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Matrix-Dosed Protease Supplementation Enhances Growth Performance, Nutrient Utilization, and Economic Return in Broiler Chickens

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Abstract

This study evaluated the effects of matrix-dosed protease supplementation on growth performance, nutrient utilization, intestinal morphology, serum biochemistry, carcass traits, and economic return in broiler chickens. A total of 240 Cobb 500 chicks were assigned to six dietary treatments (T0–T5) with four replicates of 10 birds each for 33 days. Protease supplementation, particularly with protease F at 250 g/tonne (T5), significantly increased body weight at day 7 (163.0 ± 1.4 g; $p = 0.002$) and day 21 (854.0 ± 7.0 g; $p = 0.014$), and improved the feed conversion ratio at day 33 (1.54 ± 0.01; $p = 0.002$). Birds in the T5 group consistently exhibited the highest serum total protein ($p < 0.001$ on Day 21; $p = 0.002$ on Day 33), albumin ($p < 0.001$ on both days), and creatinine ($p < 0.001$ on Day 21; $p = 0.006$ on Day 33), along with reduced low-density lipoprotein (LDL) levels ($p < 0.001$ on Day 21; $p = 0.002$ on Day 33). Intestinal morphology was also enhanced, with villus height increasing to 874.0 ± 1.0 µm at day 21 and 931.0 ± 1.0 µm at day 33, accompanied by greater villus height-to-crypt depth ratios (11.23 ± 0.02 and 12.59 ± 0.01, respectively; $p < 0.001$). Moreover, apparent ileal digestibility of dry matter, crude protein, metabolizable energy, and amino acids were improved in T5 compared with the control and other treatments. Economic analysis showed the highest profit and return on investment (7.01%) in T5, followed by T4 and T2. These findings indicate that matrix-based protease supplementation enhances growth, nutrient absorption, and gut morphology while delivering substantial economic benefits, making it a cost-effective strategy for improving broiler productivity and profitability in commercial production systems.

Keywords: serum biochemistry; feed conversion ratio; villus height; ileal digestibility; return on investment

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1. Introduction

Global demand for poultry has driven a 1.2% increase in global animal feed production, reaching approximately 1.4 billion metric tonnes in 2025 [1]. Feed accounts for 70–80% of total poultry production costs, with protein ingredients, particularly soybean meal, representing the largest share [2,3]. Soybean meal remains the preferred protein source due to its high nutritional quality, which can be further improved through modern processing and enzyme supplementation [4].

Given that dietary protein is both essential for broiler growth and the most expensive feed component, protease supplementation has gained attention as a strategy to enhance amino acid digestibility, maintain growth performance, lower feed costs, and reduce nitrogen excretion [5]. Adequate dietary amino acids, such as glutamate, glutamine, aspartate, arginine, glycine, methionine, cysteine, and threonine, are critical for maintaining intestinal integrity, mucin synthesis, immune and antioxidative responses, and microbial balance, thereby supporting gut health and nutrient utilization [6]. For example, a *Bacillus subtilis*-derived protease has been shown to improve nitrogen and crude protein digestibility, increasing amino acid availability and growth in broilers fed low-protein, condensed distillers dried grains with solubles-based diets, without adverse effects on carcass traits, energy utilization, blood biochemistry, or gut morphology [7]. However, rising soybean meal prices, driven by climate change and trade disruptions, have intensified the search for alternative protein sources and enzyme technologies to sustain poultry productivity. Enzyme supplementation enhances the nutritional value of soybean meal feed efficiency and rising costs [8].

Exogenous proteases are widely used to improve protein utilization, allow dietary protein reduction without compromising performance, and lower nitrogen and ammonia emissions, offering both economic and environmental benefits [9]. Studies have shown that protease supplementation increases metabolizable energy and amino acid digestibility in broiler and swine [10,11]. Combined phytase and protease supplementation further enhances growth, energy utilization, and amino acid digestibility in nutrient-restricted diets [12]. Commercial protease products have been reported to improve feed conversion, ileal digestible protein, dry matter digestibility, and energy utilization, effectively compensating for reduced dietary lysine and energy [13]. Nevertheless, enzyme efficacy depends on feed composition, emphasizing the need for tailored enzyme applications. Protease supplementation generally improves fat and CP digestibility and metabolizable energy across broiler diets, independent of protein or energy levels [14]. Protease use also contributes to environmental sustainability by reducing CO_2 emissions from soybean cultivation and mitigating nitrogen-related eutrophication and acidification [15].

In addition to improving nutrient utilization, protease supplementation enhances gut morphology, as indicated by increased villus height, reduced epithelial thickness, and improved ileal amino acid digestibility, collectively supporting feed efficiency [16,17]. However, excessively high protease doses may impair growth performance and nitrogen digestibility, highlighting the importance of dose optimization [18,19]. Inconsistent outcomes across studies are often linked to failure in integrating enzyme matrix values during diet formulation, leading to inaccurate nutrient supply estimates [20]. Matrix-dosed protease supplementation offers a more precise strategy to reduce reliance on costly protein ingredients like soybean meal.

Despite the well-documented benefits of protease supplementation in broiler nutrition, most existing studies have evaluated single commercial products in isolation, often under non-standardized or on-top supplementation strategies. Consequently, there remains a lack of comparative evidence on how different protease formulations, particularly standard versus next-generation enzyme blends, perform when applied under a matrix-dosed design

that accounts for nutrient release values. Matrix-dosed supplementation is of growing practical interest, as it allows feed cost reduction while maintaining performance; however, its success depends on the specific efficacy of the enzyme formulation used. Therefore, this study aims to evaluate the impact of protease supplementation as a partial substitute for soybean meal on growth metrics, nutrient absorption, intestinal structure, blood biochemical parameters, carcass traits, and overall economic viability in broiler chickens.

2. Materials and Methods

2.1. Ethical Statement

All animal care and experimental procedures complied with institutional animal welfare regulations. The protocol was reviewed and approved by the Animal Experimentation Ethics Committee, Faculty of Veterinary, Animal, and Biomedical Sciences, Khulna Agricultural University, Bangladesh (Approval No. AEEC/KAU/2024-1005).

