†	0.9[5]
Lys	87.1	90.2*	90.4*	91.2*	90.3*	90.6*	90.9*	89.1	89.2†	90.4*	90.4*	89.0	91.0*	88.9	90.8*	89.1	0.6[5]
His	84.1	89.2*	87.6*	89.8*	88.1*	87.5*	89.5*	86.9†	86.4	88.2*	89.3*	86.3	89.6*	86.1	88.7*	86.7	0.8[5]
Arg	88.0	92.4*	91.0*	92.4*	91.1*	91.4*	93.1*	90.9*	90.2	91.6*	92.4*	90.7*	92.4*	90.3†	92.3*	90.6†	0.6[5,6]
Trp	87.2	88.4	89.3	90.8*	89.6†	89.2	88.7	88.3	88.8	89.3	90.4*	87.9	90.2*	87.4	89.6†	89.3	0.7[5,6]
Starch	85.0	94.1*	82.9	84.8	85.9	87.3	89.8	84.0	82.8	81.6	87.4	84.1	85.4	87.7	87.0	85.7	1.6[5,6]

[1] Reduced nutrient density negative control.
[2] Nutrient adequate positive control.
[3] Non-essential amino acids.
[4] Essential amino acids.
[5] Significant effect of diet ($P < 0.05$). Means in the same column are significantly different from the negative control, $^{*}P < 0.05$, $^{\dagger}P < 0.10$.
[6] Significant effect of block ($P < 0.05$).

Table 7. Apparent digestible amino acid intake (g/day) of turkey poults fed reduced crude protein and amino acid diets and novel proteases from hatch to 18 days post-hatch (Experiment 1)

		Neutral protease								Acid protease							
Nutrient	NC[1]	1	2	3	4	5	6	7	8	1	2	3	4	5	6	PC[2]	SE
NEAA[3]																	
Asp	0.58	0.46*	0.69*	0.71*	0.62	0.71*	0.74*	0.69*	0.70*	0.71*	0.71*	0.72*	0.69*	0.72*	0.71*	0.83*	0.02[5]
Ser	0.24	0.19*	0.28†	0.29*	0.26	0.30*	0.32*	0.28*	0.28*	0.29*	0.29*	0.33*	0.28*	0.28*	0.28*	0.33*	0.01[5]
Glu	1.02	0.80*	1.17*	1.24*	1.09	1.21*	1.27*	1.20*	1.21*	1.23*	1.23*	1.25*	1.19*	1.24*	1.22*	1.40*	0.03[5]
Pro	0.32	0.24*	0.36	0.38*	0.33	0.37*	0.39*	0.36	0.36	0.38*	0.37*	0.37*	0.36†	0.37*	0.37*	0.41*	0.01[5]
Gly	0.22	0.18*	0.26*	0.27*	0.24	0.27*	0.28*	0.26*	0.27*	0.27*	0.27*	0.27*	0.26*	0.27*	0.27*	0.31*	0.01[5]
Ala	0.27	0.21*	0.31†	0.33*	0.29	0.32*	0.33*	0.31*	0.31*	0.33*	0.32*	0.32*	0.31*	0.31*	0.32*	0.36*	0.01[5]
Cys	0.06	0.06	0.08*	0.09*	0.07	0.08*	0.09*	0.08*	0.09*	0.09*	0.08*	0.08*	0.08*	0.09*	0.09*	0.09*	0.00[5]
Tyr	0.17	0.13*	0.20*	0.22*	0.19	0.20*	0.21*	0.19	0.20*	0.21*	0.21*	0.20*	0.20*	0.20*	0.20*	0.22*	0.01[5]
EAA[4]																	
Thr	0.21	0.17*	0.25*	0.26*	0.23	0.26*	0.27*	0.25*	0.25*	0.25*	0.26*	0.26*	0.25*	0.25*	0.26*	0.29*	0.01[5]
Val	0.26	0.21*	0.31*	0.32*	0.28	0.31*	0.32*	0.31*	0.32*	0.32*	0.31*	0.30*	0.31*	0.32*	0.32*	0.36*	0.01[5]
Met	0.14	0.11*	0.17*	0.16*	0.14	0.15	0.18*	0.16†	0.19*	0.17*	0.17*	0.17*	0.15	0.17*	0.18*	0.24*	0.00[5]
Iso	0.24	0.19*	0.29*	0.30*	0.26	0.29*	0.30*	0.29*	0.30*	0.29*	0.29*	0.28*	0.29*	0.30*	0.30*	0.34*	0.01[5]
Leu	0.49	0.39*	0.56*	0.60*	0.53	0.57*	0.60*	0.57*	0.57*	0.59*	0.59*	0.59*	0.57*	0.58*	0.58*	0.66*	0.02[5]
Phe	0.28	0.23*	0.33*	0.35*	0.30	0.34*	0.36*	0.34*	0.34*	0.34*	0.35*	0.35*	0.34*	0.35*	0.34*	0.40*	0.01[5]
Lys	0.42	0.32*	0.49*	0.48*	0.44	0.52*	0.49*	0.49*	0.52*	0.49*	0.49*	0.51*	0.48*	0.52*	0.50*	0.58*	0.01[5]
His	0.17	0.13*	0.19*	0.20*	0.18	0.19*	0.20*	0.20*	0.20*	0.20*	0.20*	0.20*	0.19*	0.20*	0.20*	0.23*	0.01[5]
Arg	0.40	0.31*	0.47*	0.48*	0.42	0.47*	0.50*	0.47*	0.48*	0.48*	0.49*	0.48*	0.46*	0.49*	0.48*	0.56*	0.01[5]
Trp	0.08	0.06*	0.09	0.09	0.08	0.09	0.08	0.08	0.09*	0.09	0.09	0.09†	0.08	0.08	0.09	0.10*	0.00[5]

