

# Effect Of Exogenous Protease Enzyme On Broiler Chickens' Performance, Gene Expression In Reduced Protein - Energy Diet

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## Abstract

This study investigated the impact of adding exogenous protease enzyme (250 g/ton) to low-protein, low-energy diets on the performance of broiler chickens. A total of 200 Ross 308 chicks were randomly assigned to four dietary treatments, including a control group, a nutrient-deficient group, and two groups supplemented with protease enzyme. The results demonstrated that protease supplementation significantly improved body weight, weight gain, feed conversion ratio, and carcass traits. Birds receiving protease showed enhanced intestinal morphology, including increased villus height and width, and deeper crypts, indicating better nutrient absorption. Additionally, IGF-1 gene expression in liver tissue was significantly higher in the protease groups, reflecting improved growth and tissue development. The immune response to common poultry viruses was also enhanced, and gut microbiota analysis showed reduced harmful bacteria (*E. coli*) and increased beneficial bacteria (*Lactobacillus*) in protease-supplemented groups. In conclusion, the addition of exogenous protease to reduced-energy and reduced-protein broiler diets improved growth performance, immunity, intestinal health, and gene expression, suggesting it as a cost-effective strategy for optimizing poultry production.

**Key word:** Protease enzyme, Growth performance, gene expression, Exogenous enzymes

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## INTRODUCTION

The chicken industry, particularly in recent years, the escalation of feed ingredient prices, especially those contributing energy, which constitutes around 70% of diet costs, has prompted the investigation of techniques to mitigate feed production expenses. Since they are the most expensive components of the diet, one tactic is to reduce the amounts of metabolizable energy (ME) and crude protein (CP). As a result, several experiments were conducted to improve the development in performance of broiler chicken and to reduce costs by reducing the proportion of specific energy components (Cozannet *et al.*, 2021).

Furthermore, diminishing protein or calorie levels in the diet may decrease nitrogen excretion and mitigate ammonia emissions, thereby contributing to a reduction in environmental effect (Liu *et al.*, 2024).

In poultry production, the nutritional quality of the diet plays a major role in the efficient utilization of nutrients by animals, ultimately influencing broiler performance and economic returns for producers (Attia *et al.*, 2022). In addition, of particular note from a sustainability standpoint is the observation that more than 60% of the raw materials used in poultry diets is in direct competition with human nutrition (e.g., corn, wheat and soybean) (Mottet *et al.*, 2017).

The use of enzymes has gained traction in the poultry sector in particular due to their ability to enhance nutrient absorption. It has been demonstrated that the most practical and economical way to increase feed consumption is using enzymes to aid the bird in absorbing vital nutrients. When enzymes are added to industrial by-products, their nutritional value increases, providing more options for diet formulation (Alabi *et al.*, 2019).

Protease catalyzes proteolysis via hydrolysis, essentially breaking down proteins into amino acids. Proteases are categorized by the optimum pH in which they are effective. Acid proteases are active in pH range 2.5-3.5 and work in the gizzard and proventriculus. Neutral proteases work in the pH range of 6.5-7 and are active in the duodenum and jejunum. Basic or alkaline proteases are active in the 7.2-7.8 pH range and work in the ileum (Sipany, 2021).

Another effective way to improve dietary protein utilization in commercial feed is by the inclusion of exogenous protease enzyme, whose effects have been verified experimentally on increasing protein digestibility and then enhancing protein use (Vieira *et al.*, 2023). Endogenous protein enzymes are secreted by the animal themselves and their concentration are not sufficient for efficient protein digestion, especially in young

animal or animal under stress. The undeveloped intestine of the young animal causes the limited secretion of digestive enzymes, which causes the incomplete digestion of dietary protein and other nutrients (Barua *et al.*, 2021).

