OPEN ACCESS
Journal of Animal Health and Production

Research Article

Assessing the Effect of Dietary Protease Supplementation with Low Amino Acid Diet on Growth, Gut Enzyme Activity, Immunity, Meat Quality and Bone Morphometry in Broiler Chicken

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Abstract | The objective of our study was to evaluate the influence of dietary protease supplementation with low amino acid diet on gut growth, gut enzyme activity, immunity, meat quality and bone morphometry in broiler chicken. Six hundred Ross 308 broilers were divided into five treatments, each with six replicates. The treatments included: a positive control (PC), a negative control (NC) with a 2% amino acid (AA) deficiency, an NC with a 4% AA deficiency, and two additional NC diets with 2% and 4% less AA supplemented with protease enzyme at a dose of 200 g/ton of feed. Broilers received NC diets with protease supplementation exhibited better feed conversion rate (FCR) and body weight (P<0.05). A significant increase in IgA and IgM level was observed in the protease-supplemented groups as compared to NC groups (P<0.05). However, no significant differences were observed in IgG levels, thyroid hormones (T3, T4), or the activity of amylase and lipase enzymes in protease-supplemented and other groups (P>0.05). Moreover, trypsin and protease activity were significantly improved in protease-supplemented groups as compared to other groups (P<0.05). Meat quality parameters showed a significant increase in crude protein content, pH, and water-holding capacity, along with a significant decrease in cooking loss in the protease-supplemented groups compared to the other groups (P<0.05). Additionally, tibia bone breaking strength was significantly improved in the protease-supplemented groups compared to the other groups (P<0.05). Based on the findings, it is concluded that protease supplementation in amino acid-deficient diets has beneficial effects on growth performance, digestive enzyme activity, immunoglobulin levels, bone strength, and, to some extent, meat quality.

Keywords | Amino acid, Bone morphometry, Gut enzyme, Immunity, Protease Supplementation

Received | March 08, 2025; **Accepted** | May 21, 2025; **Published** | June 27, 2025
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Citation | Zakria M, Leghari IH, Rajput N, Soomro SA, Pirzado SA (2025). Assessing the effect of dietary protease supplementation with low amino acid diet on growth, gut enzyme activity, immunity, meat quality and bone morphometry in broiler chicken. J. Anim. Health Prod. 13(3): 572-582.
DOI | https://dx.doi.org/10.17582/journal.jahp/2025/13.3.572.582
ISSN (Online) | 2308-2801

INTRODUCTION

The poultry industry is frequently probing innovative strategies to enhance production efficiency and ensure the health and welfare of broiler chickens. Central to this venture is the formulation of rations that accommodate the nutritional requirements of broilers at various phases of growth while minimizing production costs. Amino acids are the building blocks of proteins and are essential for multitude of physiological processes, including muscle development, immune function, and bone metabolism. Subsequently, suboptimal amino acid intake may challenge growth performance, immune responses, and overall bird health (Abou-Elkhair *et al*., 2020; Pirzado *et al*., 2022). Thus, interest in researching dietary interventions that can increase protein utilization and mitigate the negative effects of low amino acid diets is on the rise. One such intervention entails the supplementation of broiler diets with exogenous protease enzymes that catalyze the hydrolysis of peptide bonds in proteins, breaking them down into smaller peptides and amino acids that are more readily absorbed in the gastrointestinal tract. By enhancing protein digestion and absorption, protease supplementation has the potential to improve the utilization of dietary proteins, particularly in diets with reduced amino acid levels (Amer *et al*., 2021). However, the effects of protease supplementation on various aspects of broiler physiology, particularly when combined with low amino acid diets, remain to be fully elucidated.

Understanding the multifaceted impact of dietary protease supplementation on broiler physiology is essential for optimizing feed formulations and maximizing production efficiency. In recent years, various studies reported that the addition of synthetic amino acids in low-protein diets enhanced crude protein digestibility in chickens without any negative effects on production, carcass traits and lowering nitrogen emission (Amer *et al*., 2021). Supplementation of synthetic amino acids in the avian diet improves the efficacy of protein digestibility and reduces the cost of feed by adopting the ideal protein concept (Lemme, 2003).

