Supplementation of Phytase and Protease Enzymes on the Performance of KUB Chicken using the MSTN Gene Expression

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ABSTRACT

Background: The KUB chicken industry faces several problems in early growth and active biological activity. One thing that needs to be done is the addition of phytase and protease enzymes to livestock feed. One effort to increase other livestock production is through molecular or genetic markers. The purpose of this study was to determine the effect of adding phytase and protease enzymes to the ration on the performance of KUB chickens, as well as to determine the expression of the MSTN gene on KUB chicken carcass production.

Methods: A total of 35 blood samples were taken from superior Balitnak (KUB) native chickens, consisting of 8 blood samples from groups of male chickens with the same variety, age with feed added with phytase enzyme, samples were taken from male chickens with the same body weight given feed with a mixture of protease enzymes. The following methods were used to calculate body weight increase, feed intake and feed conversion ratio (FCR). Amplification of the myostatin gene (MSTN) in KUB chickens was carried out using the Polymerase Chain Reaction (PCR) technique.

Result: Phytase and protease enzyme supplementation did not show a significant effect (P>0.05) on the relative weight of chicken internal organs, but has the potential to increase digestive efficiency and metabolic function. Enzyme activity in the digestive tract is considered an important factor that can affect gut health and nutrient digestibility. MSTN gene transcript expression was detected in all enzyme doses investigated in this study. Myostatin (MSTN) gene expression in KUB chickens was associated with muscle development, growth rate, organ composition and body weight.

Key words: Agriculture, KUB chicken, MSTN, Phytase, Protease.

INTRODUCTION

The demand for animal products is rising annually in Indonesia as people become more conscious of the value of animal protein (Yulianto *et al*., 2024). The yearly consumption of chicken meat from the area contributes to the rising demand for chicken (Wahyono and Utami, 2018). The Agricultural Research and Development Agency located in Ciawi (Bogor) has conducted a selection to produce superior native chickens which are named the Superior Native Chickens of the Livestock Research Center (KUB) (Tirajoh *et al*., 2021). KUB chickens have advantages in higher egg and meat production, more uniform growth, rapid environmental adaptation and more efficient use of rations compared to native chickens in general (Zulfan *et al*., 2024).

KUB chicken industry faces several problems in initial growth and active biological activities according to market requirements to improve the socio-economic status of poultry-oriented communities in developing countries. In order to optimize production results, one of the efforts made by livestock farmers is to provide Antibiotic Growth Promoters (AGP), but in recent years the government has regulated the prohibition of the use of AGP in supporting the improvement of livestock quality and performance (Ma *et al*., 2021; Okafor *et al*., 2019). In this case, another solution that can be done is to provide alternative feed

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additives to replace AGP with the aim of increasing the absorption of nutrients in the feed (Al Arif *et al*., 2024; Warsito *et al*., 2024). One thing that needs to be done is adding phytase and protease enzymes to animal feed.

Increasing productivity by using enzymes in feed can improve the efficiency of feed use (Alagawany *et al*., 2018). One type of enzyme that is often used in animal feed is the phytase and protease enzymes (Miladinovic *et al*., 2021). The benefits of the phytase enzyme are to break down phytic acid contained in feed and release inorganic phosphorus and inositol (Rizwanuddin *et al*., 2023; Ayuti *et al.,* 2024). Phytase enzymes can also free proteins, trace minerals, amino acids and other nutrients that are attached to phytic acid (Bohn *et al*., 2008). Protease enzymes are useful for preventing the loss of amino acids, thereby improving production performance because nutrients will be more effectively used to improve livestock performance (Solanki *et al*., 2021).

One of the efforts to increase livestock production is through molecular or genetic markers. Genetic markers are also known as Marker Assisted Selection (MAS) (Collard and Mackill, 2008). One of the genes that is the main and determining gene in controlling growth traits and meat production is the Myostatin gene (MSTN) (Ceccobelli *et al*., 2022). The MSTN or Growth Differentiations Factor 8 (GDF-8) gene is a member of the Transforming Growth Factor (TGF) superfamily which functions as a regulator of skeletal muscle growth (Baig *et al*., 2022). The MSTN gene consists of one promoter, three exons and two introns (Khalil *et al*., 2017). The MSTN gene is one of the genes that is important for carcass and meat properties, one way to identify myostatin gene expression in KUB chickens is through the polymerase chain reaction (PCR) method.

