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Original Article

Effects of a Novel Protease from Bacillus Subtilis K-5 in Low Protein Corn Distiller Dried Grains with Solubles (cDDGS) Based Diets on Performance and Nutrients Digestibility in Broiler Chickens

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ABSTRACT

This experiment was conducted to evaluate the supplemental effects of a novel protease produced from *Bacillus subtilis* in low crude protein (CP) corn distiller dried grain with solubles (cDDGS) based diets on growth performance, carcass attributes, nutrients digestibility, blood chemistry, and intestinal histomorphometry of broiler chickens. One hundred and sixty, one-day-old chicks were randomly allotted to one of 4 dietary treatments. Each dietary treatment had four replicates, with 10 birds in each replicate. Two basal diets were formulated for both starter (1-21d) and finisher (22-35d) phase; (PC) a corn soybean meal based diet as per standard recommendations of Ross 308; (NC) 5% cDGGS with 5% reduction in CP with concomitant reduction in essential amino acids (EAAs) compared with PC. The negative control diet was further subdivided into 3 parts. One part was without enzyme supplementation, while the other two parts were supplemented with a novel protease (PROT1) and a commercial protease (PROT2), respectively. The same procedure was adopted for finisher diets. A digestibility assay (32-35d) was carried out using acid insoluble ash (AIA), an external digestibility marker. At the end of 35d, ileal digesta were collected from four birds per experimental unit for nutrient digestibility measurement. Tissue samples of duodenum, jejunum, and ileum were collected for villus height, villus width, crypt depth, and crypt width. Body weight gain (BWG) and feed:gain were improved ($p<0.05$) with protease supplementation. No effect was observed on carcass parameters. However, CP digestibility, apparent digestibility coefficient for nitrogen (ADC_N), nitrogen retention (N_{ret}), and apparent ileal amino acid digestibility (AIAAD) were improved ($p<0.05$). However, there was no effect on apparent metabolizable energy (AME) and apparent metabolizable energy corrected for nitrogen (AME*n*), blood glucose, total protein and cholesterol ($p>0.05$) and intestinal integrity ($p>0.05$). It was concluded that protease enzyme can improve nitrogen and CP digestibility, resulting in improved amino acids availability in low protein diets.

INTRODUCTION

Poultry meat has the potential to meet global demand for inexpensive protein source due to its comparatively low cost. Nevertheless, high dependancy of poultry diets on soybean meal could make it a limiting input (Selle *et al.*, 2020). High soybean meal prices with unpredictable supply further aggravate the problem. It has been suggested that reducing crude protein (CP) by 3 percentage points can lead to a one third reduction in soybean meal contents. In addition, it will also release the land for additional soybean cultivation, otherwise required to sustain poultry production (Chrystal *et al.*, 2020). Moreover, broiler production is often linked with large ammonia production and negative environmental impact. In this context, poultry nutritionists are

required to explore and enhance the nutritive value of alternative protein sources, along with reducing the environmental impact of poultry production. Alternative raw materials usually either have poor protein quality or contain anti-nutritive factors, such as non-starch polysaccharides (NSPs), a trypsin inhibitor. Despite these factors, the use of alternative feed ingredients is increasing, owing to their low price and local availability. The dietary inclusion rate of these feed ingredients can be increased by improving their nutritional values (Campasino *et al.*, 2015).

Corn distillers dried grains with solubles (cDDGS) is a co-product of biofuel industry that has 85% of the energy value as of corn and is a relatively good source of protein. Therefore, it could potentially replace part of corn, soybean meal, and inorganic phosphorus in poultry diets (Shim *et al.*, 2011). The use of cDDGS as a protein source could provide additional economic leverage in light of the rising prices of raw materials, especially soybean meal. Nevertheless, the anti-nutritional factors in cDDGS are one of the putative hurdles for its regular use in poultry diets.

Exogenous enzymes such as proteases, carbohydrases, and phytases, especially of microbial origin, are widely used in animal production. Protease has been suggested to increase the availability of protein from plant sources and decrease the effect of trypsin inhibitor (Freitas *et al.,* 2011), improving amino acid digestibility and protein utilization rate. It could hereby help to decrease the dietary protein levels in broiler diets (Vieira *et al.,* 2016). Protease supplementation can possibly be employed to decrease dietary protein contents without compromising broiler performance, hence leading to less protein waste and nitrogen excretion into the environment (Yu *et al.*, 2007).

Taken together, we hypothesized that supplementation of exogenous protease with the addition of cDDGS in low CP diets could improve protein utilization and broiler performance. The objectives of this study were therefore to compare the effects of a novel protease enzyme produced from *Bacillus subtilis* K-5 with a commercial protease enzyme in low protein cDDGS based diets on growth performance, carcass characteristics, nutrients digestibility, blood profile, and intestinal structure of broiler chickens.

MATERIALS AND METHODS

All procedures followed in the conduction of this experiment were approved by the Advanced Studies and Research Board, PMAS Arid Agriculture University, Rawalpindi, Pakistan.

Animal husbandry and experimental procedures

One hundred and sixty (n=160) ROSS 308 one-day-old broiler chicks were purchased from a local hatchery. These birds were randomly allotted into 16 replicates of 4 treatments with 10 birds in each replicate. Each experimental diet was allotted to four replicates with forty birds under each treatment. The experiment was conducted at the Avian Research Station, Pir Mehr Ali Shah - Arid Agriculture University, Rawalpindi, Pakistan. Birds were reared on floor with 2-inch layer of saw dust as bedding material. Each bird was allotted the floor space of $1ft^2$ in a 3 x 4 square foot pen in a semi controlled shed. The birds were vaccinated against Newcastle disease (ND) + Infectious bronchitis (IB) on day 1 via eye drops, followed by vaccination against ND and infectious bursal disease (IBD) via subcutaneous injection on day 4. The ND vaccine was applied at the 1^{st}, 2^{nd} and 3^{rd} weeks of the trial. The birds were fed the respective experimental diets *ad libitum* for the entire experimental period. Each pen was provided with round bottom feeders while water supply was made available round the clock through a manual drinking system. The farm temperature was maintained at 32^oC during the first week of the trial, with reductions of 3^oC every week till the third week of trial; there after being maintained at 25^oC till the end of trial. Provision of uninterrupted light around the clock was ensured during experimental period. All birds were reared under identical management conditions throughout the experiment.