The current study will be aimed to evaluate the effects of protease enzyme that will be supplemented to broiler chickens' diet by studying the IGF-1 gene expression, the body weight performance and intestinal morphology, by studying the following parameters: -

Productive traits: (body weight, weight gain, feed intake, feed conversion ratio). ELISA for (ND, IB, IBD, and AI) anti body titers at 4, 20 and 30 days old. Bacterial count of drop (*E. coli* form and *lactobacillus*). Gene expression (IGF-1). Intestinal morphology (villus height, villus width, villus area, and crypts depth), also will be studied the intestine muscular thickness.

### **Materials and Methods**

This study was conducted to evaluate the effect of protease enzyme on reduced protein - energy diet of broilers. Two hundred broiler chickens 1-day-old 308 Ross divide into 4 equal groups:

T1 (Group one): control group was fed on basal diet. T2 (Group two): was fed on reduced protein- energy diet (100 Kcal /kg and 1% CP). T3 (Group three): was fed basal diet with adding 250 g/ton protease enzymes. T4 (Group four): was fed in reduced protein- energy diet (100 Kcal and 1% CP) adding 250g/ton protease enzyme.

The experiment lasted for five weeks, during which feed and water were freely provided to the broilers during the study period specific programs were followed to vaccinate the birds and take care of their health according to the recommendations for raising broiler chickens. The environment and lightning were check daily, the performance traits were recorded weekly. The blood samples and liver tissue and intestinal samples were collected at the end of the study.

### **Productive Performance**

Weekly production traits were measured by (Al-Jebory *et al.*,2021).

#### **Average body weight (BW) each week (g/birds).**

Each chick was weighed on the first day of life and at the conclusion of each week using a delicate balance to determine the weight. The total weight of all the chicks divided by the number of chicks yielded the mean body weight.

#### **Mean weekly growth in weight (WG) (grams/birds)**

Weekly mean body weight gain for each replicate was calculated by substrate recording the Body weight gain at the end of the week and depending on the following equation:

Mean weekly weight gain= mean body weight at the end of the week- mean body weight at the beginning of the week.

#### **Weekly intake of feed (FI) (g /bird).**

Feed intake was calculated weekly using the equation provided by this was accomplished by weighing the leftover feed at the conclusion of each week and deducting it from the total amount provided at the start of the week.

#### **Weekly mean Feed Conversion Ratio (F.C.R) %**

Feed Conversion Ratio was calculated weekly for each group up to the end of experiment. Was reported the equation for measurement of FCR. The FCR measurement equation was described in (AL-Fayadh and Naji, 1989).

**FCR= mean weekly feed intake (gm) ÷ mean weekly body weight gain (gm)**

### **Bacterial Culturing and Enumeration**

Random sampling was conducted across all experimental groups to quantify the populations of both pathogenic and beneficial bacteria. Cecal content samples were collected from the paired ceca at 20 and 30 days of age.

### **Immunological tests**

The Immune response for ND, IB, IBD& AI were determined by use a special ELISA Kits.

### **Blood sampling**

At 4 days all blood samples were taken from the heart and 20, 30 days from the wing vein in a test tube without anticoagulant. In order to extract serum, the blood was allowed to coagulate and centrifuged for 10

minutes at 3000 rpm. The serum was refrigerated at -20°C until examination. Antibody titer against ND, IB, IBD, and AI illnesses is measured by ELISA.

### Gene expression sample

All Gene expression samples were collected at days 35 of age from all groups randomly. The sample were obtained from the liver and stored at liquid nitrogen until analysis. The samples were used for detection of gene expression from insulin-like growth factor 1 gene (IGF-1).

### Measuring the weight of internal organs after slaughter

The weight of the organs (Heart, liver, spleen and gizzard) was assessed post-mortem and after evacuation from the body using an electronic scale, and compared with the body weight.

### Histological examination

Intestine histology preparation and measuring

### Statistical analysis

SPSS 22.0 software was used to analyze the data using the general linear model (GLM) method and one-way ANOVA (Corp, 2011).