Moreover, supplementing phytase mitigates the negative effects on growth performance, bone mineralization, and apparent ileal digestibility of Phosphorus and phytate after the coccidiosis vaccine (Shi *et al*., 2022). Additionally, supplementing exogenous enzymes in poultry diet is essential for the higher availability of nutrients, removing anti-nutritional components such as trypsin inhibitors and lectins present in the feedstuffs, lower feed cost and minimum nitrogen emission in the environment (Velazquez-De *et al*., 2021; McCafferty *et al*., 2022). Besides, another practical technique to elevate protein digestion in commercial feeds is by the addition of protease enzymes (Park *et al*., 2020; Shad *et al*., 2022; Vieira *et al*., 2023). Absorption of proteins in poultry birds depends on the release of protein enzymes endogenously in the digestive tract that break down proteins into peptides and amino acids for further utilization (Recoules *et al*., 2017). Endogenous proteases are produced by the animals and their levels are not adequate for effective proteins utilization, especially in young birds. Therefore, digestive complications have been observed in grower and finisher diets in broilers due to overfeed consumption (Jabbar *et al*., 2021). Dietary protease inclusions may increase gut integrity and protein utilization and result in higher production performance of broiler chickens (Cowieson *et al*., 2018; Law *et al*., 2018). Supplementing proteases in feed produce some variations in microbial prosperity, variety and manage to alter some Bacteroidetes taxa. The microbes are beneficial because they create beneficial metabolites, such as short-chain fatty acids and these are associated with reduction of gut inflammation (Jeferson *et al*., 2020). Commercially available protease enzymes can survive and function at consistent digestive tract pH and support digestive abilities after heat treatments at different temperatures generally used during the feed process at the factory (Ravindran, 2013).

Therefore, the present study aims to comprehensively assess the effects of dietary protease supplementation alongside a low amino acid diet on various aspects of broiler chicken physiology that includes, growth performance, gut enzyme activity, immune responses, meat quality attributes, and bone morphometry.

MATERIALS AND METHODS

Study Area

The current study was conducted at the poultry experimental station, Department of Poultry Husbandry, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam, Pakistan. All protocols were followed according to the Experimental Animal Care Committee, approved by the Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam, Pakistan.

Management and Housing

A total of 600-hundred-day old Ross 308 chicks (initial weight 40 ± 1 g) were purchased from a commercial hatchery. Chicks were initially weighed and randomly distributed into 5 dietary treatments; each treatment consisted of 6 replicates (20 birds/pen). The experiment continued for 35 days. The birds were reared under the semi-heated shed, in floor pens with rice husk as litter material and equipped with one drinker and one feeder in each pen throughout the experimental period. The initial temperature was kept at 35°C±1 during brooding and gradually brought to normal temperature at 24°C±1. The lighting program consisted of 22 hours of light and 2 hours of darkness throughout the study period. Feed and water were given *ad libitum* throughout the experiment. Chicks were given feed and water *ad libitum* throughout the trial period. The birds were provided with starter feed (1-21 days) and finisher feed (22-35 d). A complete randomized design was used in this research trial.

Experimental Diets

The chicks were distributed into five dietary treatments: (1) Positive Control (PC), a basal diet formulated to meet Ross 308 requirements; (2) Negative Control 1 (NC-1), with 2%

Table 1: Ingredients and basal diet composition (kg/100kg)*.