2.2. Experimental Animals and Design

This study used 240 one-day-old male Cobb 500 broiler chicks, with an average initial body weight of 45.54 $\pm$ 3.67 g, sourced from Nourish Poultry and Hatchery Limited, Jhenaidah, Bangladesh. Chicks were reared under standard commercial hatchery conditions prior to transfer, where they received routine vaccination and brooding care in environmentally controlled facilities until collection. Following sex determination at hatch, the male chicks were uniformly distributed into six dietary treatment groups.

Protease X (OM Biopharma, Bangladesh) is a heat-stable serine protease blend containing acidic, neutral, and alkaline components, with an activity level of 1,000,000 units per gram. It is produced through submerged fermentation utilizing *Bacillus licheniformis* as the microbial source.

Protease A is a thermostable multi-protease formulation incorporating a serine protease characterized by the catalytic triad His57, Ser195, and Asp102. This advanced enzyme system facilitates rapid and efficient peptide bond cleavage, resulting in accelerated protein hydrolysis and improved protein utilization in poultry diets.

Protease F is a specialized protease designed for pelleted feed applications, formulated to withstand thermal processing while promoting rapid protein hydrolysis. It enhances nutrient digestibility and gut health, supporting cost-effective incorporation of alternative protein sources without compromising growth performance or feed efficiency.

Compared with protease X, both protease A and protease F exhibit faster protein hydrolysis rates due to their optimized catalytic activity and broader substrate specificity, enabling superior nutrient release and utilization under commercial feeding conditions.

Each treatment group (T0 to T5) consisted of four replicates, with 10 chicks per replicate, totaling 40 birds per group. The dietary treatments were as follows: T0: basal diet without protease supplementation; T1: basal diet plus 250 g/tonne of protease X as a top-up; T2: basal diet supplemented with 350 g/tonne of protease X including matrix values; T3: basal diet plus 150 g/tonne of protease A as a top-up; T4: basal diet with 250 g/tonne of protease A including matrix; and T5: basal diet supplemented with 250 g/tonne of protease F including matrix.

The experiment was conducted in two phases: the starter phase (day 0 to 14) and the grower phase (day 15 to 33). The birds were kept in environmentally controlled enclosures, each measuring 1.2 square meters and bedded with rice husk litter. The initial brooding temperature was set at 33 °C and then decreased by 2 °C each week, reaching 24 °C by the end of the trial on day 33.

2.3. Experimental Diet

Proteases were added either as top-up (direct inclusion without nutrient adjustment) or matrix (nutrient adjustments equivalent to enzyme nutrient release). Diets were pelleted for uniform intake (starter: 2 mm × 3 mm; grower: 3 mm × 5 mm) and offered ad libitum along with fresh water. The experimental feed formulations were designed to meet the nutrient requirements for broilers as specified by the Bureau of Indian Standards (BIS, 2007). The proximate composition of the basal diet, including dry matter (DM), crude protein (CP), crude fiber (CF), ether extract (EE), and total ash, was determined following the official procedures outlined by the Association of Official Analytical Chemists (AOAC, 1992). Ingredient and nutrient profiles are provided in Tables 1 and 2 and are expressed on an as-fed basis.

Table 1. Ingredient composition of experimental diets (0–14 days).

Ingredients, %	Starter (d 0–14)					
	T0	**T1**	**T2**	**T3**	**T4**	**T5**
Maize (12% M)	55.20	55.20	56.20	55.20	56.20	56.20
Soybean meal (46%)	29.00	29.00	27.50	29.00	27.50	27.50
Corn gluten meal (58%)	2.00	2.00	2.00	2.00	2.00	2.00
Poultry meal (51%)	4.00	4.00	4.00	4.00	4.00	4.00
Rice polish	3.40	3.40	3.90	3.40	3.90	3.90
Soybean oil	2.00	2.00	2.00	2.00	2.00	2.00
Limestone powder	1.20	1.20	1.20	1.20	1.20	1.20
Di-calcium phosphate	1.01	1.01	1.01	1.01	1.01	1.01
Salt	0.23	0.23	0.23	0.23	0.23	0.23
Broiler vitamins	0.10	0.10	0.10	0.10	0.10	0.10
Organic acids powder	0.20	0.20	0.20	0.20	0.20	0.20
DL methionine	0.40	0.40	0.40	0.40	0.40	0.40
L lysine	0.34	0.34	0.34	0.34	0.34	0.34
Broiler minerals	0.10	0.10	0.10	0.10	0.10	0.10
L threonine	0.10	0.10	0.10	0.10	0.10	0.10
Sodium bicarbonate	0.26	0.24	0.23	0.24	0.24	0.24
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07
Phytase	0.01	0.01	0.01	0.01	0.01	0.01
* Multi-enzymes	0.06	0.06	0.06	0.06	0.06	0.06
Emulsifier	0.05	0.05	0.05	0.05	0.05	0.05
Toxin binders	0.20	0.20	0.20	0.20	0.20	0.20
Halquinol	0.05	0.05	0.05	0.05	0.05	0.05
Anticoccidial	0.01	0.01	0.01	0.01	0.01	0.01
Antioxidant	0.01	0.01	0.01	0.01	0.01	0.01
Enzyme (protease)	0.00	0.03	0.04	0.02	0.03	0.03
Total	100.00	100.00	100.00	99.99	100.00	100.00
Calculated value (%)						
Crude protein (CP)	22.20	23.60	23.60	23.60	23.60	23.60
Metabolic energy (ME), kcal/kg	3000.00	3000.00	3000.00	3000.00	3000.00	3000.00
Crude fiber	3.50	3.50	3.50	3.50	3.50	3.50
Fat	4.70	4.70	4.70	4.70	4.70	4.70
Ca	0.88	0.88	0.88	0.88	0.88	0.88
Available P	0.47	0.47	0.47	0.47	0.47	0.47
Cl	0.25	0.25	0.25	0.25	0.25	0.25
Na	0.20	0.20	0.20	0.20	0.20	0.20
Dig lysine	1.35	1.35	1.35	1.35	1.35	1.35
DEB	255.00	255.00	255.00	255.00	255.00	255.00

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease-A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne; Ca: calcium, P: phosphorus, Cl: chloride, Na: sodium, DEB: dietary electrolyte balance. * Multi-enzymes: a commercial blend containing xylanase (10,000 U/g), amylase (2000 U/g), and phytase (500 FTU/g), included at 0.06% of diet.