Genetic improvement of KUB chickens for higher growth rates is an ongoing process, the nutritional needs of KUB chickens also change accordingly (Tona *et al*., 2022). For this reason, assessing the KUB chickens' nutritional requirements is a continuous procedure. The performance of KUB chickens is determined by the interaction of their genetic potential and feed nutrients. In recent years, nutrigenomic approaches have increased understanding of the influence of diet on gene expression (Mierziak *et al*., 2021). Based on this background, it is necessary to conduct research to determine the effect of adding phytase and protease enzymes in the ration on the performance of KUB chickens, as well as to determine the expression of the MSTN gene on KUB chicken carcass production.

MATERIALS AND METHODS

Ethical approval

The ethical clearance committee of the Faculty of Veterinary Medicine at Universitas Airlangga, Indonesia provided consent for the use of animals with number: 1. KEH.039.03.2024.

Study area and sample collection

The research was conducted from April 2024 to August 2024. This study used a completely randomized design (CRD) with 2 factors, namely factor A the addition of phytase enzyme with a dose of 0% (control), 0.02%, 0.04%, 0.06% and factor B the addition of protease enzyme with a dose of 0% (control), 0.03%, 0.05%, 0.07% consisting of 5 replications. This study used 35 KUB male chickens. Feeding was based on the needs of KUB chickens, feed was given 2 times a day, namely in the morning at 7.00 A.M and in the afternoon at 4.00 P.M. Preparation for the study and maintenance of KUB chickens were also carried out at the Faculty of Veterinary Medicine and sample analysis was carried out at the Institute of Tropical Disease, Airlangga University.

Enzyme supplementation treatment

A total of 20 blood samples were taken from superior Balitnak (KUB) native chickens, consisting of 8 blood samples from a group of male chickens with the same variety, age with feed supplemented with phytase enzyme, samples were taken from male chickens with the same body weight that were fed with a mixture of protease enzymes. Then after selection based on production performance during maintenance, 8 blood samples were taken again. As a control, 5 blood samples of chickens that were not given enzymes in their feed were also used. A total of 20 samples were used in this study.

Performance and organ sample collection

The following methods were used to calculate body weight increase, feed intake and feed conversion ratio (FCR). To calculate feed consumption, weekly weigh-ins and records of fed and unfed feed were made. The amount of feed supplied was subtracted from the amount of feed left to determine the amount of feed consumed. Weekly feed intake was computed and then recalculated for each chicken. The amount of feed consumed divided by the body weight gain during the 90-day period was used to calculate the FCR. In the meantime, the difference between the starting and final body weights up to 90 days of age, divided by the number of days of upbringing, was used to compute the average daily body weight growth (BWG). After reaching 90 days of age, the birds were slaughtered and feathered. The head, neck, calves and legs were cut off from the carcass. The internal organs, including the kidneys, liver, heart, lien and gizzard, were then taken out and weighed independently to determine how much they weighed in relation to the carcass.

MSTN gene detection

RNA extraction was performed using a promega kit (Sancko-Junyaku Co., Ltd., Tokyo, Japan). The procedure is as follows: 30 µl of blood sample plus 100 µl of solution 1 (tris buffer) was stirred slowly, then 100 µl of solution 2 (guanidine thiocyanate) was added and stirred until it came