Experimental diets

Two phase feeding; *i.e.,* starter diet (CP 23%; ME 3,000 kcal/kg) and finisher diet (CP 19.5%; ME 3,200 kcal/kg) was adopted for this trial. Two basal diets; positive control (PC) and negative control (NC) were formulated for both starter and finisher phase. The PC diet was formulated as per standard recommendation of ROSS 308. The NC diet was formulated with the inclusion of 5% cDDGS by replacing corn and soybean meal partially with reduction of 5% CP and 5% EAA. The NC diet was subdivided into 3 parts. One part was without exogenous enzyme supplementation and the remaining two parts were supplemented with novel protease (PROT1) and commercial protease (PROT2), respectively. Diets were formulated on digestible amino acid (DAA) basis, keeping lysine as the reference amino acid by using least cost feed formulation software (Brill® Feed Management System, Inc). The DAA ratio met or exceeded the ideal amino acid ratio. The ingredients

and nutrients composition of experimental diets for starter (1-21d) and finisher (22-35d) are given in Table 1 and 2, respectively.

Exogenous proteases

The commercial protease enzyme (Winzyme Pro Plus®) used in this experiment was supplied by Suntaq International Limited, China. This enzyme contained 20,000 U/g (8,000 U/g of acidic and 12,000 U/g of neutral protease). One protease unit was defined as the amount of enzyme necessary for hydrolysed casein to result in 1 µg of tyrosine in 1 minute at 40°C and 3.0 pH for acidic protease and at 30°C and 7.5 pH for neutral protease. The enzyme was supplemented at a rate of 3,000 protease units/kg of feed.

Novel alkaline protease was produced from *Bacillus subtilis* through solid state fermentation by using wheat bran as substrate in fermentation medium, which is a cheaper and easily available agro-industrial by-product. *Bacillus subtilis* was screened as a protease producing strain by using casein (1% w/v) as substrate. The proteolytic activity was detected by the presence of a clear zone of hydrolysis on casein agar. The strain was identified as *Bacillus subtilis* K-5 by genetic identification based on 16S rRNA and blast technology of the National Center of Biotechnology Information. Response Surface Methodology was used for the optimization of all culture conditions. Maximum protease production (71.18 u/mL) from *Bacillus subtilis* K-5 was obtained at a temperature of 35°C and pH 9 by incubating the fermentation medium for 37 h at a 75% moisture level. Protein concentration of 0.63 mg/mL and specific activity of 111.56 U/mg was observed in crude protease enzyme. Enzyme was partially purified in a 2-step procedure involving ammonium sulphate precipitation and Sephadex G-100 gel chromatography. Protein contents of 0.57 mg/mL (specific activity of 124.72 U/mg) and protein contents of 0.44 mg/mL (specific activity of 143.65 U/mg) were observed by 70 % saturation with ammonium sulphate and gel chromatography, respectively in partially purified protease enzyme. Line Weaver Burk plot was used to find its Vmax and Km, which were 344. 83 mg/mL/min and 100.04 mg, respectively. This partially purified and characterized protease was produced in bulk for use in poultry diets. It was added in the diet at 42.15 mL/kg of feed, providing 3,000 units/kg of feed.

Digestibility assay

A digestibility assay was carried out between 32-35 days of the bird's age. Celite® (source of AIA) was used as an external marker and mixed at 1% of the

Table 1 – Ingredients composition of experimental diets for starter (1-21d) and finisher (22-35d) phases.

Ingredients	Starter Phase (1-21 d)		Finisher Phase (22-35 d)	
	[1]PC	[2]NC	[1]PC	[2]NC
	%		%	
Maize	57.22	55.43	65.61	64.50
Soybean meal 44%	32.65	29.51	23.49	19.43
cDDGS[3]	0.00	5.00	0.00	5.00
Fish meal	5.00	5.00	5.00	5.00
Poultry fat	0.00	0.00	1.30	1.43
Limestone	1.10	1.16	0.87	0.93
MCP 22.5%	0.68	0.60	0.62	0.69
NaCl	0.31	0.32	0.21	0.21
$NaHCO_3$	0.23	0.23	0.23	0.23
Lysine sulphate 70%	0.28	0.29	0.27	0.24
DL-methionine	0.31	0.26	0.23	0.19
L-threonine	0.12	0.10	0.07	0.05
Choline chloride 70%	0.10	0.10	0.10	0.10
Broiler vitamins premix[4]	1.00	1.00	1.00	1.00
Broilers minerals premix[5]	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00

[1]PC = Standard diet as per standard nutritional recommendations of Ross 308.

[2]NC = 5% cDDGS with partial replacement of corn and soybean meal with reduction of 5% CP and 5% EAAs.

[3]cDDGS = corn distiller dried grain with solubles.

[4]Broiler vitamins premix = each kg premix contains vitamin A 12,000, D3 5,000, Vitamin K3 3.2 mg, vitamin B1 3.2 mg, vitamin B2 8.6 mg, vitamin b3 60 mg, vitamin B5 20 mg, vitamin B6 4.3 mg, vitamin B9 2.2 mg, vitamin b12 0.017 mg, vitamin H2 0.22 mg.

[5]Broiler minerals premix = each kg mineral premix contains Mn (MnSO4. H2O) 120 mg, Zn (ZnSO4. H2O) 110 mg, Cu (CuSO4. H2O) 16 mg, Fe (FeSO4. H2O) 20 mg, Iodine (KI) 1.25 mg, Se (sodium selenite) 0.30 mg.

Table 2 – Nutrients composition of experimental diets for starter (1-21d) and finisher (22-35d) phases.