Table 2. Ingredient composition of experimental diets (15–33 days).

Ingredients, %	Grower (d 15–33)					
	T0	**T1**	**T2**	**T3**	**T4**	**T5**
Maize (12% M)	55.20	55.20	56.20	55.20	56.20	56.20
Soybean meal (46%)	28.00	28.00	26.50	28.00	26.50	26.50
Corn gluten meal (58%)	2.00	2.00	2.00	2.00	2.00	2.00
Poultry meal (51%)	4.00	4.00	4.00	4.00	4.00	4.00
Rice polish	3.40	3.40	3.90	3.40	3.90	3.90
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00
Limestone powder	1.20	1.20	1.20	1.20	1.20	1.20
Di-calcium phosphate	1.00	1.00	1.00	1.00	1.00	1.00
Salt	0.22	0.22	0.22	0.22	0.22	0.22
Broiler vitamins	0.10	0.10	0.10	0.10	0.10	0.10
Organic acids powder	0.20	0.20	0.20	0.20	0.20	0.20
DL methionine	0.40	0.40	0.40	0.40	0.40	0.40
L lysine	0.34	0.34	0.34	0.34	0.34	0.34
Broiler minerals	0.10	0.10	0.10	0.10	0.10	0.10
L threonine	0.10	0.10	0.10	0.10	0.10	0.10
Sodium bicarbonate	0.23	0.23	0.21	0.23	0.22	0.22
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07
Phytase	0.02	0.02	0.01	0.02	0.01	0.01
* Multienzymes	0.06	0.06	0.06	0.06	0.06	0.06
Emulsifier	0.05	0.05	0.05	0.05	0.05	0.05
Toxin binders	0.20	0.20	0.20	0.20	0.20	0.20
Halquinol	0.05	0.05	0.05	0.05	0.05	0.05
Anticoccidial	0.05	0.05	0.05	0.05	0.05	0.05
Antioxidant	0.01	0.01	0.01	0.01	0.01	0.01
Enzyme (protease)	0.00	0.03	0.04	0.02	0.03	0.03
Total	100.00	100.03	100.00	100.02	100.00	100.00
Calculated value (%)						
Crude protein (CP)	21.30	21.80	21.80	21.80	21.80	21.80
Metabolic energy (ME), kcal/kg	3130.00	3130.00	3130.00	3130.00	3130.00	3130.00
Crude fiber	3.30	3.30	3.30	3.30	3.30	3.30
Fat	5.80	5.80	5.80	5.80	5.80	5.80
Ca	0.80	0.80	0.80	0.80	0.80	0.80
Available P	0.41	0.41	0.41	0.41	0.41	0.41
Cl	0.23	0.23	0.23	0.23	0.23	0.23
Na	0.19	0.19	0.19	0.19	0.19	0.19
Dig lysine	1.25	1.25	1.25	1.25	1.25	1.25
DEB	227.00	227.00	227.00	227.00	227.00	227.00

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease-A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne; Ca: calcium, P: phosphorus, Cl: chloride, Na: sodium, DEB: dietary electrolyte balance. * Multi-enzymes: a commercial blend containing xylanase (10,000 U/g), amylase (2000 U/g), and phytase (500 FTU/g), included at 0.06% of diet.

2.4. Growth Performance Parameters

Initial body weight was recorded at placement. Body weight (BW), body weight gain (BWG), and feed intake (FI) were measured on days 7, 14, 21, 28, and 33. Feed conversion ratio (FCR) was calculated as the ratio of FI to BWG. Mortality was recorded daily. On day 33, final performance data were collected, and selected birds processed for further analysis.

2.5. Blood Profile

On days 21 and 33, blood (3 mL) was collected from the wing vein of one randomly selected bird per replicate (n = 4 per treatment). Samples were chilled on ice, centrifuged for serum separation, and analyzed for total protein (TP), albumin, blood urea nitrogen (BUN), creatinine, glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and low-density lipoprotein (LDL) using commercial diagnostic kits (BioMérieux, Craponne, France) and an automated analyzer (Humalyzer 300, Merck®, Tokyo, Japan).

2.6. Carcass Traits and Relative Organ Weights

At 33 days of age, one bird per replicate (n = 4 per treatment) was randomly selected and humanely euthanized. Carcass components including chest, wings, legs, gizzard, intestine, liver, and heart were weighed to assess carcass traits. Absolute organ and carcass weights were recorded, and data are presented as mean ± SD.

2.7. Ileal Intestinal Morphology

Ileum samples from one bird per pen (day 33) were fixed in 10% neutral buffered formalin for 72 h, dehydrated through graded ethanol (60–100%), cleared with xylene, and embedded in paraffin. Sections (4–5 µm) were prepared using a rotary microtome and stained with hematoxylin and eosin (H&E). Intact villi and crypt structures were examined under 40× magnification (Olympus BX51, Tokyo, Japan), and six well-oriented villi were selected for measurement of villus height (VH), crypt depth (CD), and VH:CD ratio by following the protocol of Lee et al. (2023) [21].

2.8. Nutrient Digestibility

Apparent ileal digestibility was determined on days 21 and 33 using a total collection approach. Ileal digesta from four birds per treatment were pooled by replicate and freeze-dried. Dry matter was analyzed in a hot air oven at 105 °C for 24 h; CP was determined via the Kjeldahl method [22]. The gross energy (GE) content of both diet and excreta was measured using an adiabatic bomb calorimeter. The apparent metabolizable energy (AME) of the diet was subsequently calculated using the following formula described by [23]:

$$\text{AME (kcal/kg)} = \text{GE intake} - \text{GE excreta}$$

This calculation provides an estimate of AME by accounting for the difference between energy consumed and energy excreted. Amino acid composition of the diet was determined via HPLC following hydrolysis in 6 N HCl at 110 °C for 24 h. This methodology ensures accurate quantification of nutrient digestibility for each experimental treatment.