Using a "protected" Duncan's analysis at the  $P \leq 0.05$  significance level, four treatment means were determined.

## RESULTS

### Production performance

The results of current study showed an increase significantly ( $P \leq 0.05$ ) in the live body weight (BW) and weight gain (WG). The highest significant increase in productive traits (BW) in the (T3) (2169.02 g) and (T4) (2186.80 g), (WG) (T3) (2127.6) and (T4) (2144.7) The experimental groups were fed from day 1 to day 35 as follows: group T3 received a basal diet supplemented with a protease enzyme, while group T4 was fed a reduced-energy and reduced-protein diet also supplemented with a protease enzyme. However, FI (3094.22 g) (T3), (3130.52g) (T4) and FCR (1.45) (T3), (1.46) (T4) were decrease significantly ( $P \leq 0.05$ ).

**Table 1:** This table shows the final body weight of broilers at the end of the 35-day trial. The enzyme-supplemented groups, especially T3 and T4, recorded significantly higher body weights compared to the control group (T1), reflecting improved growth performance.

Impact of protease in reduced energy diet on the BW (g) of broiler chickens (Mean  $\pm$  SD)

Groups Weeks	T1	T2	T3	T4
Day1	41.4 $\pm$ 2.99 a	42.1 $\pm$ 2.76 a	41.4 $\pm$ 2.74 a	43.06 $\pm$ 2.79 a
Week 1	172.1 $\pm$ 3.00 a	158.3 $\pm$ 3.69 c	173.7 $\pm$ 3.12 a	173.1 $\pm$ 3.40 ab
Week2	425.5 $\pm$ 6.67 c	405.4 $\pm$ 4.03 d	440.4 $\pm$ 4.29 b	447.3 $\pm$ 2.69 a
Week3	953.7 $\pm$ 4.91 c	844.06 $\pm$ 3.43 d	990.3 $\pm$ 3.71 b	1019.6 $\pm$ 30.78 a
Week 4	1404.2 $\pm$ 8.88 b	1208.7 $\pm$ 7.00 c	1497.3 $\pm$ 8.80 a	1504 $\pm$ 8.96 a
Week 5	1938.2 $\pm$ 16.67 b	1768.4 $\pm$ 16.47 c	2169.02 $\pm$ 32.55 a	2186.8 $\pm$ 41.92 a

**Table 2:** This table illustrates the effect of protease supplementation on weekly and cumulative weight gain in broilers during the experimental period. Groups T3 and T4 demonstrated clear superiority in weight gain, highlighting the enzyme's role in offsetting the effects of reduced dietary protein and energy.

Effect of protease in a low protein-energy diet on weight gain (WG) of broiler chickens

Groups Weeks	T1	T2	T3	T4
Week 1	129.6 $\pm$ 2.50 b	116.1 $\pm$ 1.11 c	132.3 $\pm$ 1.53 a	131.08 $\pm$ 3.67 a
Week 2	253.4 $\pm$ 6.26 c	247.1 $\pm$ 4.45 d	266.6 $\pm$ 3.71 b	274.2 $\pm$ 2.70 a
Week 3	450.5 $\pm$ 8.94 b	364.6 $\pm$ 10.09 c	506.9 $\pm$ 11.55 a	484.4 $\pm$ 34.24 a

Week 4	528.2±7.46 b	438.6±5.06 c	549.9±6.10 a	572.2±30.79 a
Week 5	622.9±25.49 b	566.6 ±23.39 b	671.7±25.73 a	682.8±45.45 a
WG	1895.7 ±25.10b	1733.2±29.40 b	2127.6 ±33.19 a	2144.7±42.49 a

**Table 3:** This table displays the average feed intake across different dietary treatments under reduced-energy conditions, with or without protease supplementation. A slight increase in feed intake was observed in the treated groups, suggesting a positive influence of the enzyme on feed palatability and/or digestibility.