out like mucus. 700 µl of solution 3 (chloroform) and 400 µl of solution 4 (Na-acetate) were added to the solution and stirred until evenly distributed, then centrifuged. The supernatant was transferred to a new tube, 60 µl of solution 5 (acetate buffer) and 600 µl of 2-propanol were added, centrifuged. The supernatant was discarded, the sediment was cleaned with 1 ml of 70% ethanol, centrifuged. The supernatant was discarded, the RNA precipitate was dried and added with 100 µl of TE (Tris-HCL EDTA) solution. The RNA concentration was calculated using 10 ng/µl prepared for PCR, namely DNA fragments were amplified with the MSTN gene marker with forward primers: 5′- AAC GGT GTT TGT GCA GAT CC -3′ and Reverse primers: 3′- CAA TCC ATC TTC ACC CGG TCC -5′ (Azhar *et al*., 2020). With these primers, a DNA fragment of 274 bp can be produced. To produce the MSTN gene RNA fragment, a mixture solution was made consisting of 10x buffer, 2 mM dNTP, 25 mM $MgSO_4$, MSTN primers (forward and reverse primers, 200 pmol/µl), KOD plus polymerase enzyme and water. Amplification was carried out using the Gene Amp thermocycler, PCR system 9700, PE Applied Biosystems. The PCR conditions were programmed, namely denaturation at 94°C for 1 minute 15 seconds, annealing was made in 3 types: 10 cycles (15 seconds at 94°C, 30 seconds at 60°C, 1 minute at 68°C), 10 cycles (15 seconds at 94°C, 30 seconds at 55°C, 1 minute at 68°C) and 10 cycles (15 seconds at 94°C, 30 seconds at 50°C, 1 minute at 68°C) and elongation for 9 minutes at 68°C. After the cycle ended, it was continued with a storage temperature of 4°C. The results of the amplification of the MSTN RNA fragment were detected by segregating the prolactin promoter band pattern with an electrophoresis system on a 2% agarose gel stained with ethidium bromide and viewed with an Ultra Violet device. Then documentation is carried out by photographing the electrophoresis results with a camera.

RESULTS AND DISCUSSION

KUB chicken performance

The results presented in Table 1 revealed significant differences (P<0.05) in the growth performance of KUB chickens among the treatments of phytase and protease enzymes with different doses. Body weight gain (BWG) of phytase enzyme group during the starter phase (0-3 weeks) was significantly lower compared to other treatments which were not significantly different from each other. During the grower phase (4-6 weeks) significantly higher BWG was observed in the treatment given protease enzyme followed by the treatment given phytase enzyme addition in the feed which was statistically different and lower BWG was observed in the control treatment which was statistically. Overall BWG in the finisher phase (0-6 weeks) was significantly higher in the treatment given protease enzyme followed by the phytase treatment and control treatment which were statistically similar, while lower BWG was observed in the control treatment. The interval factors of 4-5 weeks and 0-6 weeks were significantly lower in the control treatment compared to the treatments given phytase and protease enzymes in the feed which were not significantly different from each other.

The control treatment showed a much better feed conversion ratio (FCR) of 4-6 weeks and 0-6 weeks compared to the treatments at 0-3 weeks which were similar to each other. The results of feed consumption showed a significant difference (P<0.05) at 6 weeks of administration. The lowest feed consumption value was found for the control treatment, which was consistent with the treatments at 0-3 weeks, namely PF2, PF4, PF6, PP3, PP5 and PP7. The highest feed consumption value was found in the treatment at 0-6 weeks, which was not significantly different from the treatment at 4-5 weeks. The results of the feed conversion ratio showed that there was an interaction (P<0.05) in the 6 weeks of administration. The lowest feed conversion ratio value was found for the PF4 treatment at 0-6 weeks, which was consistent with the PP3 treatment at 6 weeks and PF4 at 4-6 weeks. The highest feed conversion ratio value was found for the control treatment at 4-6 weeks, which was not significantly different from the PP5 and PP7 treatments at 4-6 weeks. The average performance parameters (feed consumption, feed conversion ratio, body weight gain) for the addition of phytase and protease enzymes in the feed of KUB chickens are listed in Table 1.

