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Comparative effects of proteases on performance, carcass traits and gut structure of broilers fed diets reduced in protein and amino acids

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The authors Majdi A. Abu Ishmais and Sadiq Al-Amaireh certify that they have no conflicts of interest to declare. The authors Alexandra L. Wealleans, Roba

Abstract

This study aimed to evaluate the effect of supplementing different protease enzymes on growth performance, intestinal morphology, and selected carcass traits in broilers fed diets reduced 3.5% in crude protein (CP) and amino acids (AA). One thousand one-day-old Ross 308 broilers (41 g) were assigned to five dietary treatments with ten replicates of 20 birds each: a positive control (PC) diet formulated to meet Ross 308 AA requirements, a negative control (NC) diet reformulated to provide 3.5% lower CP and AA compared to PC, NC supplemented with a multi-protease (PR1) solution, containing 3 different coated proteases produced from *Aspergillus niger*, *Bacillus subtilis* and *Bacillus licheniformis*, NC supplemented with a serine protease (PR2) produced from *Bacillus licheniformis*, and NC supplemented with an alkaline protease (PR3) produced from *Bacillus licheniformis*. At slaughter, 40 birds per treatment were used to assess the effect of the different treatments on carcass traits. At 32 days, samples of the duodenum, jejunum, and ileum of 10 birds per treatment were collected for intestinal morphology evaluation. Birds fed PC and NC supplemented with multi-protease exhibited better ($p < 0.05$) feed efficiency compared to NC and NC supplemented with all the other protease enzymes. Multi-protease supplementation was linked to the highest ($p < 0.05$) carcass weight and yield. There were significant differences ($p < 0.05$) between treatments in all gut segments, with PC, PR1, PR2, and PR3 exhibiting longer villi height (VH) compared to NC. This study demonstrates that 3.5% reduction of CP and AA negatively affected for the overall period feed efficiency, carcass yield, and intestinal morphology. The supplementation of the multi-protease restored feed efficiency and improved carcass yield.

Keywords: Broiler, Protease enzyme, Growth performance, Gut morphology, Carcass trait

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Authors' contributions
Conceptualization: Wealleans AL, Gonzalez-Sanchez D.
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Ethics approval and consent to participate
All experimental procedures were conducted in accordance with commercial practices and were approved by the institutional animal care and use committee (IACUC) of the Jordan University of Science & Technology (16/04/12/206). All experimental procedures were compliant with all local animal welfare legislation.

INTRODUCTION

With feed cost accounting for 60%–70% of the total cost of poultry production [1], the profitability of broiler production is largely driven by feed cost and by the efficiency of feed conversion. Therefore, increasing the digestibility of diets, and the use of less energy and nutrient-dense formulations without performance penalty, is of prime interest to poultry nutritionists. Crude protein (CP) digestibility is especially important, due also to the impact of excreted N on the environment. Formulating broiler diets with lower CP levels is regarded as a promising strategy to improve the sustainability of chicken meat production [2] and it has shown to improve litter quality and foot pad lesions [3]. Moreover, diets reduced in CP can reduce the amount of undigested CP reaching the hindgut, preventing pathogen proliferation, such as *Clostridium perfringens*, thus improving flock health status [4]. CP is often indirectly improved by the addition of feed additives such as phytases [5–7], non-starch polysaccharide (NSP) degrading enzymes [8–11], and biosurfactants [12,13], but direct improvements in CP and amino acids (AA) utilization are achieved through the application of protease enzymes.

Cowieson and Roos [14] reported an average improvement in AA digestibility of 3.74% following supplementation of poultry and swine diets with a mono-component protease, and further studies have also reported improvements in energy utilization [15–17]. Other studies have reported improved gut morphology [15], carcass quality [15,18], environmental impact [19], and alterations to the intestinal microbiome [18,20,21]. The addition of protease, by lowering both CP levels in diets and nitrogen excretion to the environment, can have a substantial impact on the environmental impacts of broiler production, potentially reducing the industry's Global Warming Potential [22].