Nutrients	Starter Phase (1-21 d)		Starter Phase (1-21 d)	
	[1]PC	[2]NC	PC	NC
Calculated Nutrient Contents	%		%	
Drymatter	89.38	89.50	89.65	89.77
Crude protein	23.00	21.85	19.50	18.53
Metabolizable energy (kcal/kg)	3000	3000	3200	3200
Ether extract	5.30	4.90	5.30	4.90
Crude fiber	2.85	3.02	4.50	4.60
Calcium	0.96	0.96	0.79	0.79
Total Phosphorus	0.75	0.75	0.75	0.75
Non phytate Phosphorus	0.48	0.48	0.40	0.40
Sodium	0.18	0.18	0.18	0.18
Potassium	0.60	0.60	0.73	0.70
Chlorides	0.18	0.18	0.18	0.18
DEB (mEq/kg)[3]	218	222	177	182
Digestible amino acids[4]	%		%	
Dig. Lysine	1.28	1.22	1.03	0.98
Dig. Methionine	0.51	0.48	0.43	0.41
Dig. Met + Cyst	0.95	0.90	0.80	0.76
Dig. Tryptophan	0.20	0.19	0.16	0.15
Dig. Threonine	0.86	0.82	0.69	0.66
Dig. Isoleucine	0.86	0.82	0.71	0.67
Dig. Arginine	1.37	1.30	1.10	1.05
Dig. Leucine	1.41	1.34	1.13	1.13
Dig. Valine	0.96	0.91	0.78	0.74
Analyzed values	%		%	
Dry matter	91.60	91.17	91.6	91.2
Crude protein	22.83	21.71	19.3	18.4
Ether extract	4.91	5.09	4.9	5.1
Crude fiber	3.72	4.97	4.7	5.0

[1]PC = Standard diet as per standard nutritional recommendations of Ross 308.

[2]NC = 5% cDDGS with partial replacement of corn and soybean meal with reduction of 5% CP and 5% EAAs.

[3]DEB = dietry electrolyte balance.

[4]Digestible amino acids were calculated on the basis of DM and CP contents of the ingredients from Brazilian tables of Poultry and Swine (Rostagno & Becker, 2005). The ratio of essential amino acids with reference to digestible lysine followed the ideal amino acid ratio.

diet. Celite® mixed diets were fed to experimental birds from day 32 to 35. Ileal digesta were collected from the ileum of 4 randomly selected birds from each replicate. Ileal contents were squeezed gently and collected from the lower half of the ileum, as described by Ravindran *et al.* (2005). Ileum was considered as the portion of the small intestine from the Meckel's diverticulum to a point about 40 mm proximal to the ileo-cecal junction. These ileal contents were flushed in 200 mL plastic cups and few drops of formalin were added to stop any microbial activity. Plastic cups were immediately transferred into an ice container, being subsequently placed at -20°C till further analysis. The digesta samples within the pen were pooled, oven dried at 65°C, and ground to pass through 2 mm sieve. Fecal samples were collected for 3 consecutive days from day 32-35 of the trial period by placing plastic sheets in each pen. These fecal samples were homogenously mixed and stored at -20°C. The dry matter was determined by drying in hot air oven at 105°C until reaching a constant weight (Zhong & Adeola, 2019). These samples were then ground to pass 2 mm sieve and stored for further analysis.

Chemical analysis

All experimental diets, ileal digesta, and faecal samples were analyzed for dry matter and total nitrogen (AOAC, 2005) for the estimation of crude protein (N × 6.25). Acid insoluble ash in the diets, ileal digesta, and fecal samples were analyzed using the procedure by Siriwan *et al.* (1993). The gross energy (GE) of diets and fecal samples was determined by bomb calorimeter (Parr Instrument Co. Moline, IL) in the laboratory of Sadiq Feeds Pvt Ltd, Sahiwal, Pakistan. The total amino acids (AAs) of experimental diets and ileal digesta were

analyzed using a Biochrom plus amino acid analyzer (Biochrom Ltd. Cambridge UK) through the procedure used by Palliyeguru *et al.* (2010) at the laboratory of Sadiq Feeds Pvt Ltd, Rawalpindi, Pakistan. Samples were briefly oxidized with a hydrogen peroxide-formic acid-phenol solution. Sodium disulphite was used to decompose the excess oxidation reagent. After oxidation, samples were hydrolyzed using 6 *M* HCl for 24 hours. The pH of hydrolysate was adjusted to 2.20, and it was centrifuged and filtered. The AAs in the solution were separated using an AA analyzer at 570 nm (AOAC, 2000).

Growth performance data

Data on daily feed intake (FI) and weekly body weight gain (BWG) were used to calculate the feed to gain ratio (feed:gain) for starter phase (1-21d) and finisher phase (22-35d).

Carcass data

At the end of the experiment, 4 birds were taken randomly from each pen and slaughtered to document carcass characteristics. Each bird was defeathered, and carcass yield (% of live weight), breast meat yield (% of carcass weight) and thigh meat yield (% of carcass weight) were recorded.

Tissue sampling

Samples of duodenum, jejunum and ileum were collected using the method described by Wang *et al.* (2015) for the determination of villus height, villus width, crypt depth, crypt width, and villus height to crypt depth ratio. Segments of 2-3 cm from the mid-point of the duodenum, the mid-point between the bile duct entry and Meckel's diverticulum (Jejunum), and the distal end of the ileum (ileum) were dissected. These segments were flushed with ice-cold phosphate buffer saline (pH 7.2) and preserved in 10% formalin phosphate buffer solution till further analysis. Villi were examined under light microscope (Fasina *et al.*, 2010).

Blood chemistry

At the end of the experiment, 5 mL of blood were collected aseptically from the bronchial veins of the two randomly selected birds from each replicate, and then transferred in BD vacutainer® SST™. These samples were centrifuged for 15 minutes at 3000 rpm at 4°C to isolate serum. The serum was then stored at -20°C for the analysis of blood glucose, cholesterol, and total protein (Alizadeh *et al.*, 2016).

Calculations and statistical analysis

The apparent digestibility coefficient for nitrogen (ADC_N) was calculated using the following equation (Ravindran *et al.*, 1999):

$$ADCN = \frac{\left(\frac{N}{AIA}\right) diet - \left(\frac{N}{AIA}\right) digesta}{\left(\frac{N}{AIA}\right) diet}$$

Where N and AIA represents nutrient and acid insoluble ash respectively.