Ingredients**	Starter			Finisher		
	PC	NC-2 % low AA	NC-4% low AA	PC	NC-2 % low AA	NC-4% low AA
Corn	59.61	59.115	58.83	72.59	72.18	71.79
SBM	26.95	27.52	27.87	15.65	16.1	16.57
Fish meal	3	3	3	3	3	3
PBM	2	2	2	2	2	2
RSM	3	3	3	3	3	3
Poultry oil	1.75	1.9	2	1.1	1.22	1.34
Choline	0.2	0.2	0.2	0.2	0.2	0.2
Soda	0.15	0.15	0.15	0.14	0.14	0.14
Salt	0.15	0.15	0.15	0.17	0.17	0.17
Phytase	0.01	0.01	0.01	0.01	0.01	0.01
Vit. Premix	0.05	0.05	0.05	0.05	0.05	0.05
Min. Premix	0.05	0.05	0.05	0.05	0.05	0.05
limestone	0.8	0.8	0.8	0.6	0.6	0.6
DCP	0.92	0.92	0.92	0.01	0.01	0.01
DLM	0.36	0.34	0.32	0.31	0.29	0.26
Lysine SO4	0.62	0.54	0.47	0.62	0.57	0.51
L Arginine	0.05	0.01	0	0.15	0.12	0.08
L Threonine	0.16	0.135	0.11	0.14	0.12	0.1
L Valine	0.08	0.05	0.03	0.09	0.07	0.05
L Isoleucine	0.09	0.06	0.04	0.12	0.1	0.07
Total	100	100	100	100	100	100
Calculated values of diet						
ME	2975			3100		
CP	22			18		
Ca	0.95			0.65		
AvP	0.5			0.36		
Dig. Lysine	1.26			1.02		
Dig. Met	0.53			0.45		
Met+Cys	0.96			0.82		
Thre	0.84			0.68		
Val	0.96			0.8		
Iso-leuc	0.84			0.7		
Tryp	0.2			0.16		
Arg	1.31			1.09		

***PC:** Positive control; **A.A:** Amino acid. ** **SBM:** Soybean Meal; PBM: Poultry By-product Meal; **RSM:** Rapeseed Meal; **DCP:** Dicalcium Phosphate; **DLM:** DL-Methionine; **ME:** Metabolizable Energy; **CP:** Crude Protein; **Ca:** Calcium; **AvP:** Available Phosphorus; **Thre:** Threonine; **Val:** Valine; **Tryp:** Tryptophan; **Arg:** Arginine.

less digestible amino acids; (3) Negative Control 2 (NC-2), with 4% less digestible amino acids; (4) NC-1 supplemented with protease at a dose of 200 g/ton; and (5) NC-2 supplemented with protease at a dose of 200 g/ton. A mono-component serine protease (Ronozyme® ProAct CT, Royal DSM, Turkey) was used in the dietary treatments. No matrix values were applied to the supplemental protease. The product contains 75,000 protease units per gram of enzyme. Ronozyme® ProAct CT is produced by *Bacillus licheniformis* through fermentation, incorporating a gene transcribed from *Nocardiopsis prasina*, and is classified as a mono-component protease.

The enzyme activity is defined as the amount of enzyme required to release 1 μmol of p-nitroaniline per minute from 1 μmol of the substrate Suc-Ala-Ala-Pro-Phe-p-nitroanilide at 37°C and pH 9.0. The feed formulations for all dietary treatments were presented in Table 1.

Production Performance

To compute production performance, body weight gain was measured by using a digital balance machine on days 0, 21 and 35. Meanwhile, the amount of feed intake and feed refusal was recorded and feed efficiency was calculated by feed consumed divided by weight gain.

Digestive Enzymatic Activity

On the completion of the experiment 5 chickens were chosen from each group, weighed and slaughtered to measure the digestive enzymatic activities (pepsin, lipase, amylase, and trypsin). For the determination of digestive enzymatic activities samples of jejunum digesta were collected. The digesta samples were collected aseptically in screw-caped sterile sample tubes and kept at -20 °C for further procedure. Samples were thawed at room temperature and homogenized (1:9, wt/vol) with ice-cold 154 mmol/L sodium chloride solution, then centrifuged at 1500 rpm for 15 minutes at 4 °C. Aliquot of the supernatants were carried out for the measurement of enzymatic activity assay. All measurements were collected in duplicate. A microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA) was used to evaluate the digestive enzyme activities by using different diagnostic kits (Shanghai Changjin, Biotechnology Co., Ltd., Shanghai, China) according to the direction of the manufacturer. Enzymatic activities were shown as units/gram from digesta of jejunal samples. Activity of amylase enzyme described as the quantity of enzyme that hydrolyses 1 mg substrate/3 minutes at pH 6.9. Lipase activity was measured per volume of enzyme which hydrolyses 1 μmol of olive oil/min. Trypsin activity was determined as the ratio of enzyme that caused an increase 0.003 in absorbance of product/min at 253 nm.