2.9. Economical Evaluation

Economic analysis considered total cost (feed, vaccines/medications, protease) and revenue from meat sales (170 BDT/kg live weight). Feed cost (39,687.24 BDT) and vaccine cost (2850 BDT) were constant across treatments. Protease costs varied by treatment as follows: T0—0 BDT, T1—128.98 BDT, T2—180.58 BDT, T3—79.09 BDT, T4—131.82 BDT, T5—130.40 BDT. Costs for DOC and electricity were excluded as they did not vary by treatment. Profit was calculated as revenue minus total cost, and return on investment (ROI) was derived accordingly.

2.10. Statistical Analyses

Data was analyzed using R (version 4.4.2; released 31 October 2024). Normality was tested with the Shapiro–Wilk test and homogeneity of variance with Levene's test.

As assumptions of normality were not met for several variables, treatment effects were assessed using the Kruskal–Wallis rank sum test ($p < 0.05$). Significant results were followed by Dunn's post hoc test with Bonferroni correction. Orthogonal contrasts were not applied.

3. Results

3.1. Growth Performance

Protease supplementation significantly enhanced broiler growth, improving weight gain, feed intake, and efficiency. Higher enzyme levels produced the greatest benefits, reflecting improved nutrient utilization and growth performance (Table 3). At day 7, birds in the T5 group exhibited the highest body weight (163.0 ± 1.4 g), followed closely by T4 (162.5 ± 1.3 g) and T3 (162.3 ± 1.3 g), with significant differences among groups ($p = 0.002$). This trend continued through day 21, where the T5 group maintained the highest weight (854 ± 7 g), while the T2 group showed the lowest (825 ± 8 g; $p = 0.014$). However, by day 33, no significant differences in body weight were observed across groups.

Table 3. Effects of protease on growth performance in broiler chickens.

Characteristic	T0 ($n = 4$)	T1 ($n = 4$)	T2 ($n = 4$)	T3 ($n = 4$)	T4 ($n = 4$)	T5 ($n = 4$)	*p*-Value
			Body Weight (g)				
7 Days	156.8 ± 2.1 ^{ab}	154.0 ± 0.8 ^{a}	160.5 ± 1.3 ^{bc}	162.3 ± 1.3 ^{c}	162.5 ± 1.3 ^{c}	163.0 ± 1.4 ^{c}	0.002
21 Days	847 ± 6 ^{b}	829 ± 12 ^{ab}	825 ± 8 ^{a}	845 ± 6 ^{b}	847 ± 5 ^{b}	854 ± 7 ^{b}	0.014
33 Days	1836 ± 5 ^{a}	1843 ± 7 ^{a}	1849 ± 22 ^{a}	1848 ± 11 ^{a}	1851 ± 14 ^{a}	1843 ± 10 ^{a}	0.400
			Feed Intake (g)				
7 Days	132.3 ± 2.06 ^{a}	133.0 ± 2.16 ^{a}	136.8 ± 2.22 ^{a}	133.5 ± 1.29 ^{a}	132.3 ± 2.06 ^{a}	130.8 ± 1.50 ^{a}	0.048
21 Days	1065.0 ± 5 ^{a}	1079.0 ± 20 ^{a}	1049.0 ± 17 ^{a}	1056.0 ± 3 ^{a}	1048.0 ± 9 ^{a}	1040.0 ± 11 ^{a}	0.024
33 Days	2856.0 ± 14 ^{e}	2962.0 ± 7 ^{a}	2939.0 ± 16 ^{ab}	2899.0 ± 16 ^{bc}	2874.0 ± 19 ^{cd}	2839.0 ± 16 ^{d}	<0.001
			Body Weight Gain (g)				
0–7 Days	111.8 ± 2.1 ^{ab}	109.0 ± 0.8 ^{a}	115.5 ± 1.3 ^{bc}	117.3 ± 1.3 ^{cd}	117.5 ± 1.3 ^{de}	118.0 ± 1.4 ^{e}	0.002
8–21 Days	690 ± 5 ^{b}	675 ± 12 ^{ab}	665 ± 7 ^{a}	683 ± 6 ^{ab}	684 ± 6 ^{ab}	691 ± 9 ^{b}	0.020
22–33 Days	990 ± 4 ^{b}	1015 ± 11 ^{a}	1024 ± 25 ^{c}	1003 ± 14 ^{b}	1005 ± 19 ^{b}	989 ± 7 ^{ab}	0.024

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease-A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet + with competitor protease-F with matrix 250 g/tonne. $p < 0.05$ is considered statistically significant; Results are expressed as means ± standard deviation of means. Kruskal–Wallis rank sum test. Means in the same row with different superscript letters differ significantly ($p < 0.05$).

Feed intake was also significantly affected by protease inclusion. At day 7, the highest intake was noted in T2 (136.8 ± 2.2 g; $p = 0.048$), while cumulative intake over 33 days was greatest in T1 (2962.0 ± 7.0 g; $p < 0.001$).

BWG from day 0–7 was significantly higher in protease-supplemented groups, particularly T5 (118.0 ± 1.4 g; $p = 0.002$), and this improvement persisted during the day 8–21 period (T5: 691 ± 9.0 g; $p = 0.020$) and day 22–33 period (T2: 1024 ± 25 g; $p = 0.024$).

Feed conversion ratio (FCR) improved significantly with protease supplementation (Figure 1). At day 7, FCR was lowest in T5 (0.80 ± 0.005; $p = 0.001$), indicating better efficiency, with continued improvements observed at day 21 (T5: 1.22 ± 0.010; $p < 0.001$) and day 33 (T5: 1.54 ± 0.010; $p = 0.002$). Overall mortality remained low and comparable among treatments, with values below 2% across all groups. Collectively, birds receiving protease-supplemented diets, particularly in the T5 group, demonstrated enhanced growth efficiency and feed utilization.