Impact of protease in a reduced protein-energy diet on feed intake (FI) (g) in “broiler chickens” (Mean±SD)

Groups Weeks	T1	T2	T3	T4
Week 1	167.83 ± 0.63 c	169.30 ± 0.47 b	169.30 ± 0.47 b	171.80 ± 0.09 a
Week 2	349 ± 6.28 a	338.62 ± 1.63 c	341.58 ± 4.40 bc	344.4 ± 0.04 ab
Week 3	801.22 ± 1.28 a	722.26 ± 2.49 c	743.46 ± 12.43 b	745.80 ± 0.07 b
Week 4	898.54 ± 16.56 a	832.90 ± 0.06 b	839.42 ± 16.81 b	842.54 ± 20.40 b
Week 5	1056.71 ± 9.40 a	975.90 ± 0.05 d	999.96 ± 1.11 c	1025.98 ± 32.04 b
FI	3273.3040 ± 12.94a	3038.98 ± 3.43 d	3094.22 ± 17.14 c	3130.52 ± 41.57 b

**Table 4:** This table presents the effect of adding protease enzyme to a reduced protein-energy diet on the feed conversion ratio (FCR) of broiler chickens. Four dietary treatments were compared. The results showed a notable improvement in feed efficiency in the enzyme-supplemented groups, particularly T3, indicating enhanced nutrient utilization.

Effect of protease in a reduced protein- energy diet on the FCR of broiler chickens (Mean ± SD)

Groups Weeks	T1	T2	T3	T4
Week 1	1.29 ± 0.01 c	1.45 ± 0.01a	1.27 ± 0.11 d	1.31 ± 0.06 b
Week 2	1.37 ± 0.04 a	1.37 ± 0.02 a	1.28 ± 0.03 b	1.25 ± 0.01 b
Week 3	1.77 ± 0.02 b	1.98 ± 0.02 a	1.46 ± 0.03 c	1.53 ± 0.06 c
Week 4	1.70 ± 0.04 b	1.89 ± 0.06 a	1.52 ± 0.04 c	1.54 ± 0.10 b
Week 5	1.69 ± 0.01 a	1.75 ± 0.01 a	1.45 ± 0.02 b	1.46 ± 0.02 b

### Carcass characterization in broiler chickens

**Table 5:** deals with the influence of proteas enzymes on the characteristics of the carcass, as it includes parameters distributed across different groups. The results include the average ± SE values for each parameter, and different letters indicate significant differences at a semantic level  $p < 0.05$ .

Effect proteas enzymes on carcass characteristic in reduced protein energy diet of broiler chickens (mean ±SE)

Groups Parameter (g)	T1	T2	T3	T4
Live BW	2116.00±71.31ab	1856.00±78.39b	2124.00±98.11ab	2256.00 ±148.07a
Without Feathers	1864.00±61.6ab	1688.00±68.51b	1904.00±88.35ab	2040.00 ±136.08a
Chest	636.94±18.05 b	590.14 ±2.46 c	677.80 ±5.78 a	695.08± 3.20 a
Wing	61.60± 0.50 a	58.00 ±2.02 b	62.20± 0.58 a	64.40± 0.60 a
Upper wing	50.20± 0.20 a	48.80± 2.17 a	50.70± 0.00 1a	51.60± 0.50 a
Lower wing	11.40± 0.40 b	9.20± 0.37 c	12.20± 0.58 ab	12.80±0.200 a

Feet	91.18±1.71 b	81.20 ±2.87 c	94.06± 1.84 b	103.56± 1.89 a
Thaig	250.06± 5.69 a	212.06± 3.12 b	269.02± 1.89 a	255.58±19.01 a
Drumstick	92.08± 0.90 b	87.54± 0.77 c	102.80 ±2.28 a	106.96± 1.07 a
Liver	60.36± 0.30 b	57.82± 0.56 c	69.88± 0.90 a	69.48± 0.79 a
Heart	12.42± 0.53 a	10.62± 0.81 b	12.58± 0.63 a	13.38± 0.39 a
Gizzard	41.20± 0.66 b	39.40± 0.50 c	41.80± 0.37 b	44.00± 0.70 a
Spleen	3.08 ±0.38 a	2.86 ±0.43 a	3.26± 0.34 a	3.46± 0.32 a