Digestive enzyme activity is thought to be a significant element that can affect gut health and the ability of nutrients to be absorbed (Zhang, 2022; Ayuti *et al.,* 2024). Sufficient amounts of both phytase and protease in the diet are crucial for the digestive system of poultry since phytase supplements can enhance gut health and feed efficiency while proteases can influence the digesta microbiota and the activity of gut digestive enzymes (Bohn *et al*., 2008; Solanki *et al*., 2021). Protease can increase the activity of trypsin and chymotrypsin in chicks (Zheng *et al*., 2023). Conversely, adding phytase and protease to basal, low-energy and low-protein diets can both greatly boost the activity of chymotrypsin and trypsin in the jejunum of chicks (Jiang *et al*., 2020). Therefore, by boosting the digestive and absorptive capacities of KUB chickens and consequently improving their ability to gain weight, increasing the activity of trypsin and chymotrypsin through dietary supplementation with protease and phytase may have contributed to the improvement of nutrient digestibility.

In feed and poultry development performance, non-starch polysaccharide enzymes (mostly xylanases) and phytases can support one another to enhance protein and energy use (Junior *et al*., 2024). Low-energy diets can closely combine water-soluble non-starch polysaccharides and phytic acid, which facilitates the easier access of non-starch polysaccharide enzymes to the phytate surface and accelerates the rate of phytate breakdown (Gehring *et al*., 2013). Therefore, these roles are complementary to one another and we propose that phytate and proteases may interact to affect the activity of trypsin and chymotrypsin in the digestive tract.

Table 1: Growth performance (mean±S.E) of chickens under different dietary enzyme phytase and protease levels.

Growth performance	BWG (0-3 week)	BWG (4-6 week)	BWG (0-6 week)	FI (0-3 week)	FI (4-6 week)	FI (0-6 week)	FCR (0-3 week)	FCR (4-6 week)	FCR (0-6 week)
PK	211^{c} ± 0.08	269^{c}±0.22	480^{c}±0.15	317.73^{ab}±0.52	759.75^{b}±0.25	1077.48^{a}±0.14	1.41^{b}±0.47	2.29^{c}±0.40	1.54^{bc}±0.35
PF2	255^{bc}± 0.12	297^{bc}±0.25	553^{b}±0.18	322.88^{c}±0.55	756.25^{a}±0.22	1079.13^{b}±0.17	1.65^{bc}±0.45	1.96^{b}±0.42	1.51^{bc}±0.37
PF4	286^{a}±0.09	370^{a}±0.27	657^{a}±0.18	322.70^{b}±0.57	758.25^{a}±0.23	1080.95^{bc}±0.15	1.22^{a}±0.44	1.58^{a}±0.39	1.16^{a}±0.34
PF6	271^{b}±0.10	353^{b}±0.22	624^{ab}±0.14	322.53^{a}±0.48	759.75^{b}±0.25	1082.28^{c}±0.14	1.83^{c}±0.42	1.94^{b}±0.33	1.57^{c}±0.35
PP3	287^{a}±0.09	371^{a}±0.23	660^{a}±0.16	322.80^{c}±0.50	759.25^{b}±0.27	1082.05^{c}±0.18	1.35^{a}±0.44	1.47^{a}±0.39	1.18^{a}±0.40
PP5	260^{b}±0.11	370^{a}±0.19	631^{ab}±0.14	322.58^{a}±0.56	762.35^{c}±0.28	1084.93^{c}±0.15	1.48^{b}±0.42	2.00^{c}±0.40	1.45^{b}±0.35
PP7	255^{bc}±0.13	319^{bc}±0.21	575^{b}±0.13	322.73^{ab}±0.54	761.38^{bc}±0.26	1084.10^{c}±0.19	1.47^{b}±0.46	1.98^{b}±0.37	1.44^{b}±0.39
Analysis of variance of growth performance									
Interaction effect:	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05
P value	1	1	1	1	1	1	1	1	1
df	3.12	3.94	3.90	4.97	1.17	1.09	1.15	5.93	4.68
F value									
Phytase enzyme:	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05
P value	1	1	1	1	1	1	1	1	1
df	3.44	4.93	4.18	4.14	1.02	1.12	1.04	3.95	3.09
F value									
Protease enzyme:	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05
P value	1	1	1	1	1	1	1	1	1
df	3.63	4.07	3.72	4.05	1.75	1.22	1.34	0.76	0.99
F value									