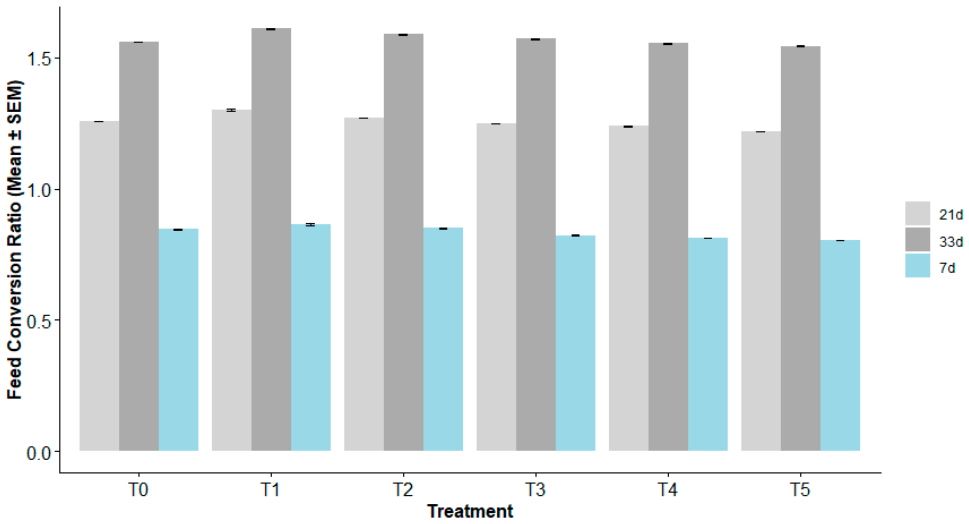

Figure 1. Effects of protease supplementation on feed conversion ratio (FCR) at days 7, 21 and 33. T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease-A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne.

3.2. Serum Biochemical Indices

Protease supplementation significantly influenced the serum biochemical parameters of broiler chickens at both 21 and 33 days of age (Table 4). Supplemented groups showed elevated TP, albumin, and creatinine concentrations, alongside reduced LDL levels, indicating improved protein metabolism and lipid regulation.

Table 4. Effects of protease on serum profile in broiler chickens.

Characteristic	T0 (n = 4)	T1 (n = 4)	T2 (n = 4)	T3 (n = 4)	T4 (n = 4)	T5 (n = 4)	p-Value
Day 21							
TP (g/dL)	2.1 ± 0.01 ^{e}	2.2 ± 0.01 ^{d}	2.1 ± 0.01 ^{e}	2.3 ± 0.01 ^{c}	2.4 ± 0.01 ^{b}	2.6 ± 0.02 ^{a}	<0.001
BUN (mg/dL)	5.5 ± 0.01 ^{f}	5.8 ± 0.01 ^{e}	6.0 ± 0.01 ^{d}	6.1 ± 0.01 ^{c}	6.3 ± 0.01 ^{b}	6.3 ± 0.02 ^{a}	<0.001
Creatinine (mg/dL)	0.4 ± 0.01 ^{d}	0.3 ± 0.01 ^{e}	0.5 ± 0.02 ^{c}	0.6 ± 0.01 ^{a}	0.5 ± 0.01 ^{bc}	0.5 ± 0.01 ^{bc}	<0.001
Albumin (g/dL)	1.0 ± 0.01 ^{d}	1.1 ± 0.01 ^{c}	1.0 ± 0.01 ^{d}	1.2 ± 0.01 ^{ab}	1.2 ± 0.01 ^{ab}	1.2 ± 0.01 ^{ab}	<0.001
GOT (IU/L)	147.0 ± 4 ^{d}	207.0 ± 2 ^{a}	199.0 ± 1 ^{b}	195.0 ± 2 ^{bc}	168.0 ± 1 ^{c}	198.0 ± 1 ^{b}	<0.001
GPT (IU/L)	8.9 ± 0.5 ^{d}	20.5 ± 2.0 ^{a}	16.6 ± 1.3 ^{b}	15.8 ± 0.9 ^{bc}	9.9 ± 0.6 ^{cd}	11.7 ± 0.8 ^{c}	<0.001
LDL (mg/dL)	33.0 ± 1 ^{b}	46.0 ± 2 ^{a}	27.0 ± 1 ^{c}	26.0 ± 2 ^{c}	21.0 ± 1 ^{d}	21.0 ± 1 ^{d}	<0.001
Day 33							
TP (g/dL)	2.8 ± 0.02 ^{c}	2.9 ± 0.02 ^{bc}	3.0 ± 0.07 ^{ab}	3.0 ± 0.06 ^{ab}	3.0 ± 0.05 ^{ab}	3.1 ± 0.03 ^{a}	0.002
BUN (mg/dL)	5.9 ± 0.24 ^{b}	6.1 ± 0.26 ^{ab}	6.2 ± 0.05 ^{ab}	6.0 ± 0.11 ^{ab}	6.3 ± 0.26 ^{a}	6.4 ± 0.07 ^{a}	0.023
Creatinine (mg/dL)	0.6 ± 0.02 ^{b}	0.7 ± 0.03 ^{a}	0.7 ± 0.03 ^{a}	0.6 ± 0.03 ^{b}	0.7 ± 0.03 ^{a}	0.7 ± 0.03 ^{a}	0.006
Albumin (g/dL)	1.0 ± 0.05 ^{d}	1.1 ± 0.02 ^{c}	1.2 ± 0.02 ^{b}	1.2 ± 0.02 ^{b}	1.2 ± 0.03 ^{b}	1.3 ± 0.03 ^{a}	<0.001
GOT (IU/L)	208.0 ± 1 ^{bc}	214.0 ± 11 ^{b}	219.0 ± 3 ^{ab}	218.0 ± 9 ^{ab}	191.0 ± 20 ^{c}	227.0 ± 2 ^{a}	0.016
GPT (IU/L)	8.9 ± 0.97 ^{e}	12.4 ± 1.47 ^{d}	16.7 ± 1.55 ^{b}	14.8 ± 3.84 ^{c}	10.1 ± 0.30 ^{e}	16.8 ± 1.31 ^{ab}	0.003
LDL (mg/dL)	40.7 ± 1.1 ^{ab}	32.9 ± 4.2 ^{cd}	42.1 ± 2.4 ^{a}	34.6 ± 4.2 ^{c}	28.2 ± 1.1 ^{d}	26.3 ± 4.6 ^{d}	0.002

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease-A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne. Additionally, TP: Total Protein; BUN: Blood Urea Nitrogen; GOT: Glutamate Oxaloacetate Transaminase; GPT: Glutamate Pyruvate Transaminase; LDL: Low-Density Lipoprotein. $p < 0.05$ is considered statistically significant; Results are expressed as means ± standard deviation of means. Kruskal–Wallis rank sum test. Means in the same row with different superscript letters differ significantly ($p < 0.05$).