The AME of the diet was determined using the following formula;

$$AME\left(kcal/_{Kg}\right) = GEdiet - \left[GEdigesta \times \left(\frac{AIAdiet}{AIAdigesta}\right)\right]$$

The catabolic compound in excreted N leads to considerable energy loss. Therefore, AME was subjected to zero N retention employing a factor of 8.22 kcal/kg (Hill & Anderson, 1958). The AME*n* = AME - (8.22 x N_{ret}), where N_{ret} is nitrogen retention in g/kg DM intake. The nitrogen retained (N_{ret}) was calculated following Bolarinwa & Adeola (2012):

$$Nret = Ndiet - \left[Ndigesta \times \left(\frac{AIAdiet}{AIAdigesta}\right)\right]$$

Apparent ileal amino acids digestibility (AIAAD) was determined by the formula suggested by Ravindran *et al.* (1999).

$$AIAAD(\%) = 1 - \left[\left(\frac{AIAdiet}{AIAdigesta}\right) \times \left(\frac{AIAdigesta}{AIAdiet}\right)\right] \times 100$$

The growth performance and nutrient digestibility data were analyzed using the General Linear Model (GLM) procedures of Minitab 17 (Minitab Inc., State College, PA). The data were subjected to analysis of variance (ANOVA). Differences were considered to be significant at $p<0.05$. The significant means were separated by Tukey's honestly significant difference test.

RESULTS

Broiler performance

The effects of protease supplementation on Feed intake (FI), body weight gain (BWG), and feed:gain from day 1-21 are presented in Table 3. Feed intake remained unaffected ($p>0.05$), but lower BWG ($p<0.05$) and poor feed:gain ($p<0.05$) was noticed in NC group as compared to PC. Protease supplementation restored BWG ($p<0.05$) and feed:gain ($p<0.05$) close to control group in starter phase.

There was no change in FI ($p>0.05$), BWG ($p>0.05$), and feed:gain ($p>0.05$)s noticed during grower phase (22-35d) with addition of protease.

Table 3 – Effect of protease enzyme supplementation on performance of broilers fed cDDGS based low protein diets.

Age (Days)	Parameters	Treatments					
		PC[1]	NC[2]	[3]NC-PROT1	[4]NC-PROT2	*SEM*	*p-Value*
1-21 Days	FI[5] (g)	1119	1138	1128	1123	6.24	0.208
	BWG[5] (g)	842[a]	812[b]	844[a]	842[a]	5.38	0.003
	Feed:gain (g:g)	1.33[b]	1.40[a]	1.34[b]	1.33[b]	0.01	0.001
22-35 Days	FI (g)	2132	2137	2146	2137	13.2	0.906
	BWG (g)	1183	1162	1177	1187	10.1	0.350
	Feed:gain (g:g)	1.80	1.84	1.82	1.80	0.01	0.363
1-35 days	FI (g)	3251	3275	3274	3260	9.91	0.296
	BWG (g)	2025[a]	1973[b]	2021[a]	2029[a]	5.94	0.001
	Feed:gain (g:g)	1.61[c]	1.66[a]	1.62[b]	1.61[c]	0.01	0.001

Means within a column lacking common superscript differ significantly ($p<0.05$).

Mean of four replicates having 10 birds per each replicate.

[1]PC = Standard diet as per standard nutritional recommendations of Ross 308.

[2]NC = 5% cDDGS with partial replacement of corn and soybean meal with reduction of 5% CP and 5% EAAs.

[3]NC-PROT1 = NC diet supplemented with novel protease enzyme.

[4]NC-PROT2 = NC diet supplemented with commercial protease enzyme.

[5]FI = feed intake, BWG = body weight gain, Feed:gain = feed conversion ratio.

The overall performance (1-35d) data indicates significant improvement. Poor BWG was noticed in NC group ($p<0.05$) as compared to PC. Protease supplementation significantly improved BWG ($p<0.05$) and feed:gain ($p<0.05$) and restored performance equal to PC group. There was no change recorded in FI ($p>0.05$).

Carcass response

Non-significant differences were noted ($p>0.05$) in carcass traits with the supplementation of protease as compared to control (Table 4).

Nutrients digestibility

Effect of protease supplementation on nutrients digestibility is reported in Table 5. There was no effect of protease addition noticed on AME ($p>0.05$) and AME*n* ($p>0.05$). However, apparent digestibility of CP was improved ($p<0.05$) with improved ADC_N ($p<0.05$) and N_{ret} ($p<0.05$) with protease supplementation as compared to NC diet. Protease supplementation significantly improved ($p<0.05$) AIAAD in comparison to the NC group. However, the improvement in AIAAD was noticed better with PROT2 than with PROT1.

Table 4 – Effect of protease enzyme supplementation on carcass characteristics of broilers fed cDDGS based low protein diets.

Parameters (%)	Treatments					
	PC[1]	NC[2]	[3]NC - PROT1	[4]NC - PROT2	*SEM*	*p*-Value
CY[5]	62.17	59.52	61.78	62.06	0.89	0.168
BMY[5]	22.97	21.10	22.32	22.35	0.43	0.061
TMY[5]	8.58	8.22	8.23	8.28	0.27	0.751

Mean of four birds per replicate (16 birds per each treatment).

[1]PC = Standard diet as per standard nutritional recommendations of Ross 308.

[2]NC = 5% cDDGS with partial replacement of corn and soybean meal with reduction of 5% CP and 5% EAAs.

[3]NC-PROT1 = NC diet supplemented with novel protease enzyme.

[4]NC-PROT2 = NC diet supplemented with commercial protease enzyme.

[5]CY = carcass yield, BMY = breast meat yield, TMY = thigh meat yield.

Blood parameters

Data on total protein, blood glucose, and cholesterol is presented in Table 6. No effect ($p>0.05$) of protease supplementation was observed on total protein, blood glucose, or blood cholesterol as compared to the NC diet group.

Intestinal Morphometry

Intestinal morphometry data is shown in Table 7. The intestinal parameters remained unchanged ($p>0.05$) with protease enzyme addition. However, improvement in ileum villus height and VH:CD ($p<0.05$) was noticed as compared to the NC diet group.

Table 5 – Effect of protease enzyme supplementation on nutrients digestibility of broilers fed cDDGS based low protein diets.