However, the reported effects of monocomponent protease supplementation have been inconsistent. In contrast to Cowieson and Roos [14], Lee et al. [23], in a meta-analysis of 67 studies, reported that supplementation of protease to monogastric diets resulted in only a 1.6% average improvement in apparent ileal AA digestibility compared to an unsupplemented control, and that this effect was further reduced or negligible in diets containing phytase and NSP-degrading enzymes. Similarly, Tari et al. [24] suggested that the lack of reported effect of serine protease supplementation on digestibility or performance was due to the high quality and inherent CP digestibility of the basal diet and the presence of NSP-degrading and phytase enzymes.

The efficacy and consistency of protease supplementation may be improved by the simultaneous supplementation of multiple proteases with different pH optima and substrate specificity. Most proteases are degraded by exposure to acid and pepsin in the stomach [25], and therefore have reduced activity in the small intestine. The incorporation of acid-stable proteases in poultry feed could expand the ability of exogenous protease supplementation to work in the upper sections of the intestinal tract, therefore allowing more time for absorption and retention. However, little data is available directly comparing the supplementation of monocomponent and multi-proteases on broiler performance. Therefore, this study aimed to compare the efficacy of three different exogenous proteases, two monocomponent and one multi-protease, with different pH optima and ranges, on the growth performance, selected carcass traits, and gut morphology of growing broilers.

MATERIALS AND METHODS

Birds, housing, and experimental diets

A 32-day study was conducted at the Broiler Research Unit of Suliman Al-Amaireh & Partners Co., Jordan. All experimental procedures were conducted in accordance with commercial practices

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and were approved by the institutional animal care and use committee (IACUC) of the Jordan University of Science & Technology (16/04/12/206). All experimental procedures were compliant with all local animal welfare legislation. A total of one thousand one-day-old Ross 308 broilers (41 g at hatch) were sourced from the commercial hatchery of Suliman Al-Amaireh & Partners Co and were feather sexed at hatch. Birds were randomly allocated to five dietary treatments with ten replicates of 20 mixed-sex broilers (10 males and 10 females) each: a positive control (PC) diet formulated to meet Ross 308 AA requirements as per 2019 nutrient specifications [26], a negative control (NC) diet reformulated to provide 3.5% lower CP and AA compared to PC, NC supplemented with 3,000 U/kg (300 mg/kg) of a multi-protease (PR1) solution, containing 3 different coated acidic, neutral and alkaline proteases produced from *Aspergillus niger*, *Bacillus subtilis* and *Bacillus licheniformis* respectively (Kemzyme Protease, Kemin Europa N.V., Herentals, Belgium), NC supplemented with 15,000 U/kg (200 mg/kg) of a commercial serine protease (PR2) produced from *Bacillus licheniformis*, and NC supplemented with 30,000 U/kg (500 mg/kg) of a commercial alkaline protease (PR3) produced from *Bacillus licheniformis*. The selection of the proteases evaluated in this trial was based on their commercial relevance and their application dose match the commercial recommendation of their suppliers. Analysis of enzyme recovery was not performed in the present experiment, due to feed spoilage in transit between the trial site and experimental laboratory.

Birds were reared on a solid floor covered with clean wood shavings. The temperature and ventilation of the building were monitored daily and were managed according to the breed recommendations [26]. A regular lighting program (0–3 days 24 h/light, 4–7 days 23 h/light, and 8–final age 20 h/light) was provided by fluorescent bulbs placed above the pens.

Two dietary phases were provided: starter (d 0–14) and grower (d 15–32). All diets were produced according to commercial practices and fed as pellets (2 mm diameter and 3 mm length in starter diet; and 3 mm diameter and 5 mm length in grower diets). Pelleting conditions were acceptable for the heat-stability of all the proteases (< 60℃). All diets contained background phytase, NSP-degrading enzymes, and biosurfactants. The composition of the experimental diets is listed in Table 1. Dry matter, CP, ash, ether extract and crude fiber from experimental feeds were determined with NIRS (NIRS DS2500 F, FOSS, Hillerød, Danmark) and are shown in Table 2. Feed and water were provided *ad libitum* throughout the study.