True protein levels increased progressively across treatments, with the highest value observed in the T5 group at both time points ($p < 0.001$ at day 21; $p = 0.002$ at day 33). Blood urea nitrogen (BUN) concentrations also showed a significant elevation with increasing protease levels, particularly at day 21 ($p < 0.001$), and a modest but significant variation

by day 33 ($p = 0.023$). Creatinine levels rose consistently with enzyme supplementation, peaking in the T5 group at both ages ($p < 0.001$ at day 21; $p = 0.006$ at day 33). Similarly, albumin concentrations significantly improved in treated groups compared to the control, showing the highest values in T5 on both days ($p < 0.001$).

Liver enzymes GOT and GPT exhibited marked changes in response to protease inclusion. GOT levels peaked in the T1 group at day 21 and in T5 at day 33 ($p < 0.001$ and $p = 0.016$, respectively), while GPT was highest in T1 at day 21 and in T5 at day 33 ($p < 0.001$ and $p = 0.003$, respectively). Interestingly, LDL concentrations decreased in higher protease-supplemented groups, especially T4 and T5, at both 21 and 33 days, indicating a possible lipid-lowering effect of protease ($p < 0.001$ and $p = 0.002$, respectively).

3.3. Carcass Traits and Organ Weights

Protease supplementation had minimal impact on overall carcass composition in 33-day-old broilers, with most carcass and organ weights remaining unaffected. Notably, gizzard weight increased significantly in the highest supplementation group, suggesting enhanced digestive organ development and potential improvements in feed utilization. The effects of protease supplementation on the carcass traits of 33-day-old Cobb 500 broiler chickens are summarized in Table 5. No significant differences were observed among treatment groups for most carcass parameters, including carcass weight, chest, wings, legs, intestine, liver, and heart weights, indicating that dietary protease had minimal influence on these traits. However, a significant difference ($p = 0.020$) was noted in gizzard weight, with the highest value observed in the T5 group (2.87%), suggesting a potential impact of protease supplementation on gizzard development.

Table 5. Effects of protease supplementation on relative carcass traits (expressed as % of carcass weight) in 33 days old Cobb 500 broiler chickens.

Trait (% of Carcass)	T0	T1	T2	T3	T4	T5	*p*-Value
Carcass weight (g)	1319.0 ± 4.0 [a]	1324.0 ± 5.0 [a]	1328.0 ± 16 [a]	1328.0 ± 8.0 [a]	1330.0 ± 10.0 [a]	1324.0 ± 7.0 [a]	0.400
Chest	37.13 [a]	37.12 [a]	37.12 [a]	37.11 [a]	37.12 [a]	37.12 [a]	0.500
Wings	13.94 [a]	13.93 [a]	13.93 [a]	13.94 [a]	13.94 [a]	13.93 [a]	0.500
Legs	34.80 [a]	34.81 [a]	34.82 [a]	34.81 [a]	34.82 [a]	34.80 [a]	0.400
Gizzard	2.79 [b]	2.76 [b]	2.79 [b]	2.79 [b]	2.83 [b]	2.87 [a]	0.020
Intestine	8.35 [a]	8.36 [a]	8.36 [a]	8.36 [a]	8.36 [a]	8.35 [a]	0.400
Liver	4.47 [a]	4.46 [a]	4.47 [a]	4.47 [a]	4.47 [a]	4.50 [a]	0.200
Heart	0.83 [a]	0.84 [a]	0.84 [a]	0.84 [a]	0.85 [a]	0.85 [a]	0.090

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne. Mean ± SD; Kruskal–Wallis rank sum test; $p < 0.05$ is considered statistically significant. Means in the same row with different superscripts differ significantly ($p < 0.05$).

3.4. Intestinal Morphology

Protease supplementation significantly improved ileal morphology in broiler chickens at both 21 and 33 days of age (Table 6; Figure 2). Across all treatments, protease increased villus height, reduced crypt depth, and enhanced the villus height-to-crypt depth (VH:CD) ratio ($p < 0.001$), indicating a more efficient absorptive surface and improved intestinal function.

At day 21, villus height increased from 749 ± 1 µm in the control (T0) to 874 ± 1 µm in the highest supplementation group (T5), while crypt depth decreased from 89.6 ± 0.4 µm to 77.9 ± 0.1 µm. This resulted in a marked improvement in VH:CD ratio (8.36 ± 0.04 to 11.23 ± 0.02). Similar trends were observed at day 33, with villus height increasing from 814 ± 1 µm (T0) to 931 ± 1 µm (T5), crypt depth declining from 86.4 ± 0.3 µm to 74.0 ± 0.1 µm, and VH:CD ratio rising from 9.42 ± 0.03 to 12.59 ± 0.01.

Table 6. Effects of protease supplementation on ileal intestinal morphology on days 21 and 33 in broilers.