Nutrients (%)	Treatments					
	[1]PC	[2]NC	[3]NC - PROT1	[4]NC - PROT2	*SEM*	*p*-Value
CP[5]	82.72[c]	82.74[c]	85.30[b]	86.75[a]	0.34	0.001
ADC_N[5]	0.85[ab]	0.81[b]	0.86[a]	0.87[a]	0.01	0.005
N_{ret}[5]	2.62[a]	2.59[b]	2.63[a]	2.64[a]	0.01	0.003
AME[5]	3075	3056	3090	3107	15.4	0.174
AME*n*[5]	3053	3035	3068	3085	15.4	0.180
Lysine	87.11[b]	86.12[b]	88.57[a]	89.23[a]	0.271	0.001
Methionine	89.09[b]	88.19[c]	89.87[b]	91.09[a]	0.223	0.001
Met+Cys	86.54[b]	85.20[c]	87.11[b]	88.21[a]	0.146	0.001
Threonine	82.13[bc]	81.56[c]	82.89[ab]	83.54[a]	0.235	0.001
Valine	82.97[a]	81.29[b]	82.96[a]	82.94[a]	0.222	0.001
Arginine	88.32[a]	87.01[b]	89.11[a]	89.03[a]	0.204	0.001
Isoleucine	82.39[b]	81.17[c]	82.78[b]	84.27[a]	0.152	0.001
Leucine	80.95[ab]	79.08[c]	80.61[b]	81.34[a]	0.162	0.001
Cysteine	82.54[a]	80.23[b]	82.19[a]	83.15[a]	0.276	0.001
Tryptophan	87.685[ab]	85.67[c]	87.21[b]	88.19[a]	0.147	0.001
Histidine	81.63[a]	79.76[b]	81.12[a]	81.18[a]	0.139	0.001

Means within a column lacking common superscript differ significantly ($p<0.05$).

Mean of 4 samples collected per replicate (16 samples per treatment).

[1]PC = Standard diet as per standard nutritional recommendations of Ross 308.

[2]NC = 5% cDDGS with partial replacement of corn and soybean meal with reduction of 5% CP and 5% EAAs.

[3]NC-PROT1 = NC diet supplemented with novel protease enzyme.

[4]NC-PROT2 = NC diet supplemented with commercial protease enzyme.

[5]CP = crude protein, ADC_N = apparent digestibility coefficient for nitrogen, N_{ret} = nitrogen retention, AME = apparent metabolizable energy, AME*n* = apparent metabolizable energy corrected for nitrogen.

Table 6 – Effect of protease enzyme supplementation on blood chemistry of broilers fed cDDGS based low protein diets.

Parameters (%)	Treatments					
	[1]PC	[2]NC	[3]NC-PROT1	[4]NC-PROT2	*SEM*	*p*-Value
Total Protein, g/dL	3.30	3.41	3.43	3.37	0.031	0.183
Blood Glucose, mg/dL	217	226	224	228	4.52	0.360
Cholesterol, mg/dL	153	147	146	146	3.04	0.297

Mean of 4 samples collected per replicate (16 samples per treatment).

[1]PC = Standard diet as per standard nutritional recommendations of Ross 308.

[2]NC = 5% cDDGS with partial replacement of corn and soybean meal with reduction of 5% CP and 5% EAAs.

[3]NC-PROT1 = NC diet supplemented with novel protease enzyme.

[4]NC-PROT2 = NC diet supplemented with commercial protease enzyme.

DISCUSSION

Numerous factors can cause a variation in nutritive composition of cDDGS, for example variability in the corn, the milling and thermal process used for cDDGS production, composition of soluble fractions added in the manufacturing process, the type of ethanol producing plant, and time of the year (Salim *et al.*, 2010; Belyea *et al.*, 2010). It is also well documented that during the drying process of cDDGS, amino acid composition is compromised due to heat treatment (Amezcua & Parsons 2007; Bandegan *et al.*, 2009; Olukosi *et al.*, 2010).

The possibility of exogenous protease supplementation in poultry diets has been seen as an opportunity to enhance feed efficiency, reduce feed cost and, lower impacts on the environment (Zakaria *et al.*, 2010). However, inconsistent reports have been found in literature, for instance, some past studies (Ghazi *et al.*, 2002; Cowieson *et al.*, 2006; Cowieson & Ravindran, 2008) noted a positive impact of enzyme addition in poultry diets while others studies (Naveed, 1998; Saleh *et al.*, 2004) reported negative effects of exogenous enzyme addition.

The results of the present study showed that addition of 5% cDDGS in NC diet by decreasing CP and EAAs

Table 7 – Effect of protease enzyme supplementation on apparent ileal amino acids digestibility of broilers fed cDDGS based low protein diets.

Parameters		Treatments					
[1]PC		[2]NC	[3]NC-PROT1	[4]NC-PROT2	*SEM*	*p*-Value	
Villus Height (µm)	Duodenum	1219	1176	1178	1251	19.9	0.059
	Jejunum	972	1037	1043	1106	29.6	0.051
	Ileum	825[c]	908[b]	914[b]	997[a]	12.0	0.001
Villus Width (µm)	Duodenum	123	123.25	123.75	126	7.39	0.991
	Jejunum	110	106	109	110	4.55	0.911
	Ileum	110	106	108	111	5.66	0.903
Crypt Depth (µm)	Duodenum	152	155	156	150	5.34	0.893
	Jejunum	139	140	139	137	5.21	0.982
	Ileum	115	113	110	110	4.21	0.747
Crypt Width (µm)	Duodenum	44.75	42.25	42.75	44.78	4.32	0.963
	Jejunum	44.11	42.25	42.50	43.08	2.69	0.968
	Ileum	40.50	40.75	39.50	39.53	1.80	0.938
[5]VH/CD	Duodenum	8.02	7.62	7.60	8.40	0.343	0.34
	Jejunum	7.03	7.46	7.58	8.11	0.404	0.348
	Ileum	7.17[b]	8.11[ab]	8.40[ab]	9.12[a]	0.331	0.001

Means within a column lacking common superscript differ significantly ($p<0.05$).

Mean of 4 samples collected per replicate (16 samples per treatment).

[1]PC = Standard diet as per standard nutritional recommendations of Ross 308.

[2]NC = 5% cDDGS with partial replacement of corn and soybean meal with reduction of 5% CP and 5% EAAs.

[3]NC-PROT1 = NC diet supplemented with novel protease enzyme.

[4]NC-PROT2 = NC diet supplemented with commercial protease enzyme.