Growth performance

Birds were weighed individually on arrival from the hatchery. Pen bird body weight (BW), body weight gain (BWG) and feed intake (FI) were recorded at 14, 28 and 32 days. Feed conversion ratio (FCR) was calculated by dividing pen FI by pen BWG. Daily mortality was recorded per pen. Final BWG, FI and FCR were calculated on day 32, and all birds were slaughtered.

Carcass traits and meat characteristics

At slaughter, 4 male birds from each replicate with an average BW of 2,050 g, were selected and euthanized for carcass traits evaluation. The male gender selection was decided due to higher and therefore closer BW to the average 2,050 g. Birds were slaughtered as per the Halal method according to Jordanian law. The skin along with the feathers was removed after slaughtering, carcasses were eviscerated by hand and individual carcasses were weighed. The whole breast as well as the abdominal fat were removed from the carcass and weighed individually. Carcass traits were expressed as a percentage of the carcass weight.

Table 1. Ingredients and nutrient composition of the experimental diets

Variable	0–14 days		14–32 days	
	PC	NC[1)]	PC	NC
Ingredients (g/kg)				
Corn	547.9	568.2	490.6	512.8
Soybean meal (46%)	411.3	391.0	354.7	335.2
Wheat	-	-	100.0	100.0
Limestone	12.3	12.4	12.3	12.4
Soybean oil	7.0	7.0	22.9	20.0
Monocalcium phosphate	7.6	7.8	6.4	6.6
Sodium chloride	2.3	2.3	2.0	2.2
Sodium bicarbonate	1.1	1.1	0.1	0.1
L-Lysine HCl	1.9	1.9	2.2	2.2
DL-Methionine	3.4	3.2	3.0	2.9
L-Threonine	1.3	1.2	0.9	0.9
Vitamin and mineral premix[2)]	2.0	2.0	2.0	2.0
Choline chloride 60%	0.7	0.7	0.7	0.7
Coccidiostat	0.6	0.6	0.6	0.6
Bio-emulsifier[3)]	0.5	0.5	0.5	0.5
NSP enzyme[4)]	0.05	0.05	0.05	0.05
Phytase[5)]	0.1	0.1	0.1	0.1
Calculated nutrient composition (%, as fed basis)				
Dry matter	88.54	88.51	88.78	88.72
ME (kcal/kg)	2940	2950	3075	3075
Crude protein	23.80	23.00	21.93	21.18
Crude fat	3.16	3.20	4.63	4.40
Crude fibre	2.64	2.62	2.55	2.56
Digestable lysine	1.28	1.23	1.18	1.14
Digestable methionine	0.64	0.61	0.58	0.55
Digestable methionine + cysteine	0.95	0.91	0.87	0.84
Digestable threonine	0.87	0.83	0.76	0.73
Digestable arginine	1.50	1.44	1.35	1.30
Digestable tryptophan	0.26	0.25	0.24	0.23
Ca	0.87	0.87	0.84	0.84
Digestable phosphorous	0.40	0.40	0.37	0.37
Na	0.16	0.16	0.14	0.15
Cl	0.22	0.22	0.21	0.22

[1)]To create the experimental treatments, the different proteases were added at the expense of corn as follows: PR1, 300 g/t (3,000 U/kg, where 1 U of protease activity is defined as the amount of enzyme that releases 1 μg of trichloroacetic acid-soluble azo-casein peptides from a 1% azo-casein substrate solution per minute in the assay at pH 7.5 and at a temperature of 37℃); PR2, 200 g/t (15,000 U/kg, where 1 U of protease activity is defined as the amount of enzyme that releases 1 μmoL of p-nitroaniline from 1 mM substrate (Suc-Ala-Ala-Pro-Phe-pNA) per minute at pH 9.0 and temperature 37℃); PR3, 500 g/t (30,000 U/kg, where 1 U of protease activity is defined as the amount of enzyme that liberates 1 micromole of para-nitroaniline (pNA) from the Succinyl-Ala-Ala-Pro-Phe-pNA ($C_{30}H_{36}N_6O_9$) substrate per minute at pH 8.0 and 37℃).