[1] Reduced nutrient density negative control.
[2] Nutrient adequate positive control.
[3] Non-essential amino acids.
[4] Essential amino acids.
[5] Significant effect of diet ($P < 0.05$). Means in the same column are significantly different from the negative control, *$P < 0.05$, †$P < 0.10$.
[6] Significant effect of block ($P < 0.05$).

Table 8. Growth performance of broiler chicks fed reduced nutrient density diets and novel proteases from hatch to approximately 17 days post-hatch (Experiment 2)

Diet	Protease	Feed intake, g	BW gain, g	FCR, g:g	Mortality, %
Negative control		724.8	525.4	1.388	0.0
Neutral					
	1	461.0*	332.7*	1.388	6.0
	2	666.1	485.4	1.389	2.0
	3	684.3	493.9	1.357	4.0
	4	639.3*	466.7	1.373	6.0
	5	679.3	481.5	1.418	8.0
	6	602.1*	439.8*	1.377	10.0
	7	675.1	471.4	1.384	4.0
	8	718.2	523.6	1.366	2.0
Acid					
	1	690.1	477.8	1.448	2.0
	2	689.9	490.8	1.398	6.0
	3	669.5	461.7	1.438	2.0
	4	666.2	463.6	1.432	6.0
	5	671.2	485.8	1.391	0.0
	6	668.4	481.8	1.402	4.0
Positive control		710.8	540.9	1.332	2.0
	SE	18.3	16.2	0.03	0.03
	Diet P-value	< 0.0001	< 0.0001	0.52	0.75
	Block P-value	0.0271	0.0100	0.10	0.71

Means in the same column are significantly different from the negative control, $^{*}P < 0.05$, $^{\dagger}P < 0.10$.

Table 9. Apparent ileal amino acid digestibility and apparent excreta starch retention of broiler chicks fed reduced crude protein and amino acid diets and novel proteases from hatch to 17 days post-hatch (Experiment 2)