Meat Quality Parameters

On the 35th day of trial, 3 broilers from each replicate were selected, weighed, and slaughtered for the evaluation of meat quality parameters. Samples of thigh and breast meat were collected from the birds. The dry matter, protein, fat, and ash of meat samples were analyzed by the procedure of AOAC (2007). A 10 grams of meat sample was mixed with 90 ml of distilled water to standardize the sample for 30 s using a blender (400 Lab Blender, Seward, UK). The pH level of meat was assessed by the pH meter (pH-2004; Selecta, Barcellona, Spain) as earlier defined by (Liao *et al*., 2018). The water-holding capacity of meat was estimated by the procedure used by Delezie *et al*. (2007). The meat samples from each group were kept in a centrifugation tube with filter units, heated for 10 minutes at 90°C then cooled for 15 minutes. The samples were centrifuged (Eppendorf Centrifuge 5804R, Taufkirchen, Germany) at 4°C for 10 minutes at 2000 rpm. Water holding capacity values were estimated as the quantity of water kept after the initial sample weight. Cooking loss of meat was calculated by the modified procedure of Saleh *et al*. (2019). Breast and thigh meat cuts were weighed and kept in a polypropylene bag, cooked for 30 minutes at 85 °C in a water bath then cooled at room temperature for 25 minutes. Cooking loss refers to the loss of weight during the cooking process. The color of the meat was measured by a Minolta colorguide (BYK-Gardener GmbH, Geretsried, Germany). Meat color was assessed as L* (lightness) a* (redness) and b* (yellowness).

Immunoglobulins and Thyroid Hormones

At the end of the research study, two broilers per replicate were chosen for blood collection. Samples of blood were obtained from the wing vein into EDTA tubes. Blood samples were placed at room temperature for coagulation. The serum was obtained from the blood samples through centrifuge at 2500 rpm for 18 minutes then transferred into Eppendorf tubes and stored at -20 °C till further examination. Serum IgA, IgG and IgM levels were analyzed by applying commercial kits (Nanjing Bioengineering Research Institute. Co. Ltd, Nanjing, China) according to manufacturer instructions. The serum level of thyroid hormones was evaluated by using different diagnostic kits (My BioSource, Inc., San Diego, CA AMS Biotechnology Ltd, UK) according to the directions of the manufacturer.

Bone Morphometry

On the 35th day of the trial, 2 broilers from each replicate were randomly selected, weighed, and slaughtered to measure bone parameters. After slaughtering the legs were isolated and de-boned to get tibia bones. After that the tibial bones were de-fatted, dried, and kept at -20 °C for further processing. For the calculation of bone traits, the tibial bones were boiled in deionized water for 12 minutes to clear sticky tissues and weight, length, and width were determined by using a digital caliper (Mitutoyo, Japan). After that samples were crushed and kept in a muffle furnace at a temperature of 600 °C for 4 hours to obtain the ash contents. After that ash was evaluated for bone mineralization i.e., Calcium (Ca) and phosphorus (P) through atomic absorption and spectrophotometer. Tibial bone Ca, P, and ash contents were evaluated by the procedure of AOAC (2019). The breaking strength of the tibial bone was assessed after thawing the bones at room temperature. The bones were thawed at room temperature to measure the tibia bone-breaking strength. The tibia bones were kept in the epiphysis region. A force load of 200 kg was applied at a speed of 5 mm/s to the central area of each bone using the TA-TPS probe with a CT3 Texture Analyzer (AMETEK Brookfield, Middleboro, Massachusetts, USA).

Statistical Analysis

The collected data was statistically analyzed by ANOVA using SPSS Statistics 19.0 software. The significant differences among means of treatments were compared by Tukey's test and statistical difference was set at ($P<0.05$).

Table 2: Effect of protease supplementation with low amino acids diets on the growth performance of broiler chickens.

Parameters	Groups					S.E	P. value
	PC	NC-2% low A.A	NC-4% low A.A	NC-2% low A.A+prot	NC-4% low A.A+prot		
Starter period (0-21 d)							
FI (g)	1076.84	1058.93	1061.33	1083.37	1088.33	8.58	0.206
BW (g)	773.44[a]	726.72[b]	722.62[b]	780.86[a]	765.82[a]	9.24	0.027
FCR	1.39[b]	1.45[a]	1.46[a]	1.38[b]	1.42[a]	0.012	0.005
Finisher period (22-35 d)							
FI (g)	2097.64	2136.31	2145.32	2104.28	2127.55	18.103	0.163
BW (g)	1131.76[a]	1069.84[b]	1085.65[b]	1138.87[a]	1128.23[a]	10.23	0.018
FCR	1.85[b]	1.99[a]	1.97[a]	1.84[b]	1.88[b]	0.022	0.005
Overall (0-35 d)							
FI (g)	3174.48	3195.24	3206.65	3187.65	3215.88	26.35	0.296
BW (g)	1905.2[a]	1796.56[b]	1808.27[b]	1919.73[a]	1894.05[a]	17.94	0.022
FCR	1.66[b]	1.778[a]	1.773[a]	1.64[b]	1.69[ab]	0.017	0.007