[2)]Provided per kilogram diet: retinyl acetate, 3.50 mg; cholecalciferol, 0.1 mg; α-tocopherol acetate, 25 mg; menadione, 3 mg; thiamine, 2.0 mg; riboflavin, 7 mg; pyridoxine, 4.0 mg; cobalamin, 0.020 mg; niacin, 50 mg; calcium pantothenate: 15 mg; Cu (from copper sulphate), 9.0 mg; Fe (from ferrous sulphate), 35 mg; I (from potassium iodate): 1 mg; Mn (from manganese sulphate), 85 mg; Se (from sodium selenite), 0.35 mg; Zn (from zinc oxide), 80 mg.

[3)]LYSOFORTE® EXTEND, a proprietary combination of lysolecithin, synthetic emulsifier, and monoglycerides manufactured by Kemin Europa NV, Herentals, Belgium.

[4)]Endo-1,4-beta-xylanase 350,000 U/g, Endo-1,3(4)-beta-glucanase 23,500 U/g, Endo-1,4-beta-glucanase 180,000 U/g, Alpha-amylase 4,000, and Bacillolysin 17,000 U/g; KEMZYME Plus concentrate dry, Kemin Europa NV (Herentals, Belgium).

[5)]6-Phytase 10,000 FTU/g. KINGPHOS 10,000 FTU/g. Qingdao Vland Biotech Group (Qingdao, China).

PC, positive control; NC, negative control.

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Table 2. Determined nutrient composition[1] (%, as fed basis) of the experimental diets

	PC	NC	PR1	PR2	PR3
0–14 days					
Dry matter	88.75	89.09	88.96	89.05	88.35
Crude protein	24.40	23.17	22.95	23.50	23.54
Crude fat	3.45	3.33	3.30	3.42	3.27
Ash	5.95	5.52	5.58	5.41	5.35
Crude fiber	2.61	2.465	2.43	2.60	2.50
14–32 days					
Dry matter	88.29	87.93	88.23	88.3	88.29
Crude protein	22.35	21.31	21.39	21.44	21.45
Crude fat	3.97	4.06	4.08	4.15	4.15
Ash	5.82	5.40	5.52	5.63	5.37
Crude fiber	2.27	2.26	2.53	2.37	2.39

[1]Determined with NIRS (NIRS DS2500 F, FOSS, Hillerød, Danmark).

PC, positive control; NC, negative control; PR1, NC supplemented with a multi-protease; PR2, NC supplemented with a serine protease; PR3, NC supplemented with an alkaline protease.

Intestinal morphology

At 32 days 1 male bird per pen (a total of 50 birds: 10 birds per treatment) was randomly selected and euthanized and samples of the duodenum, jejunum, and ileum were collected and fixed in 10% neutralized formalin for three days and sent to the Histopathology lab of The Jordan University of Science and Technology (Irbid, Jordan). The samples were then dehydrated through ascending concentrations of alcohol starting from 60% concentration, 70%, 80%, 90%, and absolute (100%) ethanol. They were transferred to xylene for one hour and then soaked in liquid paraffin and embedded in paraffin using specialized molds. The mold was left to cool down at room temperature. The embedded samples were sectioned at 4–5 μm thickness using a rotary microtome and subsequently were stained with hematoxylin and eosin. They were then examined by a light microscope connected with a camera (Olympus BX51, Olympus, Tokyo, Japan). Each slide of the three parts of the small intestine was pictured at 40X magnification. The morphometric measurements of each sample, namely villi height (VH), villus width (VW), and crypts depth (CD) were taken using image J software (https://imagej.nih.gov/ij/). Only well-oriented sections were considered for measurements. At least six readings of each slide (total of 11 slides of each intestinal segment) of well-oriented sections were taken and considered for statistical analysis. The ratio of villus height: crypt depth (VH:CD) for each replicate was calculated from the average measurement.