VH/CD = villus height/crypt depth.

resulted in a reduction of BWG by 3.56% and feed:gain (1.33 vs 1.40) as compared to the control group in starter phase. The depression in growth performance from the addition of cDDGS in young birds could likely be due to their inability to utilize this protein source because of deficiencies in innate endogenous enzymes availability, which are required in high amounts in young growing birds. Rapid feed passage rate in starter period can also result in lower protein digestibility (Uni *et al.*, 1999). Supplementation of PROT1 and PROT2 in NC diets improved BWG by 3.79% and 3.56%, respectively. Likewise, feed:gain was also improved with PROT1 (1.34 vs. 1.40) and PROT2 (1.33 vs. 1.40) from day 1-21. During early bird age, endogenous protease and peptidase are inadequate to hydrolyze proteins so supplementation of exogenous protease improved protein digestion. It is likely that in our study enzyme supplementation could have improved protein digestion and subsequent growth performance. The present findings are in line with Dosković *et al.* (2013), who suggested that exogenous enzymes complemented naturally produced enzymes to increase intestinal and pancreatic protease production in young birds. Essentially, this phenomenon clearly suggest that endogenous production of endogenous enzymes may be inadequate in the initial post-hatch period in poultry. Overall growth performance from day 1-35 was also improved with the addition of protease enzyme. Body weight gain was improved by 2.37% and 2.75% with PROT1 and PROT2 supplementation, respectively; however, FI remained unaffected. The feed:gain from day 1-35 was also improved with the addition of PROT1 (1.62 vs. 1.66) and PROT2 (1.61 vs. 1.66), hence restoring bird's performance to a level comparable to the control group. Similar findings have been reported by Moran & Lehman 2008 and Angel *et al.* (2011), who reported similar results with graded supplementation of mono-component protease enzyme in broilers. Putatively, this improvement in BWG and feed:gain with protease supplementation is due to its direct effect in protein digestibility, liberating more amino acids for protein synthesis (Freitas *et al.*, 2011). Gao *et al.* (2008) reported that supplementation of protease enzymes could change the nutritional and physiological status of the broilers and improve growth rate. These results are also in agreement with Loar *et al.* (2010) and Wang *et al.* (2008), who reported that feed conversion ratio was not affected by increasing level of cDDGS with addition of protease enzyme; so cDDGS could be used at up to 20% if diets are formulated on digestible amino acids basis.

We did not notice any effect of protease supplementation on carcass traits. Many studies in literature (Gao *et al.,* 2007; Hajati, 2010; Opoku *et*

al., 2015; Dalolio *et al.,* 2016; Mahmood *et al.*, 2018; Hussain *et al.*, 2019) also demonstrated similar effects of protease on carcass attributes.

The inherent digestibility of dietary protein depends on the quality of the raw material used in poultry diets. Therefore, undigested protein represents an important chance for using protease enzyme in poultry diets. In the present study, the apparent CP digestibility was 82.74%, which indicates the presence of a 17.26% indigestible protein fraction in cDDGS based NC diets. However, the indigestible protein fraction decreased to 14.7 and 13.25% with the addition of PROT1 and PROT2, respectively. In other words, it could be speculated that PROT supplementation brought about an average 19% improvement in the indigestible protein fraction of the diet, which would lead to less nitrogen excretion. The apparent digestibility of CP in cDDGS based NC diets was improved by 3.00% and 4.62%; hence, improvement in N_{ret} was of 1.52% and 1.89% with PROT1 and PROT2, respectively. It has been suggested that protease supplementation may help neutralize anti-nutritional factors such as antigenic proteins, trypsin inhibitors, and lectins; improving protein digestibility (Douglas *et al.*, 2000). Previous studies (Amerah *et al.*, 2017; Cowieson & Ravindran, 2008) also found that supplementary protease improved protein digestibility and nitrogen retention. Nevertheless, protease addition not always resulted in improvement in CP digestibility. In one such study, Campasino *et al.* (2015) reported that protease supplementation did not improve protein digestibility at 10% inclusion level of cDDGS. Surprisingly, CP digestibility was improved at 15% cDDGS inclusion level of.

Supplementation of protease has been employed in diets to reduce CP levels in order to minimize dietary-protein waste, nitrogen excretion, and nitrogen emission into the environment without compromising bird performance (Yu *et al.*, 2007). In our study, the apparent digestibility coefficient for nitrogen (ADC_N) was also improved by 5.81% and 6.89% with the addition of PROT1 and PROT2, respectively. It is most likely that the inclusion of cDDGS has provided a bigger undigested nitrogen fraction for protease to digest. In the past, (Opoku *et al.*,2015; Hussain *et al.*, 2019; Olukosi *et al.*, 2015) protease addition has led to improved N digestibility when a alternate raw material bringing in more indigestible fraction was added.

In the present study, we did not find any improvement in AME and AME*n* in response to protease addition. Contrarily, Olukosi *et at.* (2015) found an improvement in metabolizable energy in low pro cDDGS based diets. One possible explanation for this difference could be the inclusion level of cDDGS which was only 5% in our study. However, Min *et al.* (2009) observed that addition of an enzyme complex (protease, phytase, amylase and xylanase) in diets containing 30% cDDGS did not have any effect on the AME values of broilers.

Protease supplementation significantly improved AIAAD in cDDGS based NC diets. The improvement in AIAAD with protease was not the same for all amino acids. It may be due to the inherent digestibility of amino acids. The importance of inherent digestibility of nutrients in the control diet as a predictor of enzyme effect has been reported previously (Cowieson & Bedford, 2009; Coweison, 2010). There are certain steps in ethanol and cDDGS production such as cooking, liquefaction, saccharification, and drying that are associated with high temperatures. Thus, it is possible that there are amino acids complexes with reducing sugars that could potentially make them recalcitrant to digestion. The possibility of beneficial impact of exogenous protease in liberating amino acids from reducing complex sugars cannot be ignored. It has been suggested that exogenous protease acts by releasing peptides from anti-nutritional factors present in the feed ingredients, cleaving linkage between amino acids-starch complexes, supplementing the endogenous production of peptidases, and reducing enzymatic secretions and protein turnover; thereby providing amino acids for protein synthesis and deposition (Freitas *et al.*, 2011; Cowieson & Roos, 2013).