Characteristic	T0 $n = 4$	T1 $n = 4$	T2 $n = 4$	T3 $n = 4$	T4 $n = 4$	T5 $n = 4$	*p*-Value
			21 days				
Villi height (VH, µm)	749 ± 1^{f}	771 ± 1^{e}	801 ± 1^{d}	823 ± 1^{c}	852 ± 1^{b}	874 ± 1^{a}	<0.001
Crypt depth (CD, µm)	89.6 ± 0.4^{a}	85.4 ± 0.2^{b}	81.4 ± 0.2^{c}	79.3 ± 0.2^{d}	78.2 ± 0.1^{e}	77.9 ± 0.1^{f}	<0.001
VH:CD	8.36 ± 0.04^{f}	9.03 ± 0.01^{e}	9.84 ± 0.04^{d}	10.38 ± 0.02^{c}	10.91 ± 0.02^{b}	11.23 ± 0.02^{a}	<0.001
			33 days				
Villi height (µm)	814 ± 1^{f}	861 ± 1^{e}	904 ± 1^{c}	884 ± 1^{d}	923 ± 1^{b}	931 ± 1^{a}	<0.001
Crypt depth (µm)	86.4 ± 0.3^{a}	70.2 ± 0.2^{f}	72.5 ± 0.1^{d}	71.1 ± 0.1^{e}	73.8 ± 0.1^{c}	74.0 ± 0.1^{b}	<0.001
VH:CD	9.42 ± 0.03^{f}	12.27 ± 0.03^{e}	12.47 ± 0.01^{c}	12.44 ± 0.01^{d}	12.50 ± 0.01^{b}	12.59 ± 0.01^{a}	<0.001

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease-A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne. Mean ± SD; Kruskal–Wallis rank sum test; $p < 0.05$ is considered statistically significant. Means in the same row with different superscripts differ significantly ($p < 0.05$).

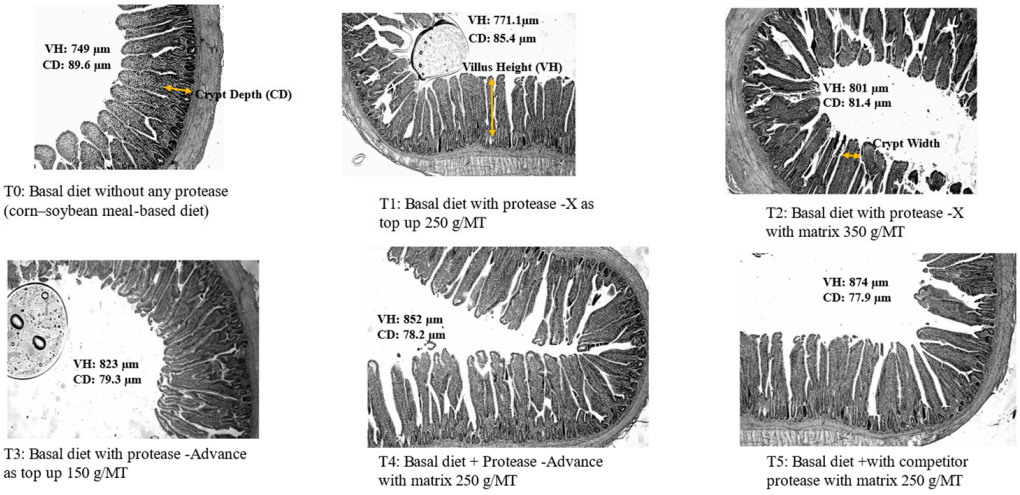

Figure 2. Representative histological section of ileum showing villus height and crypt depth in broilers.

3.5. Nutrient Digestibility

Dry matter and CP levels in digesta increased with higher protease inclusion, indicating more efficient nutrient breakdown and absorption (Table 7). On day 33, dietary DM content differed among treatments ($p < 0.001$), while digesta dry matter increased from 77.13% in T0 to 77.6% in T5. Although dietary CP varied slightly across groups ($p < 0.001$), CP content in digesta rose markedly with supplementation, reaching 20.10% in T5. Apparent metabolizable energy in the diet remained consistent (~3130 kcal/kg), but AME in digesta increased significantly ($p < 0.001$), from 2768 kcal/kg in T0 to 2803 kcal/kg in T5. Amino acid levels also differed among treatments ($p = 0.001$), with the lowest value recorded in T3 (2.19%) and the highest in T0 (2.26%).

Table 7. Effects of protease on nutrient digestibility in 33 days in broiler chickens.

Characteristic (33 Days)	T0 $n = 4$	T1 $n = 4$	T2 $n = 4$	T3 $n = 4$	T4 $n = 4$	T5 $n = 4$	p-Value
DM in diet (%)	88.94 ± 0.01^{b}	88.14 ± 0.01^{c}	89.52 ± 0.01^{a}	88.84 ± 0.01^{b}	89.42 ± 0.01^{a}	88.90 ± 0.01^{b}	<0.001
DM in digesta (%)	77.13 ± 0.01^{c}	77.22 ± 0.01^{c}	77.52 ± 0.01^{ab}	77.28 ± 0.01^{c}	77.37 ± 0.01^{bc}	77.61 ± 0.01^{a}	<0.001
CP in diet (%)	22.11 ± 0.01^{a}	21.52 ± 0.01^{b}	21.22 ± 0.01^{c}	21.09 ± 0.01^{c}	21.33 ± 0.01^{c}	21.40 ± 0.01^{bc}	<0.001
CP in digesta (%)	19.59 ± 0.01^{c}	19.80 ± 0.01^{b}	20.01 ± 0.01^{a}	19.95 ± 0.01^{ab}	20.02 ± 0.01^{a}	20.10 ± 0.01^{a}	<0.001
AME in diet (kcal/kg)	3130.2 ± 0.01^{a}	3130.1 ± 0.01^{a}	3130.1 ± 0.01^{a}	3130.2 ± 0.01^{a}	3130.1 ± 0.01^{a}	3130.1 ± 0.01^{a}	<0.001
AME in digesta (kcal/kg)	2768 ± 0^{c}	2774 ± 0^{bc}	2787 ± 0^{ab}	2781 ± 0^{bc}	2794 ± 0^{a}	2803 ± 0^{a}	<0.001
Amino acids in diet (%)	2.26 ± 0.008^{ab}	2.23 ± 0.008^{b}	2.22 ± 0.008^{b}	2.19 ± 0.008^{c}	2.25 ± 0.008^{ab}	2.26 ± 0.008^{a}	0.001

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne. Mean ± SD; Kruskal–Wallis rank sum test; $p < 0.05$ is considered statistically significant; DM: dry matter, CP: crude protein, AME: apparent metabolizable energy. Different superscripts (a–c) within a row indicate significant differences among treatments.