Statistical analysis

Data are presented as means with overall SEM and were analyzed in the Fit Model platform of JMP 15 (SAS Institute, Cary, NC, USA) with protease supplementation as the main factor. The pen was considered the experimental unit for performance. The individual broiler sampled was considered the experimental unit for carcass traits and histology. No outlier data were identified or excluded from the dataset. In all statistical analyses, differences were considered significant at $p < 0.05$.

RESULTS

Growth performance

Mortality was considered low (<5%) and was not different among the different treatments. Performance results per feeding period and overall (0 to 32 days) are shown in Table 3. By day 14, broilers from PR1 and PR2 showed higher ($p < 0.05$) BW compared to PR3, while no difference ($p > 0.05$) was found compared to PC and NC. Between days 14 and 28, broiler fed NC diets supplemented or not with any of the protease enzymes (PR1, PR2, PR3, and NC) showed better ($p < 0.05$) FCR than broilers fed the PC diet. Across the total trial period (0 to 32 days), the best feed efficiency ($p < 0.05$) was realized when feeding birds with the PC diet and the NC diet supplemented with 300 mg/kg of the multi-protease solution (PR1).

Intestinal morphology

The morphometric changes in the duodenal, jejunal, and ileal villi of birds from the different dietary treatments at 32 days are presented in Table 4. There were significant differences between treatments in all gut segments. Briefly:

- Duodenum: VH was longer ($p < 0.05$) for PR3 compared to PR2, NC, and PC. VW was higher ($p < 0.05$) for PR3 and PC compared to all other treatments. CD was longer ($p < 0.05$) for PR3 compared to all other treatments. VH:CD was higher ($p < 0.05$) for PR2 compared to PC and PR3.
- Jejunum: VH was longer ($p < 0.05$) for PR3 compared to all other treatments. VW was higher ($p < 0.05$) for PC compared to NC. CD was longer ($p < 0.05$) for PR3 compared to all other

Table 3. Effect of the dietary supplementation of different proteases on the growth performance of broilers in each experimental group measured at different growth stages

	PC	NC	PR1	PR2	PR3	SEM	*p*-value
0–14 days							
BW (day 14)	485^{ab}	493^{ab}	503^{a}	501^{a}	482^{b}	4.5018	0.0056
BWG (g)	444^{ab}	452^{ab}	462^{a}	460^{a}	441^{b}	4.5018	0.0056
FI (g)	430	439	440	440	425	6.4400	0.3688
FCR	0.967	0.972	0.951	0.956	0.963	0.0069	0.2342
14–28 days							
BW (day 28)	1,782	1,779	1,813	1,819	1,788	14.1348	0.1583
BWG (g)	1,296	1,286	1,310	1,318	1,306	10.2540	0.2356
F, (g)	1,987	1,937	1,952	1,987	1,952	15.7609	0.0988
FCR	1.532^{a}	1.506^{b}	1.490^{b}	1.508^{b}	1.495^{b}	0.0058	< 0.0001
0–32 days							
BW (day 32)	2,089	2,103	2,140	2,106	2,072	20.6572	0.2333
BWG (g)	2,048	2,062	2,099	2,065	2,031	20.6572	0.2333
FI (g)	2,821	2,938	2,872	2,911	2,886	35.5427	0.2071
FCR	1.377^{b}	1.425^{a}	1.368^{b}	1.410^{a}	1.420^{a}	0.0062	< 0.0001
Adjust. FCR (2.1 kg BW)	1.379	1.424	1.359	1.409	1.426	-	-

$^{a-c}$Values with different superscripts in the same row were significantly different ($p < 0.05$).