[a,b,c] Mea without a common superscript were determined to be significantly different (P<0.05); df: Degree of freedom; S.E: Standard error the values bearing different superscripts within the column differ significantly; BWG: Body weight gain; FI: Feed intake; FCR: Feed conversion ratio; PK: Feed without multienzyme; PF2: Feed with the addition of phytase enzyme dose of 0.02%/kg feed; PF4: Feed with the addition of phytase enzyme dose of 0.04%/kg feed; PF6: Feed with the addition of phytase enzyme dose of 0.06%/kg feed; PP3: Feed with the addition of protease enzyme dose of 0.03%/kg feed; PP5: Feed with the addition of protease enzyme dose of 0.05%/kg feed; PP7: Feed with the addition of protease enzyme dose of 0.07%/kg feed.

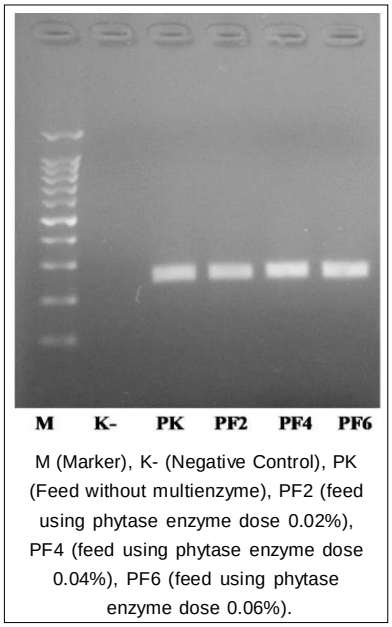
Table 2: Organ weight and per cent (mean±S.E) of chickens under different dietary enzyme phytase and protease levels.

Organ weight	Liver		Heart		Gizzard		Kidney	
and per cent	(g)	(%)	(g)	(%)	(g)	(%)	(g)	(%)
PK	25.95±6.28	18.93±2.16	6.88±0.29	5.13±0.72	29.88±5.51	22.52±6.11	7.88±1.78	5.83±2.33
PF2	22.38±2.73	17.70±1.17	6.85±0.59	5.46±0.68	32.40±2.76	25.69±1.38	5.90±1.22	4.72±1.88
PF4	25.08±5.93	18.70±2.34	5.75±0.45	4.28±1.87	30.35±5.06	22.79±1.23	5.75±.21	4.34±1.72
PF6	29.10±5.92	17.69±1.83	7.43±2.86	4.52±1.45	39.90±6.30	24.37±4.30	8.55±2.11	5.39±2.21
PP3	28.75±4.22	20.54±3.10	4.98±1.66	3.62±1.12	34.63±3.44	24.64±4.56	7.75±1.33	5.41±2.25
PP5	24.98±5.72	17.52±1.22	6.58±0.49	4.61±1.67	32.45±2.93	22.83±1.88	6.85±1.12	4.94±1.58
PP7	25.20±5.47	17.43±1.24	6.73±0.82	4.76±1.82	35.38±3.73	25.00±1.57	5.90±1.24	4.27±1.77
Analysis of variance of growth performance								
Interaction effect:	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
P value	1	1	1	1	1	1	1	1
df	2.32	4.14	2.90	3.07	1.37	1.69	1.25	4.13
F value								
Phytase enzyme:	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
P value	1	1	1	1	1	1	1	1
df	4.74	3.73	3.28	3.74	1.52	1.44	1.64	3.25
F value								
Protease enzyme:	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
P value	1	1	1	1	1	1	1	1
df	2.43	3.02	3.22	3.25	1.25	1.82	1.24	0.78
F value								

[a,b,c] Mea without a common superscript were determined to be significantly different (P<0.05); df: Degree of freedom; S.E: Standard error the values bearing different superscripts within the column differ significantly; PK: Feed without multienzyme; PF2: Feed with the addition of phytase enzyme dose of 0.02%/kg feed; PF4: Feed with the addition of phytase enzyme dose of 0.04%/kg feed; PF6: Feed with the addition of phytase enzyme dose of 0.06%/kg feed; PP3: Feed with the addition of protease enzyme dose of 0.03%/kg feed; PP5: Feed with the addition of protease enzyme dose of 0.05%/kg feed; PP7: Feed with the addition of protease enzyme dose of 0.07%/kg feed.