		Neutral protease								Acid protease							
Nutrient	NC[1]	1	2	3	4	5	6	7	8	1	2	3	4	5	6	PC[2]	SE
NEAA[3]																	
Asp	85.6	85.3	86.5	85.8	85.5	87.4	84.7	86.6	86.9	83.7	86.5	84.0	86.3	87.1	87.8	84.5	0.7[5]
Ser	86.9	87.9	88.7	88.6	87.5	89.5†	86.7	89.0	88.9	86.1	88.4	87.4	88.1	88.7	89.4†	88.4	0.7[5,6]
Glu	92.1	91.7	92.1	92.6	92.2	92.6	91.6	92.0	92.4	91.4	93.2	91.6	92.2	92.6	93.5	91.9	0.5
Pro	84.3	86.1	85.2	84.8	84.2	85.2	82.7	83.4	85.6	83.1	84.3	81.9	83.7	86.1	85.1	84.8	0.7[5]
Gly	82.0	81.3	81.8	81.3	80.7	83.4	80.6	82.3	83.8	79.8	82.6	79.7	82.6	82.9	83.4	82.2	0.7[5]
Ala	82.6	83.6	82.8	82.4	81.9	83.8	81.5	83.0	84.4	80.9	82.8	80.4	82.9	83.5	84.7	83.9	0.8[5]
Cys	78.9	79.7	79.3	78.9	77.6	80.0	77.3	78.1	80.4	75.0†	79.1	73.8*	78.7	80.4	80.2	77.8	1.0[5]
Tyr	84.6	87.8	86.9	87.2	86.2	84.5	83.1	84.1	84.6	81.6	86.7	83.2	85.2	84.4	85.6	85.9	0.9[5]
EAA[4]																	
Thr	82.1	81.0	81.6	81.3	81.0	84.0	81.1	83.7	84.4	80.6	82.8	80.2	83.3	83.3	84.3	83.8	0.7[5]
Val	81.7	81.9	82.1	81.5	81.4	83.5	80.9	82.2	83.6	79.9	82.5	80.0	82.9	83.6	83.7	82.0	0.7[5]
Met	93.6	94.7	94.5	94.5	93.8	94.3	93.2	93.6	94.8	93.2	94.0	93.2	93.2	95.1*	94.8	95.0*	0.4[5]
Iso	85.3	84.5	84.3	84.0	84.1	86.7	84.2	85.7	86.4	83.1†	86.0	83.1†	85.9	86.4	86.9	86.0	0.6[5]
Leu	86.1	86.6	85.5	85.6	85.2	86.6	84.8	85.7	87.1	83.7	86.5	85.0	86.0	86.8	87.2	86.7	0.7[5]
Phe	86.7	86.5	86.0	86.0	86.4	87.1	85.2	86.2	87.2	84.0*	86.8	84.9	86.5	87.2	87.4	87.5	0.6[5]
Lys	89.8	89.9	90.4	89.6	89.7	92.9*	89.7	91.2	91.6	90.3	90.9	89.9	90.6	91.6	92.3*	91.4	0.6[5,6]
His	88.7	87.4	87.6	87.4	87.5	89.2	87.7	89.1	89.7	87.6	89.6	86.9	89.2	88.9	90.2	89.1	0.7[5]
Arg	91.6	93.0	91.2	92.3	91.2	92.5	90.7	91.4	93.5	89.7	92.7	92.4	91.2	92.2	92.6	93.1	0.7[5,6]
Trp	88.2	89.0	89.3	88.4	88.3	87.4	86.7	88.4	88.1	87.0	87.4	88.5	87.4	89.1	88.0	86.0*	0.5[5,6]
Starch	97.6	99.4*	98.1	98.9†	98.1	98.8	97.0	97.4	97.5	97.3	98.3	96.7	97.2	96.9	97.3	98.3	0.3[5]

[1] Reduced nutrient density negative control.
[2] Nutrient adequate positive control.
[3] Non-essential amino acids.
[4] Essential amino acids.
[5] Significant effect of diet ($P < 0.05$). Means in the same column are significantly different from the negative control, *$P < 0.05$, †$P < 0.10$.
[6] Significant effect of block ($P < 0.05$).

Table 10. Apparent digestible amino acid intake (g/day) of broiler chicks fed reduced crude protein and amino acid diets and novel proteases from hatch to 17 days post-hatch (Experiment 2)