PC, positive control diet formulated to meet Ross 308 AA requirements; NC, negative control diet reformulated to 3.5% lower digestible AA compared PC; PR1, NC supplemented with 3,000 U/kg (300 mg/kg) of a multi-protease solution, containing 3 different coated proteases produced from *Aspergillus niger*, *Bacillus subtilis* and *Bacillus licheniformis*; PR2, NC supplemented with 15,000 U/kg (200 mg/kg) of a serine protease produced from *Bacillus licheniformis*; PR3, NC supplemented with 30,000 U/kg (500 mg/kg) of an alkaline protease produced from *Bacillus licheniformis*; SEM, n = 10 replicates per treatment (20 birds per replicate); BW, body weight; BWG, body weight gain; FI, feed intake; FCR,feed conversion ratio; Adjust. FCR (2.1 kg BW), Adjusted FCR at 2.1 kg of final BW calculated according to Ross Broiler Management Handbook 2014.

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Table 4. Effect of dietary supplementation of the different proteases on the intestinal morphology of broilers in each experimental group at 32 days of age

	PC	NC	PR1	PR2	PR3	SEM	*p*-value
Duodenum							
Villus height	226.37^{cd}	223.61^{d}	280.05^{ab}	259.88^{bc}	299.56^{a}	7.8988	< 0.0001
Villus width	32.02^{a}	25.55^{b}	26.24^{b}	26.31^{b}	32.22^{a}	1.1154	< 0.0001
Crypt depth	26.74^{b}	25.44^{b}	29.18^{b}	28.94^{b}	36.86^{a}	1.1979	< 0.0001
VH:CD	9.01^{b}	9.06^{ab}	9.75^{ab}	10.66^{a}	9.01^{b}	0.4230	0.0173
Jejunum							
Villus height	187.83^{bc}	163.84^{c}	200.13^{b}	191.17^{bc}	230.68^{a}	7.5963	< 0.0001
Villus width	35.26^{a}	27.33^{b}	28.25^{ab}	28.87^{ab}	28.64^{ab}	1.8999	0.0603
Crypt depth	22.52^{b}	23.68^{b}	25.39^{b}	21.76^{b}	35.11^{a}	0.9437	< 0.0001
VH:CD	8.74^{a}	7.34^{bc}	8.50^{ab}	9.28^{a}	6.62^{c}	0.3570	< 0.0001
Ileum							
Villus height	182.40^{a}	147.53^{b}	160.28^{ab}	158.52^{ab}	162.56^{ab}	6.0764	0.0059
Villus width	38.32^{a}	35.50^{ab}	30.81^{bc}	28.58^{c}	31.79^{abc}	1.5900	0.0004
Crypt depth	21.11^{b}	25.92^{ab}	22.88^{b}	22.63^{b}	31.48^{a}	1.5513	< 0.0001
VH:CD	9.47^{a}	5.82^{c}	7.16^{bc}	7.52^{b}	6.22^{bc}	0.3800	< 0.0001

$^{a-c}$Values with different superscripts in the same row were significantly different ($p < 0.05$).

PC, positive control diet formulated to meet Ross 308 AA requirements; NC, negative control diet reformulated to 3.5% lower AA compared PC; PR1, NC supplemented with 3,000 U/kg (300 mg/kg) of a multi-protease solution, containing 3 different coated proteases produced from *Aspergillus niger*, *Bacillus subtilis* and *Bacillus licheniformis*; PR2, NC supplemented with 15,000 U/kg (200 mg/kg) of a serine protease produced from *Bacillus licheniformis*; PR3, NC supplemented with 30,000 U/kg (500 mg/kg) of an alkaline protease produced from *Bacillus licheniformis*. SEM, n = 10 replicates per treatment (1 male bird per replicate). VH:CD, villus height to crypt depth ratio.

treatments. VH:CD was higher ($p < 0.05$) for PC and PR2 compared to NC and PR3, whilst VH:CD for PR1 was higher ($p < 0.05$) than PR3.