#### **lactobacillus and E. coli counts in broiler droppings at 20 and 30 days of age**

**Table 6:** This table presents the differences in E. coli colony counts and lactobacillus among the treatment groups after 20 and 30 days of feeding. A significant reduction in E. coli populations was observed in the protease-treated groups, particularly T3 and T4, compared to the untreated group T2, which showed the highest bacterial load

Groups / CFU/ml	E. coli		Lactobacillus	
	Days 20	Days 30	Days 20	Days 30
T 1	5.4 ± 0.01	13.01 ± 1.00	4.96 ± 0.02	4.90 ± 0.02
T 2	6.79 ± 0.52	54.33 ± 11.02	4.72 ± 0.12	5.07 ± 0.04
T 3	4.56 ± 0.05	11.33 ± 1.53	5.18 ± 0.03	4.86 ± 0.03
T 4	3.33 ± 0.08	4.67 ± 3.79	5.04 ± 0.06	6.19 ± 0.03

#### **Effect of protease enzyme in a reduced protein- energy diet on anti- body titers against ND, IB, IBD and AI**

Immune response was enhancing by increase of Antibody titer against ND and IBD vaccines. Antibody titer was increased significantly ( $P<0.05$ ) of ND and IBD in the (T3) and (T4) compared with other groups as show in table 7

**Table 7:** Effect of protease enzyme in a reduced protein- energy diet on the immunological of broiler chickens (Mean±SD)

Groups	T1	T2	T3	T4
In 4 days				
ND	18330.00± 1.41 c	17783.20± 1.01 d	22247.00 ± 0.70 b	22604.00± 1.41 a
AI	1114.40 ±1.72 a	1104.40± 1.46 b	1116.80 ± 1.35 a	1114.00 ± 1.51 a
IB	4514.00 ±1.41 d	6543.60 ± 1.16 c	7404.40 ± 1.74 b	8064.60 ± 1.32 a
IBD	10874.40± 1.02 c	10793.80 ± 1.74 d	10894.80 ± 1.71 b	10904.40 ± 1.72 a
In 20 days				
ND	1193.80± 1.35 d	2205.00 ± 1.22 c	2335.60 ± 1.02 b	2415.80 ±0.86 a
AI	3975.60 ±0.92 d	6563.00 ± 1.18 c	7754.40 ± 1.36 b	8234.60 ± 1.24 a
IB	694.20 ± 1.56 d	886.00 ± 1.22 c	1795.20± 1.28 b	1833.60 ± 1.16 a

IBD	304.60 ± 1.36 d	404.60 ± 1.36 c	776.00 ± 1.30 b	984.20 ± 1.15 a
In30days				
ND	5214.80 ± 1.39 d	7084.60 ± 1.20 c	8545.20±1.15 b	8662.80±0.86 a
AI	14885.80±0.86 d	1874.00 ± 1.00 c	17764.80 ± 1.46 b	17834.20 ± 1.31 a
IB	1584.80± 1.65 d	1794.00 ± 1.30 c	2364.60 ± 1.28 b	3404.20 ± 1.42 a
IBD	154.20 ± 1.42 d	194.60 ± 1.07 c	255.20 ± 1.06 b	267.00 ± 1.14 a