Positive control (PC), negative control (NC), amino acids (AA), protease (prot), feed intake (FI) body weight (BW), feed conversion ratio (FCR). [a,b,c] with different superscripts in the same row were significantly (p<0.05) different.

Table 3: Effect of protease supplementation with low amino acids diets on the immunoglobulins (mg/dL), thyroid hormones (ng/dl), and digestive enzymes (U/mg) of broiler chickens.

Parameters	Groups					S.E	P. value
	PC	NC-2% low A.A	NC-4% low A.A	NC-2% low A.A+prot	NC-4% low A.A+prot		
IgM	93.57[ab]	85.86[b]	80.71[b]	104.66[a]	97.14[a]	2.08	0.047
IgA	80.88[a]	68.03[b]	67.27[b]	80.45[a]	78.23[a]	2.11	0.031
IgG	357.45	344.44	341.92	353.7	350.28	6.84	0.086
T_3	6.93	6.81	7.02	7.08	6.87	0.541	0.998
T_4	2.12	1.97	2.03	1.95	2.08	0.049	0.985
Amylase	147.29	145.23	141.16	138.82	137.5	4.64	0.633
Lipase	419.81	392.02	384.36	431.25	426.11	5.79	0.267
Trypsin	451.93[ab]	391.83[b]	403.48[b]	521.44[a]	503.84[a]	6.91	0.005
Protease	17.93[ab]	13.43[b]	13.21[b]	20.56[a]	19.43[a]	0.37	0.036

Positive control (PC), negative control (NC), amino acids (AA), protease (prot), triiodothyronine (T3), thyroxine (T4). [a,b,c] with different superscripts in the same row were significantly (p<0.05) different.

RESULTS

Growth Performance

The effects of protease supplementation with low amino acid diets are illustrated in Table 2. The birds fed with protease-supplemented diet significantly (P<0.05) increased body weight and feed gain ratio as compared to negative control groups. However, a non-significant difference was reported between supplemented and positive control group. Additionally, dietary protease enzyme supplementation didn't show a significant impact on feed intake among the groups.

Immunoglobulins, Thyroid Hormones and Digestive Enzymes

The results of immunoglobulins, thyroid hormones and digestive enzymes are given in Table 3. There was a significant (P<0.05) influence of protease supplementation on IgA and IgM as compared to non-supplemented groups. However, triiodothyronine (T_3) and thyroxine hormone (T_4) were not affected by the dietary addition of protease enzyme. Trypsin and protease activity was significantly (P<0.05) decreased in the low amino acids supplemented groups as compared to protease treated groups and PC group.

Meat Quality

Table 4 shows the effect of protease enzyme supplementation on meat quality parameters. Statistical analysis indicated that moisture, fat and ash of meat were not significantly influenced by the dietary addition of protease enzyme except crude protein as compared to other treated groups. However, pH, water holding capacity, and cooking loss of meat showed significant differences (P<0.05) among

Table 4: Effect of protease supplementation with low amino acids diets on the proximate analysis of meat and meat quality traits of broiler chickens.