		Neutral protease								Acid protease							
Nutrient	NC[1]	1	2	3	4	5	6	7	8	1	2	3	4	5	6	PC[2]	SE
NEAA[3]																	
Asp	0.68	0.40*	0.68	0.67	0.62	0.68	0.55*	0.63	0.69	0.58*	0.68	0.55*	0.64	0.64	0.67	0.72	0.02[5,6]
Ser	0.33	0.21*	0.34	0.34	0.30	0.35	0.28*	0.32	0.35	0.29*	0.34	0.28*	0.31	0.31	0.33	0.35	0.01[5,6]
Glu	1.31	0.76*	1.28	1.28	1.16*	1.28	1.07*	1.19	1.31	1.13*	1.30	1.10*	1.21	1.21	1.25	1.35	0.04[5,6]
Pro	0.41	0.26*	0.41	0.41	0.36†	0.40	0.33*	0.35*	0.41	0.38	0.39	0.34*	0.37	0.40	0.37	0.41	0.01[5,6]
Gly	0.28	0.16*	0.27	0.27	0.24*	0.28	0.22*	0.26	0.29	0.24*	0.28	0.23*	0.26	0.26	0.27	0.29	0.01[5,6]
Ala	0.34	0.20*	0.33	0.33	0.30*	0.34	0.28*	0.31	0.35	0.30†	0.33	0.29*	0.31	0.31	0.33	0.34	0.01[5,6]
Cys	0.10	0.06*	0.10	0.10	0.08*	0.10	0.08*	0.09	0.10	0.08*	0.09	0.07*	0.09	0.10	0.09	0.09	0.00[5,6]
Tyr	0.23	0.13*	0.23	0.23	0.21*	0.21	0.18*	0.21†	0.23	0.19*	0.23	0.19*	0.21	0.20*	0.22	0.24	0.01[5]
EAA[4]																	
Thr	0.27	0.16*	0.27	0.27	0.25	0.28	0.22*	0.26	0.29	0.24†	0.28	0.23*	0.26	0.26	0.27	0.29	0.01[5,6]
Val	0.31	0.19*	0.31	0.30	0.28	0.31	0.25*	0.29	0.32	0.27*	0.31	0.26*	0.29	0.29	0.30	0.32	0.01[5,6]
Met	0.20	0.13*	0.21	0.21	0.18	0.21	0.17*	0.18†	0.22	0.17*	0.21	0.17*	0.18*	0.23*	0.21	0.23*	0.01[5,6]
Iso	0.29	0.17*	0.28	0.28	0.26†	0.29	0.23*	0.27	0.29	0.25*	0.29	0.23*	0.27	0.27	0.28	0.30	0.01[5,6]
Leu	0.61	0.36*	0.58	0.57	0.53*	0.58	0.49*	0.54†	0.60	0.52*	0.59	0.50*	0.55	0.55	0.57	0.61	0.02[5,6]
Phe	0.36	0.20*	0.33	0.33	0.32†	0.34	0.28*	0.31*	0.34	0.29*	0.34	0.28*	0.32*	0.32†	0.33	0.37	0.01[5,6]
Lys	0.47	0.30*	0.48	0.46	0.45	0.53†	0.40	0.45*	0.53	0.48	0.49	0.43	0.45	0.48	0.52	0.50	0.01[5,6]
His	0.19	0.11*	0.18	0.18	0.16†	0.17	0.15*	0.17	0.19	0.16†	0.18	0.15*	0.17	0.17	0.18	0.19	0.01[5,6]
Arg	0.48	0.28*	0.46	0.47	0.43	0.47	0.38*	0.44	0.50	0.41*	0.47	0.39*	0.44	0.44	0.46	0.51	0.01[5,6]
Trp	0.09	0.06*	0.09	0.09	0.08*	0.08†	0.07*	0.09	0.09	0.08	0.08	0.09	0.08*	0.08	0.08	0.08*	0.00[5,6]

[1] Reduced nutrient density negative control.
[2] Nutrient adequate positive control.
[3] Non-essential amino acids.
[4] Essential amino acids.
[5] Significant effect of diet ($P < 0.05$). Means in the same column are significantly different from the negative control, *$P < 0.05$, †$P < 0.10$.
[6] Significant effect of block ($P < 0.05$).

Table 11. Growth performance of broilers fed reduced nutrient density diets and novel proteases from hatch to 18 days post-hatch (Experiment 3)

Diet	Protease	Phytase, FTU/kg	Feed intake, g	BW gain, g	FCR, g:g	Mortality, %
Negative control		500	807.6	508.7	1.573	2.0
	Neutral					
	1	500	724.8	374.5^{*}	1.801^{*}	4.0
	2	500	781.5	486.0	1.620	2.0
	3	500	771.7	483.8	1.616	0.0
	4	500	789.2	797.9	1.613	6.0
	5	500	806.0	512.8	1.575	6.0
	6	500	832.9	517.9	1.617	0.0
	7	500	812.0	498.1	1.645	4.0
	8	500	834.6	522.9	1.609	4.0
	Acid					
	1	500	760.2	476.0	1.609	0.0
	3	500	752.5	460.9	1.646	0.0
	4	500	813.9	471.0	$1.739^{\dagger}$	0.0
	5	500	789.6	488.0	1.640	2.0
		1500	830.7	529.9	1.577	4.0
		3000	837.0	521.8	1.617	2.0
Positive control		500	879.7	556.3	1.588	2.0
SE			23.3	17.3	0.04	0.02
Diet P-value			0.0021	< 0.0001	0.0553	0.59
Block P-value			0.32	< 0.0001	< 0.0001	0.98

Means in the same column are significantly different from the negative control, $^{*}P < 0.05$, $^{\dagger}P < 0.10$.