### IGF\_1 gene expression in Broiler Chickens

This study aimed to evaluate the expression of the IGF-1 gene in the liver tissue of broiler chickens under the influence of protease enzyme supplementation and a reduced protein-energy diet. The results demonstrated that IGF-1 gene expression was significantly upregulated in both the T3 and T4 groups. Specifically, the T3 group showed an (83.23 ± 6.76), while the T4 group exhibited a (155.0 ± 20.11) fold increase, compared to the T2 group (reduced protein-energy diet without enzyme supplementation) and the control group, as shown in Table 8

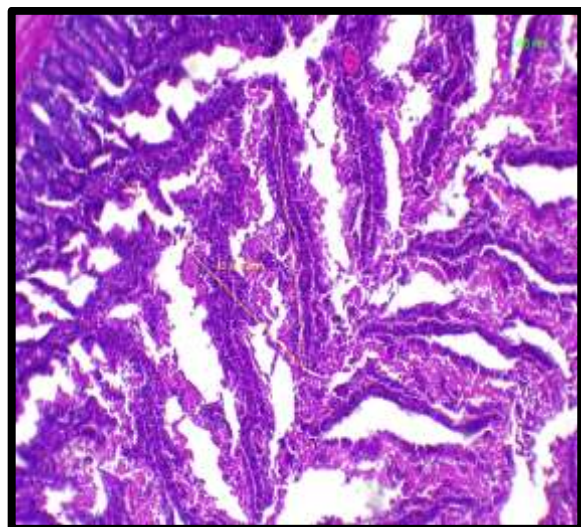
**Table 8:** Effect of protease enzyme in reduced protein energy diet on IGF\_1 gene expression in Broiler Chickens (Mean±SD)

Parameters	T1		T2		T3		T4	
	Mean	±S. D	Mean	±S. D	Mean	±S. D	Mean	±S. D
IGF-1	1	0.000	53.59	5.83	83.23	6.766	155.0	20.11

### Intestine Histology morphology

There was increase significant (P≤0.05) Length, width, and area villi and crypt depth in duodenum.

The current findings demonstrate that supplementation with protease enzyme has a significant positive effect on the morphology of duodenal, jejunum villi. The longest villi were recorded in group T3, which received a basal diet supplemented with protease enzyme. This was followed by group T4, which was fed a reduced protein-energy diet in combination with protease enzyme supplementation



**Figure 1:** histological section in the duodenum of control broiler chicken (T1) showed irregular network of villi formed by anastomosis (yellow arrow) with villous height (1.427.6 mm red arrow) (H&E stain X10)

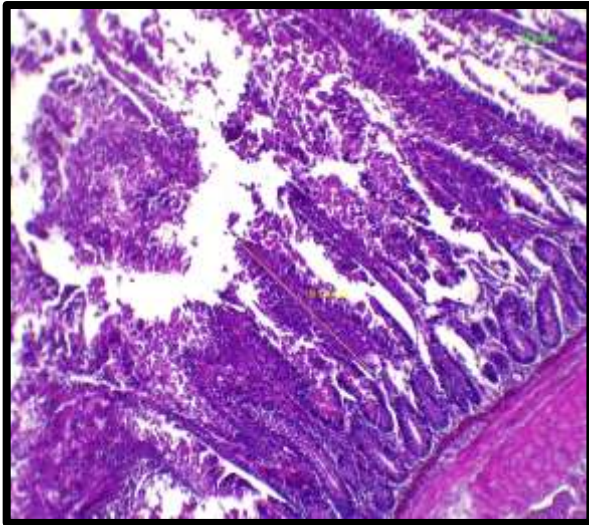


Figure 2: histopathological section in the duodenum of T2 broiler chicken showed ulceration of intestinal mucosa & lamina propria with sever necrotic debris (yellow arrow) & villous height 528.4 mm (black arrow) (H&E stainX10)



Figure 3: histopathological section in the duodenum of T3 broiler chicken showed hypertrophy of villi length (1.645.5mm red arrow) with increase surface area (H&E stainX10)