Parameters	Groups					S.E	P. value
	PC	NC-2% low A.A	NC-4% low A.A	NC-2% low A.A+prot	NC-4% low A.A+prot		
Moisture	74.21	70.04	69.82	74.89	74.56	0.85	0.085
CP	20.07[ab]	19.87[b]	19.69[b]	20.87[a]	21.13[a]	0.16	0.047
EE	3.62	3.48	3.34	3.23	3.17	0.09	0.73
Ash	2.23	2.205	2.19	2.17	2.14	0.13	0.993
pH	5.87[ab]	5.63[b]	5.58[b]	6.13[a]	6.06[a]	0.07	0.048
WHC	43.87[ab]	38.86[b]	39.07[b]	44.68[a]	44.32[a]	0.21	0.023
DL	5.06	5.31	5.24	4.59	4.67	0.08	0.041
CL	22.31[ab]	25.57[a]	25.96[a]	20.67[b]	20.23[b]	0.72	0.036
L*	53.72	52.93	53.27	54.06	54.32	1.26	0.25
b*	11.45	11.38	10.87	10.94	11.16	0.62	0.63
a*	3.02	2.86	2.77	2.94	2.73	0.35	0.18

Positive control (PC), negative control (NC), amino acids (AA), protease (prot), crude protein (CP), ether extract (EE), water holding capacity (WHC), drip loss (DL), cooking loss (CL) lightness (L*) yellowness (a*), redness (b*). [a,b,c] with different superscripts in the same row were significantly ($p<0.05$) different.

Table 5: Effect of protease supplementation with low amino acids diets on the bone morphometry of broiler chickens.

Parameters	Groups					S.E	P. value
	PC	NC-2% low A.A	NC-4% low A.A	NC-2% low A.A +prot	NC-4% low A.A + prot		
TWT (g)	10.64	10.26	10.18	10.59	10.47	0.257	0.164
TL (mm)	88.26	84.11	82.83	86.96	87.46	1.328	0.089
TW (mm)	10.02	9.31	9.37	9.88	9.83	0.297	0.187
TBS (kg)	19.18[ab]	17.64[b]	17.31[b]	20.87[a]	21.23[a]	0.434	0.038
Ash%	45.26	45.45	43.88	47.51	46.47	1.126	0.091
Ca%	16.48	15.63	15.44	17.11	17.21	0.236	0.075
P%	7.93	7.63	7.75	8.15	8.26	0.302	0.096

Positive control (PC), negative control (NC), amino acids (AA), protease (prot), tibia weight (TWT), tibia length (TL), tibia width (TW), tibia breaking strength (TBS), calcium (Ca), phosphorus (P). [a,b,c] with different superscripts in the same row were significantly ($p<0.05$) different.

the protease supplemented and low amino acids treated groups. Moreover, protease supplementation revealed no impact on the drip loss and meat color among the treatments.

Bone Morphometry

The findings of bone morphometry indices are given in Table 5. Results showed that dietary protease added groups showed non-significant effects on weight, length, and width of tibial bone. However, the breaking strength of tibial bone was significantly ($P<0.05$) enhanced by the protease-supplemented groups when compared to negative control groups. Moreover, calcium, phosphorus and ash contents of tibial bone also revealed non-significant ($P<0.05$) effects among the groups.

DISCUSSION

Dietary proteins have a vital role in the development of the digestive system and production performance. They are mostly present in the soybean meal, which is the costlier ingredient in the food. Therefore, it is essential to maximize the digestion and absorption of soybean meal particles in the intestine by enhancing intestinal activity through exogenous enzyme supplementation. Moreover, digestive performance depends on the development of intestine which is less developed in the early weeks than at the late stage in chicken and it requires supporting enzymes in the form of supplements to boost digestion and absorption (Pirzado *et al*., 2020; Soomro *et al*., 2024). It has been reported that supplemented enzymes may affect immunity, bone morphometry and other physiological aspects of chicks.