Protease supplementation increased the villus height of ileum, hence ileum VH:CD also improved. Corn DDGS also contains yeast cell wall, the supplementation of which is reported to improve villus height, since it contains mannan and glucan contents that could bind and block enteropathogens (Savage *et al.*, 1997; Shane, 2001). Further research is required to clearly establish the role of yeast cell wall of cDDGS on the intestinal histomorphometry of broiler chickens.

In conclusion, supplementation of protease in low protein cDDGS based diet improved performance, apparent digestibility of dietry protein, nitrogen retention, and apparent ileal amino acids digestibility in broiler chicken.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

REFERENCES

Alizadeh M, Rodriguez-Lecompte JC, Rogiewicz A, Patterson R, Slominski BA. Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance, gut morphology, and gene expression of pattern recognition receptors and cytokines in broiler chickens. Poultry Science 2016;95:507-517.

Amerah AM, Romero LF, Awati A, Ravindran V. Effect of exogenous xylanase, amylase, and protease as single or combined activities on nutrient digestibility and growth performance of broilers fed corn/soy diets. Poultry Science 2017;96(4):807-816.

Amezcua CM, Parsons C. Effect of increased heat processing and particle size on phosphorus bioavailability in corn distillers dried grains with solubles. Poultry Science 2007;86(2):331-337.

Angel CR, Saylor W, Vieira SL, Ward N. Effect of monocomponent protease on performance and protein utilization in 7-to-22-day-old broiler chickens. Poultry Science 2011;90(10): 2281-2286.

AOAC - Association of Official Analytical Chemists. Official methods of analysis. Gaithersburg; 2000

AOAC – Association of Official Analytical Chemists. Official methods of analysis. Gaithersburg; 2005

Bandegan A, Guenter W, Hoehler D, Crow G, Nyachoti C. Standardized ileal amino acid digestibility in wheat distillers dried grains with solubles for broilers. Poultry Science 2009;88(12):2592-2599.

Belyea RL, Rausch KD, Clevenger TE, Singh V, Johnston DB, Tumbleson ME. Sources of variation in composition of DDGS. Animal Feed Science and Technology 2010;159(3-4):122–130

Bolarinwa OA, Adeola O. Energy value of wheat, barley, and wheat dried distiller grains with solubles for broiler chickens determined using the regression method. Poultry Science 2012;91(8):1928-1935.

Campasino A, Williams M, Latham R, Bailey CA, Brown B, Lee JT. Effect of increasing dried distillers' with solubles and non-starch polysaccharide degrading enzyme inclusion on growth performance and energy digestibility in broilers. Journal of Applied Poultry Research 2015;24: 135-144.

Chrystal PV, Moss AF, Khoddami A, Naranjo VD, Selle PH, Liu SY. Effects of reduced crude protein levels, dietary electrolyte balance, and energy density on the performance of broiler chickens offered maize-based diets with evaluations of starch, protein, and amino acid metabolism. Poultry Science 2020;99(3):1421-1431.

Cowieson AJ, Bedford MR. The effect of phytase and carbohydrase on ileal amino acid digestibility in monogastric diets: complimentary mode of action? World's Poultry Science Journal 2009;65(4):609-624.

Cowieson AJ, Ravindran V. Effect of exogenous enzymes in corn-based diets varying in nutrient density for young broilers: growth performance and digestibility of energy, minerals and amino acids. British Poultry Science 2008;49(1):37-44.

Cowieson AJ, Roos FF. Bioefficacy of a mono-component protease in the diets of pigs and poultry: a meta-analysis of effect on ileal amino acid digestibility. Journal of Applied Animal Nutrition 2013;2:1-8

Cowieson AJ, Singh DN, Adeola O. Prediction of ingredient quality and the effect of a combination of xylanase, amylase, protease and phytase in the diets of broiler chicks. Growth performance and digestible nutrient intake. British Poultry Science 2006;47(4):477-489.

Cowieson AJ. Strategic selection of exogenous enzymes for corn/ soy-based poultry diets. The Journal of Poultry Science 2010;47(1):1-7.

Dalolio FS, Moreira J, Vaz DP, Albino LFT, Valadares LR, PIRES AV, *et al.* Exogenous enzymes in diets for broilers. Revista Brasileira de Saude e Produção Animal 2016;17(2):149-161.

Dosković V, Bogosavljević-Bosković S, Pavlovski Z, Milošević B, Škrbić Z, Rakonjac S, *et al.* Enzymes in broiler diets with special reference to protease. World's Poultry Science Journal 2013;69(2):343-60.

Douglas MW, Parsons CM, Bedford MR. Effect of various soybean meal sources and Avizyme on chick growth performance and ileal digestible energy. Journal of Applied Poultry Research 2000;9(1):74-80.

Fasina YO, Hoerr FJ, McKee SR, Conner DE. Influence of *Salmonella enterica* serovar Typhimurium infection on intestinal goblet cells and villous morphology in broiler chicks. Avian Diseases 2010;54(2):841-847.

Freitas DM, Vieira SL, Angel CR, Favero A, Maiorka A. Performance and nutrient utilization of broilers fed diets supplemented with a novel mono component protease. Journal of Applied Poultry Research 2011;20(3):347-352.

Gao F, Jiang Y, Zhou G, Han Z. The effects of xylanase supplementation on growth, digestion, circulating hormone and metabolite levels, immunity and gut microflora in cockerels fed on wheat-based diets. British Poultry Science 2007;48(4):480-488.

Gao F, Jiang Y, Zhou GH, Han ZK. The effects of xylanase supplementation on performance, characteristics of the gastrointestinal tract, blood parameters and gut microflora in broilers fed on wheat-based diets. Animal Feed Science and Technology 2008;142(1-2):173-184.

Ghazi S, Rooke JA, Galbraith H, Bedford MR. The potential for the improvement of the nutritive value of soybean meal by different proteases in broiler chicks and broiler cockerels. British Poultry Science 2002;43(1):70-77.

Hajati H. Effects of enzyme supplementation on performance, carcass characteristics, carcass composition and some blood parameters of broiler chicken. American Journal of Animal and Veterinary Sciences 2010;5(3):221-227.