3.6. Economic Evaluation

Protease supplementation improved the economic efficiency of broiler production, with higher enzyme levels yielding the greatest financial return (Table 8). Although feed and health-related costs were consistent across treatments, total production costs increased slightly in supplemented groups due to the added protease. Despite similar total income across treatments, profitability varied.

Table 8. Profit and return on investment (ROI) comparison.

Group	Total Feed Cost (BDT)	Medicine/Vaccine Cost (BDT)	Protease Cost (BDT)	Total Cost (BDT)	Total Income (BDT)	Profit (BDT)	ROI (%)
T0	39,687	2850	0	54,037	57,776	3600	6.67
T1	39,687	2850	129	54,166	57,776	3551	6.55
T2	39,687	2850	181	54,218	57,776	3700	6.83
T3	39,687	2850	79	54,116	57,776	3650	6.74
T4	39,687	2850	132	54,169	57,776	3741	6.89
T5	39,687	2850	130	54,168	57,776	3801	7.01

T0: Basal diet without any protease (corn–soybean meal-based diet); T1: Basal diet with protease-X as top up 250 g/tonne; T2: Basal diet with protease-X with matrix 350 g/tonne; T3: Basal diet with protease-A as top up 150 g/tonne; T4: Basal diet + protease-A with matrix 250 g/tonne; T5: Basal diet +with competitor protease-F with matrix 250 g/tonne.

The highest economic benefit was achieved in T5, which generated the greatest net profit (BDT 3801) and return on investment (7%). T4 and T2 also showed improved ROI compared with the control. In contrast, the control group exhibited the lowest ROI (6.67%), and T1 recorded the lowest profit among supplemented groups.

4. Discussion

The present study demonstrated that dietary protease supplementation significantly enhanced broiler growth performance during the early rearing phase, as reflected by higher body weight at 7 and 21 days of age. These improvements can be attributed to enhanced protein digestibility and more efficient amino acid absorption [24]. Previous studies have similarly reported that broilers receiving a combination of acid and neutral protease exhibited improved body weight gain and nitrogen retention compared with negative controls [25]. Moreover, increasing dietary protease levels from 0% to 0.09% has been shown to yield linear improvements in average daily gain and nutrient digestibility [26]. Supplementation with non-starch polysaccharide enzymes combined with protease further enhanced growth rate and crude protein digestibility [27]. Protease supplementation has also been linked to the upregulation of genes associated with nutrient transport and growth regulation, thereby supporting improved growth performance. Nonetheless, inconsistent effects observed in some parameters are in agreement with previous reports, where vari-

ability in enzyme efficacy has been attributed to factors such as bird age, enzyme source, dosage, and diet composition [28].

Intestinal morphology was markedly improved in birds receiving protease, particularly through increased villus height, which enlarges the absorptive surface area and enhances nutrient uptake efficiency. These findings align with earlier studies demonstrating that protease supplementation improves jejunal villus architecture, supporting greater intestinal integrity and digestive capacity [29–32]. Such morphological adaptations are crucial for sustaining growth performance, as they directly contribute to improved nutrient absorption and gastrointestinal health.

The digestibility trial corroborated these outcomes, revealing significant improvements in dry matter, crude protein, and apparent metabolizable energy utilization, especially in birds receiving higher protease inclusion (T5 group). These results are consistent with prior reports showing enhanced ileal digestibility and nitrogen retention in broilers supplemented with protease [30,33]. Specifically, supplementation with *Bacillus licheniformis*-derived protease at 300–400 mg/kg has been shown to increased protein digestibility, jejunal trypsin activity, and villus height, thereby improving nutrient assimilation [32]. Similarly, supplementation with 50 g/tonne protease improved amino acid digestibility, gut morphology, and cecal microbiota balance [31]. Notably, protease efficacy appears to be influenced by dietary protein content, with benefits being more pronounced under optimal protein levels, while diets with markedly reduced crude protein may limit protease effects [30,33]. Collectively, enhanced nutrient utilization likely underpins the improvements in growth performance and feed efficiency observed in the current study.

Protease supplementation also elicited favorable metabolic responses, as evidenced by increased concentrations of lysine, methionine, and threonine in breast muscle, indicative of more efficient protein utilization and synthesis [34]. Concurrently, reductions in LDL cholesterol alongside increases in HDL suggest beneficial modulation of lipid metabolism, consistent with improved physiological status and overall health in broilers [21]. These metabolic changes further highlight the role of protease in optimizing nutrient assimilation and systemic functions beyond growth performance.

Despite improvements in growth performance, feed intake, and feed conversion ratio, protease supplementation did not significantly alter carcass yield or relative organ weights. This observation aligns with previous research indicating that early growth advantages do not necessarily translate into changes in carcass composition at market age [21,26]. Such outcomes may reflect compensatory growth mechanisms, whereby early gains are balanced over the course of the rearing period, resulting in comparable carcass characteristics across treatments.

From an economic standpoint, protease supplementation improved profitability by enhancing feed efficiency and nutrient utilization without substantially increasing feed costs. These improvements translated into higher returns on investment and profit margins in supplemented groups. Such outcomes are consistent with previous reports that highlighted enzyme supplementation as a cost-effective strategy to improve productivity in intensive poultry production systems [21,35].

5. Conclusions

Matrix-dosed protease supplementation improved broiler growth, nutrient digestibility, gut morphology, and profitability. Protease F at 250 g/tonne, a rapidly protein-hydrolyzing enzyme, achieved the highest body weight, feed efficiency, CP digestibility, and AME, offering the greatest economic benefit under the study conditions. Protease X at 350 g/tonne also enhanced performance, particularly in the finisher phase. Although the study did not directly quantify matrix nutrient release values, the consistent responses

under matrix-dosed conditions support their potential practical activity. Overall, protease strategies enabling efficient protein hydrolysis can enhance broiler productivity and cost-effectiveness, though economic gains may vary with production context.

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