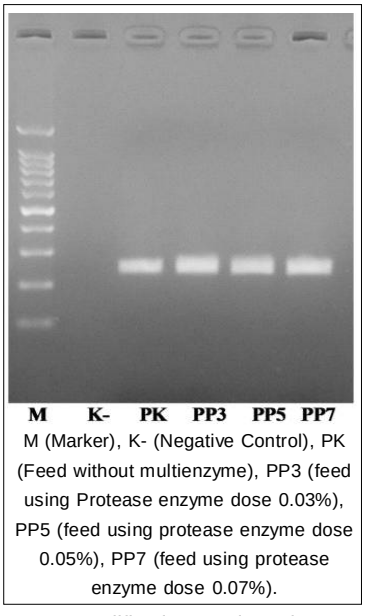
Effect of phytase and protease enzymes on organ

The main results of carcass characteristics are presented in Table 2. Internal organs showed a non-significant increase (P>0.05) in chickens fed with phytase and protease enzyme supplements compared to the control group. The results of earlier studies that found that the administration of mixed enzymes did not alter relative liver weight are consistent with our conclusion (Tüzün *et al.,* 2020). Additionally, according to Salazar-Villanea *et al*. (2023), the relative weight of internal organs such the liver, gizzard and heart was unaffected by the injection of phytase and protease. According to a study that found a negative interaction between amylase and protease that decreased the relative weight of the gizzard at 21 days of age but did not show this difference at 7 days, the maximum weight of these organs was reached before 9 days of age (Radhi *et al.,* 2023). This is believed to be the result of the feed's high insoluble fiber content, which promotes gizzard development. As a result, the feed's composition and the chicken's age at observation have a significant impact on how the digestive organs react to enzyme supplementation.



M (Marker), K- (Negative Control), PK (Feed without multienzyme), PF2 (feed using phytase enzyme dose 0.02%), PF4 (feed using phytase enzyme dose 0.04%), PF6 (feed using phytase enzyme dose 0.06%).

Fig 1: MSTN gene amplification results using PCR technique on 1.5% agarose gel.



M (Marker), K- (Negative Control), PK (Feed without multienzyme), PP3 (feed using Protease enzyme dose 0.03%), PP5 (feed using protease enzyme dose 0.05%), PP7 (feed using protease enzyme dose 0.07%).

Fig 2: MSTN gene amplification results using PCR technique on 1.5% agarose gel.

These results suggest that enzyme supplementation may hasten the development of immunological organs. However, Baloch *et al*. (2021) found that adding protease to male hens' feed between the ages of 7 and 21 days considerably raised the spleen's relative weight. They link this to enhanced nutrition absorption, better digestive processes and better metabolic hormone management, all of which can tangentially strengthen the immune system (Kaimkhani *et al.,* 2025). The relative weight of chicken liver in this investigation was not significantly impacted by the addition of phytase and protease enzymes. These outcomes corroborate those of Abd El Latif *et al*. (2023), who found that adding enzymes to diet, such as phytase or mixed enzymes, had no effect on the relative weight of the liver. This implies that while feed enzymes can increase the effectiveness of digestion and nutrient absorption, their effect on liver growth is negligible, particularly when chickens are in their typical physiological state (Walk *et al.,* 2018). Although the increase in the enzymes' metabolic efficiency may indirectly improve the chicken's overall performance, this lack of influence can be interpreted as meaning that the feed enzymes have no direct structural impact on the heart or cardiovascular system (Alagawany *et al.,* 2021). There were no appreciable changes in the relative weights of the kidney and gizzard, which likewise responded similarly (Sozcu, 2019). The amount of crude fiber and the physical characteristics of the meal have a significant impact on the development of the gizzard, an organ crucial to the mechanical process of digestion. According to Maynard *et al*. (2023), a high insoluble fiber content can promote the development of the gizzard, though this effect was not consistently seen at all age stages.