According to Saleh *et al.* (2020) digestibility of dietary proteins enhanced by protease enzyme supplementation results in better growth performance and Ndazigaruye *et al.* (2019) confirmed that the addition of protease enzyme during the starter phase leads to enhanced production performance of chicken. A study associated with enzyme efficacy in corn-soya meal-based diets indicated that specific enzyme activities can improve nutrient absorption in the digestive system of broiler chickens (Kocher *et al.*, 2000). Studies have shown that the addition of proteases results in better production and enhanced CP digestibility (Moftakharzadeh *et al.*, 2017; Saleh *et al.*, 2019). In the present study, we have reported an increase in growth performance and FCR after protease enzyme supplementation. Thus, our findings are consistent with the results of various studies (Xu *et al.*, 2017; Aderibigbe *et al.*, 2020; Jabbar *et al.*, 2021; McCafferty *et al.*, 2022) indicating supplementing proteases enhance the production performance of broilers. This might occur because during the early stages of broiler life endogenous enzymatic activities and the gut microbiota in the digestive tract may not be fully developed, potentially indicating a lack of substantial growth response in chickens and the addition of enzymes have provoked greater outcomes in the amino acid deficient diets. A possible reason for this may be that the amount of amino acid reduction in the negative control diet could have possibly provoked greater outcomes to enzyme addition, thereby improving the performance. Additionally, supplementation of enzymes may regulate the hormonal signals related to digestion and result in better nutrient utilization. Cholecystokinin (CCK) contents were increased in the intestine by the addition of enzymes in the diet. The release of digestive enzymes and bile from the pancreas into the intestinal lumen is regulated by the hormone known as cholecystokinin and the release of CCK is controlled by dietary proteins (Cahu *et al.*, 2004; Jiang *et al.*, 2014). Cholecystokinin plays an important role in the digestive process by modulating pancreatic exocrine release and enhancing the production of crucial digestive enzymes such as trypsin, chymotrypsin, and amylase.

Gut health and nutrient digestibility might be influenced by enzymatic activities in the digestive tract (Petterson and Aman, 1989). Walk *et al.* (2018) stated that adequate protease levels in the feed are beneficial for a healthy gut system because protease enzymes regulate the gut microbiota and activities of intestinal digestive enzymes in poultry. Dietary supplementation of proteases enhanced the trypsin activities and increased nutrient utilization by promoting chickens' digestive and absorptive function, consequently better production performance of poultry birds. Indeed, the enhancement of trypsin activities proposes that a maximum level of proteins is available for absorption by using exogenous protease enzymes in poultry diets. According to Mirzaie *et al.* (2012) the activity of intestinal enzymes depends on the origin of dietary ingredients and quantity or quality of anti-nutritional factors. Current results are in line with the outcomes of (Enberg *et al.*, 2004; Wang *et al.*, 2008) they noted that the inclusion of multi-enzymes potentially increased trypsin, chymotrypsin, protease, lipase and amylase activities in pancreas and duodenal contents.

The digestive process mainly depends on the activities of endogenous enzymes. Thus, it may be supposed that the improvement in pancreatic enzymes and pepsin activity could be linked with the increase in nutrient digestibility (Pubols, 1991; Hubener *et al.*, 2002).

Various research reports demonstrated that different proteases (i.e., alkaline, neutral, and acid) have vigorous roles by promoting amino acid absorption, regulating gut integrity, and enhancing digestive enzyme activities (Stefanello *et al.*, 2016; Cowieson *et al.*, 2018). Recent findings are in align with the results of (Yuan *et al.*, 2017; Alagawany *et al.*, 2017; Jiang *et al.*, 2020; Yaqoob *et al.*, 2022), who indicated that digestive enzymatic activities were linearly improved by the addition of enzymes in the broiler diet.

Microbial protease supplementation has no significant impact on the proximate analysis of meat except crude protein. These findings are supported by (Sarica *et al.*, 2020; Lee *et al.*, 2023) who claimed that dietary protease addition enhanced crude protein levels in broiler meat. In the poultry industry meat quality is a significant aspect that is mostly valued by the producer and consumer. Poultry meat possesses sensory characteristics, which are crucial for consumer acceptance. The results of the present study observed that the addition of protease enzymes in the diet increased meat pH, and water holding capacity and reduced drip loss and cooking loss of chicken meat. The outcomes of present study align with those (Calvo *et al.*, 2017; Saleh *et al.*, 2020; Lee *et al.*, 2023; Li *et al.*, 2023) observed that proteases improved pH value, water holding capacity and reduced the drip loss of breast meat. On the contrary (Dalolio *et al.*, 2015; Habib *et al.*, 2016; Hussein *et al.*, 2020; Duque-Ramirez *et al.*, 2023) they didn't find a significant impact of enzymes on meat characteristics. Meat pH shows a direct impact on water holding capacity i.e., meat with high pH has more water holding capacity than low pH and higher meat pH is also associated with lower drip loss. Cooking yield depends on cooking loss, while the reduction in cooking loss enhances water holding capacity of meat. Water holding capacity relates to the capacity of meat to hold its water when external force is applied and is the main indicator of meat juiciness (Omojola *et al.*, 2014). This is ascribed to the hydrolyzing activities of proteases. Meat quality may be improved due to the microbial proteases from non-pathogenic microbes (Alaa *et al.*, 2014).