Hill FW, Anderson DL. Comparison of metabolisable energy and productive energy determination with growing chicken. Journal of Nutrition 1958;64:587-603.

Hussain M, Mirza MA, Nawaz H, Asghar M, Ahmed G. Effect of exogenous protease, Mannanase and xylanase supplementation in corn and Hi protein corn DDGS based diets on growth performance, Intestinal morphology and nutrients digestibility in broiler chickens. Brazilian Journal of Poultry Science 2019;21(4):1-10.

Krogdahl A, Sell JL. Influence of age on lipase, amylase and protease activities in the pancreatic tissue and intestinal contents of young turkeys. Poultry Science 1989;68(11):1561-1568.

Loar RE, Moritz JS, Donaldson JR, Corzo A. Effects of feeding distillers dried grains with solubles to broilers from 0 to 28 days posthatch on broiler performance, feed manufacturing efficiency, and selected intestinal characteristics. Poultry Science 2010;89(10):2242- 2250.

Mahmood T, Mirza M, Nawaz H, Shahid M. Exogenous protease supplementation of poultry by-product meal-based diets for broilers: Effects on growth, carcass characteristics and nutrient digestibility. Journal of Animal Physiology and Animal Nutrition 2018;102(1):233-241

Min YN, Hancock A, Yan F, Lu C, Coto C, Karimi A, *et al.* Use of combinations of canola meal and distillers dried grains with solubles in broiler starter diets. Journal of Applied Poultry Research 2009;18(4):725-733.

Moran ET, Lehman R. Response to combined amylase-phytase-protease xylanase supplementation when 8-week broiler males had received corn-soybean meal feeds devoid of antimicrobials with/without alfalfa and/or DDGS. Poultry Science 2008;87(suppl 1):158.

Naveed A. Effect of enzyme supplementation of UK-grown *Lupinus albus* on growth performance in broiler chickens. British Poultry Science 1998;39:S36–S37.

Olukosi O, Beeson L, Englyst K, Romero L. Effects of exogenous proteases without or with carbohydrases on nutrient digestibility and disappearance of non-starch polysaccharides in broiler chickens. Poultry Science 2015;94(11):2662-2669.

Olukosi O, Cowieson A, Adeola O. Broiler responses to supplementation of phytase and admixture of carbohydrases and protease in maize-soybean meal diets with or without maize distillers' dried grain with solubles. British Poultry Science 2010;51(3):434-443.

Opoku EY, Classen HL, Scott TA. Evaluation of inclusion level of wheat distillers dried grains with solubles with and without protease or β-mannanase on performance and water intake of turkey hens. Poultry Science 2015;94(7):1600-1610.

Palliyeguru MW, Rose SP, Mackenzie AM. Effect of dietary protein concentrates on the incidence of subclinical necrotic enteritis and growth performance of broiler chickens. Poultry Science 2010;89(1):34–43.

Ravindran V, Cabahug S, Ravindran G, Bryden WL. Influence of microbial phytase on apparent ileal amino acid digestibility of feedstuffs for broilers. Poultry Science 1999;78(5):699-706.

Ravindran V, Hew LI, Ravindran G, Bryden WL. Apparent ileal digestibility of amino acids in dietry ingredients for broiler chickens. Animal Science 2005;81:85-97.

Saleh F, Ohtsuka A, Tanaka T, Hayashi K. Carbohydrases are digested by proteases present in enzyme preparations during in-vitro digestion. The Journal of Poultry Science 2004;41(3):229-235.

Salim HM, Kruk ZA, Lee BD. Nutritive value of corn distillers dried grains with solubles as an ingredient of poultry diets: a review. World's Poultry Science Journal 2010;66(3):411–432.

Savage TF, Zakrzewska EI, Andreasen JR. The effects of feeding mannan oligosaccharide supplemented diets to poults on performance and the morphology of the small intestine. Poultry Science 1997;76(suppl 1):139.

Selle PH, Paula Dorigam JC de, Lemme A, Chrystal PV, Liu SY. Synthetic and crystalline amino acids: Alternatives to soybean meal in chicken-meat production. Animals 2020;10(4):729.

Shane SM. Mannan oligosaccharides in poultry nutrition: mechanisms and benefits. Science and Technology in the Feed Industry 2001;65-77

Shim MY, Pesti GM, Bakalli RI, Tillman PB, Payne RL. Evaluation of corn distillers dried grains with solubles as an alternative ingredient for broilers. Poultry Science 2011;90(2):369-376.

Siriwan P, Bryden WL, Mollah Y, Annison EF. Measurement of endogenous amino acid losses in poultry. British Poultry Science 1993;34(5):939–949.

Uni ZE, Noy YA, Sklan, DA. Posthatch development of small intestinal function in the poult. Poultry Science 1999;78(2):215-222.

Vieira SL, Stefanello C, Cemin HS. Lowering the dietry protein levels by the use of synthetic amino acids and the use of a mono component protease. Animal Feed Science and Technology 2016;221:262-266.

Vogtmann H, Pfirter HP, Prabucki AL. A new method of determining metabolisability of energy and digestibility of fatty acids in broiler diets. British Poultry Science 1975;16:531-534.

Wang X, Peebles ED, Morgan TW, Harkess RL, Zhai W. Protein source and nutrient density in the diets of male broilers from 8 to 21 d of age: Effects on small intestine morphology. Poultry Science 2015;94(1):61-67.

Wang Z, Cerrate S, Coto C, Yan F, Waldroup PW. Evaluation of high levels of distillers dried grains with soluble (DDGS) in broiler diets. International Journal Poultry Science 2008;7(10):990-996.

Yu B, Wu ST, Liu CC, Gauthier R, Chiou PWS. Effects of enzyme inclusion in a maize-soybean diet on broiler performance. Animal Feed Science and Technology 2007;134(3-4):283-294.

Zakaria HAH, Jalal MAR, Ishmais MAA. The influence of supplemental multi-enzyme feed additive on the performance, carcass characteristics and meat quality traits of broiler chickens. International Journal of Poultry Science 2010;9(2):126-133.

Zhong R, Adeola O. Energy values of solvent-extracted canola meal and expeller-derived canola meal for broiler chickens and growing pigs determined using the regression method. Journal of Animal Science 2019;97(8):3415-3425.