Table 12. Apparent ileal amino acid and starch digestibility of broiler chicks fed reduced crude protein and amino acid diets and novel proteases from hatch to 18 days post-hatch (Experiment 3)

		Neutral protease								Acid protease				Phytase, FTU			
Nutrient	NC[1]	1	2	3	4	5	6	7	8	1	3	4	5	1500	3000	PC[2]	SEM
NEAA[3]																	
Asp	84.2	83.9	83.2	82.8	82.7	83.2	83.4	82.2	81.1*	83.5	82.7	84.2	83.2	83.0	83.2	85.2	0.6[5]
Ser	85.0	87.4†	84.2	82.4*	84.5	83.8	83.7	82.0*	80.6*	84.4	84.0	84.0	83.7	83.1	83.3	84.6	0.6[5]
Glu	92.2	92.3	92.0	91.8	92.1	92.1	91.9	91.1	90.6*	92.5	92.0	92.3	92.3	91.5	91.8	91.8	0.3[5]
Pro	89.8	91.7	89.0	88.5	88.9	89.1	89.6	88.3	88.5	89.6	89.1	89.1	90.0	89.2	89.4	90.1	0.6
Gly	83.0	85.3	81.1	81.4	80.6	81.4	82.0	80.4†	78.8*	81.5	81.7	81.8	82.5	81.0	81.6	83.7	0.6[5]
Ala	83.9	82.6	82.3	82.0	81.5	82.4	83.3	81.6	80.7*	82.9	82.6	82.8	83.7	82.1	82.5	84.7	0.7[5]
Cys	80.1	77.0	76.5	77.0	76.4	77.3	77.8	76.2	75.2	77.9	77.7	78.1	79.0	78.7	77.4	77.2	1.1[6]
Tyr	83.8	88.2	85.0	85.5	86.2	85.8	86.8	85.0	80.8	84.5	83.4	85.7	86.0	82.9	83.8	86.2	1.4[5,6]
EAA[4]																	
Thr	84.0	86.2	82.6	81.3*	82.5	83.0	83.9	81.6†	81.3*	82.8	83.0	83.4	83.5	82.5	83.1	84.3	0.6[5]
Val	84.3	86.6	83.0	82.8	82.3	82.9	83.6	82.4	81.7†	83.1	83.1	84.1	84.4	82.8	82.7	85.2	0.7[5]
Met	94.1	95.1	93.8	93.3	94.1	94.1	94.7	94.0	93.5	94.2	94.1	94.5	94.4	93.7	94.5	94.8	0.4[6]
Iso	85.4	88.0*	84.6	84.3	84.2	84.8	84.8	84.6	82.6*	85.4	84.5	85.5	85.5	84.5	84.5	86.1	0.6[5,6]
Leu	86.8	89.9*	86.2	86.2	85.9	86.4	86.4	85.4	84.1*	86.9	86.0	86.8	86.9	85.7	86.3	87.1	0.6[5,6]
Phe	87.7	89.7	85.7	85.9	86.0	86.7	87.3	85.7	84.4*	86.8	84.9	87.0	86.8	86.0	86.5	86.9	0.8[5,6]
Lys	91.1	92.7*	90.5	90.0	89.8	91.1	90.7	90.1	89.6	90.9	90.6	90.6	91.2	90.1	90.9	91.3	0.4[5]
His	88.0	90.4*	86.5	86.4	86.8	87.6	88.1	86.5	85.8†	86.7	87.2	87.8	88.7	86.6	87.0	88.5	0.6[5]
Arg	89.0	88.8	87.8	87.3	86.0*	88.2	88.9	87.5	86.4*	88.4	88.1	88.9	89.1	87.8	88.5	89.8	0.5[5]
Starch	82.4	71.9	78.4	83.6	80.1	80.0	83.0	76.0	79.2	82.5	79.4	86.6	86.6	85.1	82.6	76.1	4.6[6]

[1] Reduced nutrient density negative control.
[2] Nutrient adequate positive control.
[3] Non-essential amino acids.
[4] Essential amino acids.
[5] Significant effect of diet ($P < 0.05$). Means in the same column are significantly different from the negative control, *$P < 0.05$, †$P < 0.10$.
[6] Significant effect of block ($P < 0.05$).