The purpose of adding phytase and protease enzymes to poultry feed is to improve the efficiency of nutrient use, particularly for protein and minerals, which can affect organ function and growth directly or indirectly (Saleh *et al.,* 2025). Increased liver metabolic performance may result from improved digestive efficiency, even if the liver's relative weight may not usually alter much (Gautier *et al.,* 2018). Enzyme administration also helps the kidneys and heart, particularly with regard to body homeostasis and metabolic stability (Sprigg *et al.,* 2022). Since energy and vital nutrients like minerals and amino acids are more readily available, circulatory function in the heart can be strengthened by improved nutritional status (Hakami *et al.,* 2022).

Even though research indicates that the size of the kidneys and heart does not typically alter much, a better nutrient balance can nevertheless have a good effect on these two organs' physiological function through the use of enzyme supplements (Huyan *et al.,* 2022). The physical makeup and composition of the diet have a significant impact on the gizzard, a mechanical digesting organ (Ghosh *et al.,* 2016). Although they don't have a direct mechanical impact on the gizzard, proteases and phytoses can alter the digesta's texture and fermentability, which can alter the gizzard's ibility to grind feed (Hao *et al.,* 2018). Enzyme administration has been demonstrated in multiple trials to decrease the gizzard's relative weight because it reduces insoluble crude fiber, which increases the gizzard's mechanical action (Ren *et al.,* 2015). Nevertheless, the gizzard continues to function at its best physically in the digestive system and the effectiveness of nutrient absorption rises as a result of enzymes that assist in the breakdown of complicated substrates into simpler molecules that the small intestine can absorb more readily.

MTSN gene expression

The results of MSTN gene amplification in KUB chickens and the administration of phytase and protease enzymes in feed with different doses at the 4842 bp base position using a Thermal Cycler machine, obtained a PCR product with a length of 247 base pairs (bp). Based on Fig 1 and 2. the results of RNA extraction electrophoresis show that the RNA band looks quite clear and even so that there is no need for repetition. Many factors affect the quality of RNA in addition to the dose of enzyme use in feed and the difference in the amount of enzyme dose used, one of which affects the quality of RNA is the lysis process, which is a process of breaking down the cell nucleus and the concentration of RNA rehydration given. The cell nucleus can be lysed properly, so the resulting RNA has a high enough concentration so that it affects the quality and quantity of RNA (Table 3).

MSTN gene expression in phytase enzyme treatment with different doses was significantly (P<0.05) upregulated in PF2 feed treatment and feed treatment without phytase enzyme with respect to PF4 and PF6 treatments (Fig 3). In protease enzyme treatment with different doses was significantly (p<0.05) upregulated in PP7 and PP3 feed treatments gene expression was significantly upregulated compared to PP5 treatment and feed treatment without protease enzyme was significantly different from each other (Fig 4). In protease enzyme treatment gene expression in PP7 and PP3 was significantly higher than other treatments, followed by phytase enzyme treatment in PF2 treatment, while the lowest expression was observed in feed treatment with phytase enzyme administration in PF6

Table 3: dCT, ddCT and dddCT values of phytase enzyme treatment of MSTN genes.

Sample	Exon2	GAPDH	DCt	ddCt	2^ddCt	Expression
PK	26.03	29.67	-3.63991	0	1	1^{a}±0.39
PF2	25.88	29.74	-3.85731	-0.2174	0.86	1.16^{a}±0.42
PF4	25.23	28.66	-3.42325	0.216653	1.16	0.86^{b}±0.32
PF6	25.17	28.20	-3.03365	0.606258	1.52	0.66^{c}±0.25

^{a,b,c} Mea without a common superscript were determined to be significantly different (P<0.05); PK (Feed without multienzymes), PF2 (feed using phytase enzyme dose of 0.02%), PF4 (feed using phytase enzyme dose of 0.04%), PF6 (feed using phytase enzyme dose of 0.06%).