- Ileum: VH was longer ($p < 0.05$) for PC compared to NC. VW was longer ($p < 0.05$) for PC compared to PR1 and PR2. CD was longer ($p < 0.05$) for PR3 compared to PC, PR1, and PR2. VH:CD was higher ($p < 0.05$) for PC compared to all other treatments. VH:CD was higher ($p < 0.05$) for PR2 compared to NC.

Selected carcass traits

Table 3 presents the effect of dietary supplementation of the different proteases on the selected carcass traits of male broilers at 32 days of age. Carcass weight as well as carcass yield was higher ($p < 0.05$) for PR1 treatment compared to all other treatments, and was higher ($p < 0.05$) for PR2 compared to NC and PR3. Breast weight was higher ($p < 0.05$) for PR1 and PR2 compared to NC and PR3. Breast % was higher ($p < 0.05$) for PR2 compared to NC and PR1. No effects ($p > 0.05$) were detected for the rest of the carcass traits evaluated.

DISCUSSION

Growth performance

Broiler growth performance is largely influenced by the supply of high levels of digestible AA [27–29] and several previous studies have shown growth performance impairment following their dietary reduction [30–34]. However, meeting these requirements involves formulating diets with highly digestible proteinaceous, and very often expensive, feed ingredients. Keeping the balance between broiler growth performance and production profitability is not an easy task for the modern poultry industry, and the sustained rise of in the cost of feed raw materials remains a primary

challenge [35], especially in an increasingly volatile and disrupted global supply chain environment. Maximizing the utilization of CP and AA at the lowest possible feed cost can offer nutritionists a window of opportunity to improve overall broiler profitability. Reformulating diets to lower cost and reduced CP and AA levels with the use of proteases has proven to be an effective strategy in this respect [36–40]. Growth performance improvements following the application of proteases to broiler diets have been previously reported [14–18,20,21,34,37,41–43] and are in line with the magnitude of FCR improvement (5.7 points) achieved in the current study by the supplementation of the multi-protease to the diet reduced in CP and AA. However, the supplementation of the serine and the alkaline monocomponent proteases did not result in improved growth performance in our study. Many of the previous studies that showed growth performance improvements of dietary protease supplementation were performed with monocomponent proteases [14,16–18,20,21,34,41–43], which is not aligned with the lack of growth performance effect seen in the present study. On the other hand, several previous studies where the application of protease had limited effect on growth or nutrient digestibility ascribed this to the presence of other feed additives in the diet, in particular high levels of phytase and NSP-degrading enzymes [23,24,43]. Both classes of enzymes are known to improve nitrogen retention and AA digestibility through indirect mechanisms: phytase reduces the anti-nutritive and protein-binding effect of phytate [5], while NSP-degrading enzymes reduce the caging effect of fiber, allowing endogenous enzymes access to dietary CP [10,11]. The diets in the present study were formulated along with commercial guidelines and contained 1000 FTU/kg of phytase, a combination of 3 NSP-degrading enzymes, amylase, and neutral protease, as well as a biosurfactant also known to have indirect effects on nitrogen utilization [12]. It could be hypothesized that the addition of the multiprotease with a wide effective pH range could have been determinant to improve feed efficiency in the presence of multiple performance enhancing additives.

Intestinal morphology

The benefits of the dietary supplementation of protease on growth performance are ascribed to increased CP digestibility [44–47], and they may be attributed to changes in intestinal morphology [38,48], reductions in the impact of antinutrients including trypsin inhibitors [49–51], and shifts in the microbiome [20,21]. The specific mode of action of dietary protease supplementation on intestinal morphology has not been well understood. Our results showed that protease supplementation had significant impact on intestinal morphology. All tested proteases provided a longer VH in the duodenum and jejunum compared to the NC. Longer VH is closely linked with greater capacity for nutrient absorption [52,53]. VH was depressed between the PC and NC, with the reductions in AA level linked to significantly shorter villi in the ileum, and near significant reductions in the duodenum and jejunum. The same pattern is seen for the reduction in VH:CD ratio in the different intestinal segments: the earlier segments showed no (duodenum) or minimal (–16.0%, jejunum) reductions, whilst a severe reduction in VH:CD was recorded in the ileum (9.47 PC vs. 5.82 NC, –38.5%), the primary site of nutrient absorption. This will provide a smaller area available for the absorption of nutrients, likely contributing to depressed feed digestibility and efficiency. It is possible that the ileum is most heavily affected by the gut health effects of undigested CP, as this is often the site where *Clostridium perfringens*, a pathogen which benefits from poor CP digestibility, is recovered from infected broilers [54].