Neves *et al.* (2014) stated that meat quality parameters are directly associated with the environmental and managemental conditions, mostly the feed given. Meat color was not influenced by the dietary protease supplementation. These findings agreed with (Wang *et al.*, 2022; Oyeagu *et al.*, 2023; Duque-Ramirez *et al.*, 2023) they reported no impact of enzymes on meat color.

The results of the current trial also indicated that protease enzyme didn't change weight, length, width, calcium, phosphorus, and ash contents of the tibia bone. However, protease enzyme supplementation enhanced the breaking strength of tibial bone. These outcomes are consistent with the previous findings of (Gautier *et al.*, 2018; Al-Qahtani *et al.*, 2021; Broch *et al.*, 2021; Hakamai *et al.*, 2022). Additionally, Abdulla *et al.* (2017) showed significantly higher breaking strength of bone by using enzymes in poultry diets. Protease enzyme implied their beneficial impact on breaking strength of tibia bone might be due to altering the bone matrixes rather than mineralization. Concurrently, recent results disagree with (Zeng *et al.*, 2015; Abu-Tayyeb *et al.*, 2019; Rahimi *et al.*, 2020; Hafeez *et al.*, 2021) they indicated that phytase and multi-enzyme inclusion in broilers feed significantly enhance the tibia weight, ash and mineral contents.

In the present study protease enzyme addition significantly affects blood immunoglobulins such as, IgM and IgA. Similar results were observed by Jiang *et al.* (2020), Amir *et al.* (2021), Asare *et al.* (2022) they indicated that enzyme supplementation increased blood immunoglobulins levels in broilers. Moreover, Attia *et al.* (2020) noted that multienzyme supplementation enhanced chickens' immunity by increasing blood immunoglobulin concentration. The expression and production of blood immunoglobulins have an essential role in the health of any organism (Qiu *et al.*, 2023). The higher concentration of IgM and IgA supplemented with protease enzyme suggests a potential improvement in the bird's capability to combat the pathogens. The increased blood IgA and IgM of broiler chickens in the current study may be linked with the immunomodulatory properties of protease. Therefore, the immune response stimulated by exogenous enzymes may be interlinked with dietary nutrient breakdown, which alters the internal organs weight and intestinal characteristics to regulate the gut microbiota to improve nutrient absorption which promotes the immunity of the bird (Cowieson *et al.*, 2019; Attia *et al.*, 2020). The inclusion of protease enzymes did not indicate a significant influence on thyroid (i.e., T3 and T4) hormones. Our results are collaborating with the (Shi *et al.*, 2018) who stated that enzyme supplementation had non-significant impact on blood thyroid hormones and metabolic hormones. There is limited information regarding the effects of enzyme addiction on the thyroid hormone concertation in poultry birds.

CONCLUSIONS AND RECOMMENDATIONS

It is concluded that exogenous enzyme protease significantly increased body weight and feed conversion ratio in the amino acid deficient group in broiler chickens. Moreover, protease supplementation significantly affected immunoglobulin IgA and IgM concentrations without disturbing thyroid hormone, i.e. triiodothyronine (T3) and thyroxine (T4). Additionally, breaking strength of tibial bone was significantly improved in protease supplemented group without any significant change in bone morphometry and calcium and phosphorus contents. Besides, protease supplementation showed significant change in meat quality parameters including pH, water holding capacity and cooking loss in broilers.

ACKNOWLEDGEMENTS

The authors would like to thank Sindh Agriculture University Tandojam for providing research facilities.

NOVELTY STATEMENT

This study is the first to comprehensively assess dietary protease supplementation with low amino acid diets in broilers, focusing on growth, gut enzymes, immunity, meat quality, and bone morphometry. It provides new insights into sustainable feed strategies while ensuring bird health and performance.

AUTHOR'S CONTRIBUTIONS

Imdad Hussain Leghari conceptualized the idea, Muhammad Zakria Pirzado implemented the research design in his experiments, Nasir Rajput and Saeed Ahmed Soomro helped in writing the manuscripts and Dr Shoaib Ahmed Pirzado helped in feed formulation, and statistical analysis.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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