Table 13. Apparent digestible amino acid intake (g/day) of broiler chicks fed reduced crude protein and amino acid diets and novel proteases from hatch to 18 days post-hatch (Experiment 3)

		Neutral protease								Acid protease				Phytase, FTU			
Nutrient	NC[1]	1	2	3	4	5	6	7	8	1	3	4	5	1500	3000	PC[2]	SEM
NEAA[3]																	
Asp	0.64	0.55*	0.57	0.54*	0.57	0.59	0.64	0.60	0.59	0.55*	0.52*	0.64	0.56*	0.62	0.60	1.04*	0.02[5]
Ser	0.35	0.33	0.33	0.29*	0.34	0.32	0.35	0.32	0.31	0.32	0.29*	0.34	0.30*	0.33	0.33	0.50*	0.01[5]
Glu	1.62	1.43†	1.51	1.47	1.56	1.58	1.65	1.56	1.55	1.49	1.43*	1.63	1.51	1.59	1.61	2.16*	0.05[5]
Pro	0.47	0.40*	0.42*	0.41*	0.44	0.47	0.51	0.45	0.49	0.41*	0.43	0.48	0.43	0.50	0.48	0.72*	0.01[5,6]
Gly	0.29	0.26	0.25†	0.25*	0.25†	0.26	0.28	0.27	0.26	0.24*	0.24*	0.28	0.26	0.28	0.27	0.40*	0.01[5]
Ala	0.28	0.24*	0.25	0.24*	0.24*	0.25	0.29	0.27	0.27	0.24*	0.24*	0.28	0.25	0.27	0.26	0.41*	0.01[5,6]
Cys	0.12	0.10*	0.10*	0.10*	0.10*	0.11	0.11	0.11	0.11	0.10*	0.10*	0.11	0.10*	0.12	0.11	0.15*	0.00[5,6]
Tyr	0.18	0.18	0.18	0.18	0.19	0.17	0.21*	0.17	0.17	0.17	0.15*	0.18	0.18	0.17	0.18	0.26*	0.01[5]
EAA[4]																	
Thr	0.30	0.27*	0.28	0.25*	0.28	0.29	0.33	0.29	0.30	0.27†	0.26*	0.31	0.29	0.31	0.30	0.42*	0.01[5,6]
Val	0.30	0.27*	0.27	0.26*	0.27	0.27	0.31	0.29	0.29	0.26*	0.25*	0.31	0.29	0.30	0.28	0.46*	0.01[5]
Met	0.22	0.19	0.22	0.19	0.23	0.23	0.27*	0.24†	0.24†	0.21	0.21	0.26*	0.24	0.23	0.26*	0.36*	0.01[5]
Iso	0.28	0.25	0.25	0.23*	0.25	0.25	0.29	0.27	0.26	0.25	0.23*	0.28	0.26	0.27	0.26	0.43*	0.01[5]
Leu	0.50	0.46	0.47	0.45†	0.47	0.47	0.52	0.49	0.47	0.45	0.43*	0.51	0.46	0.49	0.49	0.74*	0.01[5]
Phe	0.35	0.31*	0.31*	0.29*	0.31	0.33	0.37	0.33	0.33	0.30*	0.29*	0.35	0.32	0.34	0.34	0.50*	0.01[5]
Lys	0.52	0.45*	0.48	0.44*	0.46†	0.52	0.53	0.51	0.52	0.46†	0.44*	0.53	0.48	0.50	0.52	0.75*	0.02[5]
His	0.18	0.16*	0.16*	0.15*	0.16	0.17	0.19	0.18	0.18	0.15*	0.16*	0.18	0.18	0.18	0.18	0.27*	0.01[5]
Arg	0.45	0.39*	0.40†	0.37*	0.38*	0.42	0.48	0.44	0.43	0.38*	0.38*	0.46	0.41	0.45	0.44	0.71*	0.01[5]

[1] Reduced nutrient density negative control.
[2] Nutrient adequate positive control.
[3] Non-essential amino acids.
[4] Essential amino acids.
[5] Significant effect of diet ($P < 0.05$). Means in the same column are significantly different from the negative control, *$P < 0.05$, †$P < 0.10$.
[6] Significant effect of block ($P < 0.05$).