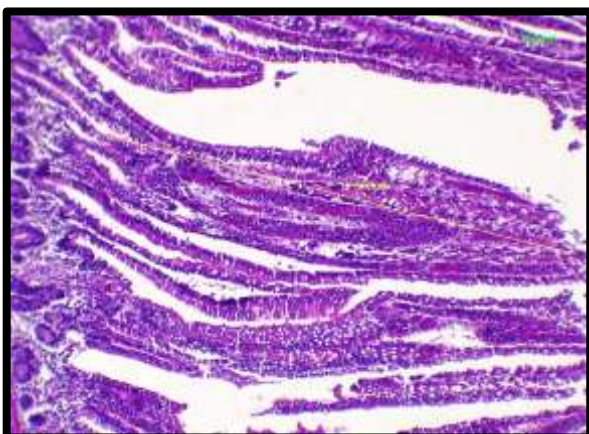


Figure 4: histopathological section in the duodenum of T4 broiler chicken showed regeneration of villi by increase length (1,500.0 mm yellow arrow) & with goblet cell proliferation (red arrow) (H&E stain 10X)

## **DISCUSSION**

The findings of this study demonstrate a significant positive impact of protease enzyme supplementation on the performance of broiler chickens, particularly under reduced protein and energy dietary conditions. The T3 and T4 groups, which received diets supplemented with protease, showed the highest values in body weight, weight gain, and feed conversion ratio, with lower feed intake compared to the T2 group (low-protein, low-energy diet without enzyme). This indicates enhanced growth performance and feed efficiency due to protease inclusion (Cowieson et al., 2019).

Carcass traits also improved notably in the T3 and T4 groups, including increased dressing percentage, breast muscle weight, and organ weights (liver, heart, and gizzard), suggesting better protein deposition and organ development (Walk et al., 2018).

Microbiologically, protease supplementation led to a significant increase in beneficial *Lactobacillus* populations and a decrease in pathogenic *E. coli*, indicating improved gut health and microbial balance (Sipany, 2021).

Immune response was enhanced, as evidenced by significantly higher antibody titers against ND, IB, and IBD vaccines in the T3 and T4 groups, reflecting improved humoral immunity under enzyme treatment (Lourenço et al., 2020; Monier, 2020).

At the molecular level, IGF-1 gene expression in liver tissue was markedly upregulated in T3 and T4, supporting the role of protease in stimulating growth-related pathways (Vaccaro et al., 2021).

Histological analysis of intestinal tissues revealed significant improvements in villus length, width, and surface area, along with thicker muscularis layers in the enzyme-treated groups, indicating enhanced absorptive capacity and overall intestinal integrity (Kraieski, 2017).

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### **Novelty Statement**

This study provides a novel insight into the synergistic effects of exogenous protease supplementation within reduced-energy and reduced-protein broiler diets. Unlike previous studies, it simultaneously investigates productive performance, gut morphology, immune status, microbial balance, and hepatic IGF-1 gene expression. The integration of molecular, immunological, and histological assessments in a single trial highlights a holistic approach to evaluating feed enzyme efficiency under nutritionally constrained conditions. This comprehensive methodology contributes new evidence for optimizing poultry nutrition while reducing production costs and environmental impact.

### **Author Contributions:**

Dr. Yasser Jamal Jamel and Dr. Latif Ibrahim Kadhim conceptualized and supervised the study, contributed to the experimental design, and provided scientific guidance throughout the research process.

Zaid Riyadh Abdul Wahid conducted the laboratory experiments, including Effect of exogenous protease enzyme on broiler chickens' performance, gene expression in reduced protein - energy diet.

Dr. Yasser Jamal Jamel and Dr. Latif Ibrahim Kadhim analyzed the experimental data, reviewed related literature, and prepared the initial draft of the manuscript.

All authors contributed to the interpretation of results, revised the manuscript critically, and approved the final version for submission.

**Conflict of Interest:** The authors declare that there is no conflict of interest regarding the publication of this paper.

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