Ding et al. [55] reported that CP reduction led to decreased trypsin activity in the pancreas and duodenum content with both VH and VH:CD ratio reduced in the duodenum, jejunum, and ileum. However, protease supplementation reverted these effects and increased the activity of trypsin in the pancreas and VH in the duodenum, jejunum and VH:CD ratio in the ileum.

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Similarly, in the present study VH in the duodenum and jejunum was significantly improved by all proteases, with the alkaline protease linked to the longest villi in all segments. This finding is also in line with other previous research studies [15,24,55,56]. This suggests that the monocomponent alkaline protease tested in the present study was the most effective at increasing gut health and absorptive capacity in the small intestine. However, this did not translate into the best growth performance. It could be hypothesized that protease supplementation may provide more AA to be utilized by certain beneficial bacterial groups that are known to stimulate the production of mucin and the proliferation of epithelial cells [57], and that this effect could differ between proteases. Further studies evaluating the effect of the different proteases in the microbiome composition and intestinal morphology would be needed to validate this hypothesis.

Selected carcass traits

Growth performance of broilers from modern genetics is more responsive to CP and AA compared to energy [27], and protein deposition in the chicken carcass is increased with additional AA intake [58]. Increases in overall BWG were not significantly different between treatments in the present study, including between the nutritionally adequate PC and the reformulated NC. However, there were significant differences between treatments on carcass weight. The reformulation to lower AA content significantly reduced the carcass weight and carcass yield of the NC, compared to the PC. This difference was partially ameliorated by the addition of the alkaline monocomponent protease and totally recovered by the addition of the serine monocomponent protease, while addition of the multi-protease was able to significantly increase carcass weight and yield above the level of the PC. This is in line with the findings of Cho et al. [37], who saw increased carcass weights and breast yields following supplementation of multi-protease to both nutritionally adequate and reformulated diets. Xu et al. [15], also found that the dietary supplementation of a multi-protease increased breast muscle weight. This effect was possibly linked to higher slaughtering weight because it was not translated to higher breast muscle yield. Similarly, in the present study, higher carcass weight resulted in higher breast weight but not in higher breast meat yield following the multi-protease supplementation. These improvements in efficiency and carcass weight and yield are likely largely driven by improvements in AA digestion and protein deposition, however, a digestibility assay was not performed in the present study and this hypothesis could not be confirmed. Previous studies have demonstrated that both monocomponent and multi-proteases improve precaecal AA digestibility of all [7,38] or some AA [16,20,59]. Protease supplementation can also improve energy retention, though results are inconsistent between and even within studies [17,60]. Improved nutrient absorption following protease supplementation has been previously linked with reduced FI [14,36], though significant differences in FI were not seen in the present study.

In conclusion, the addition of a multi-protease to broiler diets reduced in CP and AA improved feed efficiency compared to all other tested proteases. Both the multi-protease and the monocomponent serine protease increased carcass weight, carcass yield, and breast weight and supported gut health and morphology. The monocomponent serine protease also improved breast %. Our findings show that a multi-protease can be supplemented to broiler diets reduced in CP and AA to enhance feed efficiency in the presence of 3 NSP-degrading enzymes, amylase, neutral protease, phytase, and biosurfactants. The addition of monocomponent proteases with a more limited pH range was not able to achieve the same performance improvements. Further studies should be performed to elucidate the effects of the different proteases in CP and AA digestibility, and microbiome composition.

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