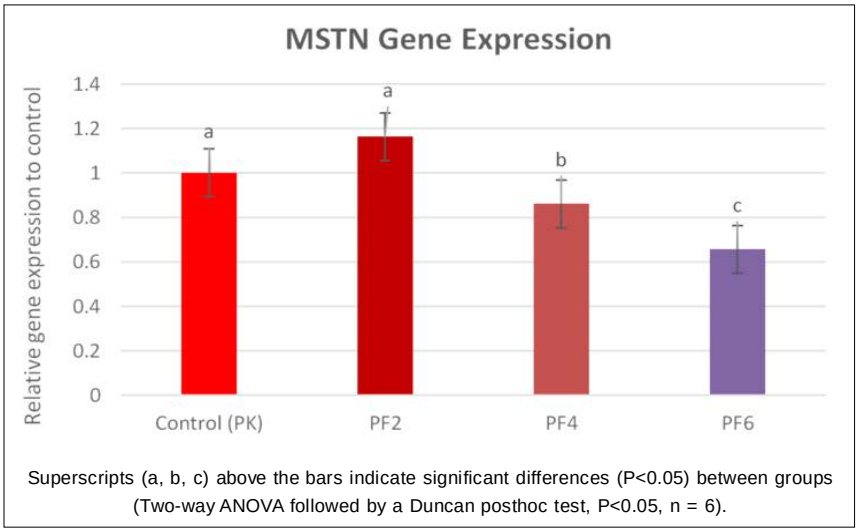


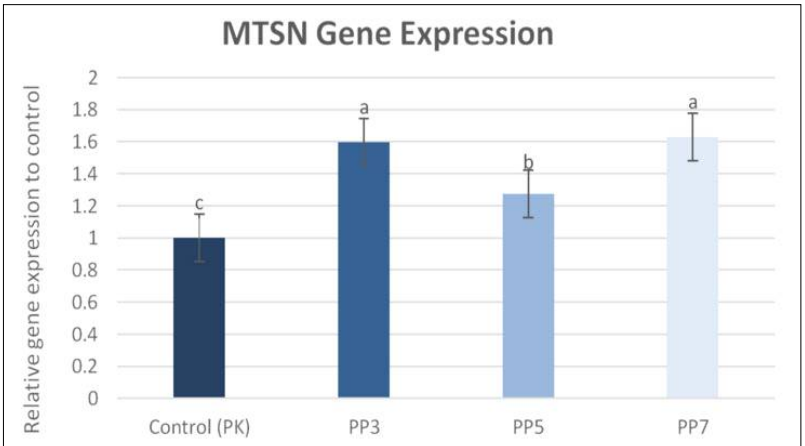
Fig 3: MSTN expression profile in KUB chicken at different doses of phytase enzyme under different dietary groups (mean±SEM).

followed by PF4 treatment. In general, fold expression was higher in PP7 protease enzyme administration treatment with different doses than phytase enzyme administration treatment (Table 4).

MSTN gene transcript expression was detected in all enzyme doses investigated in this study. Regardless of the type of enzyme used in the feed with different doses, in relation to the reference gene, the mRNA expression levels of the target gene varied significantly at different enzyme doses. In the administration of protease enzyme, MSTN gene transcripts had relatively higher expression, namely in PP7 (1.63), PP3 (1.59), PP5 (1.28) and the treatment without protease enzyme. When compared to the expression in the feed given phytase enzyme, MSTN gene expression was regulated in the phytase enzyme treatment by PF2 (1.16), PF4 (0.86), PF6 (0.66) and the treatment without phytase enzyme. However, among all enzyme doses, those with higher and more gene expression were in the treatment with protease enzyme administration at a dose of 0.07% (PP7).

The Myostatin (MSTN) gene is a negative regulator that is predominantly expressed in skeletal muscle (Chen *et al*., 2021). Instructions for producing the protein myostatin, which is active in skeletal muscle before and after birth, are provided by this gene (Baig *et al*., 2022). Normally, this protein regulates muscle growth to prevent excessive muscle growth (Ramasamy *et al*., 2017). Treating myoblasts (muscle cells) with myostatin results in reduced cell proliferation and differentiation (McFarlane *et al*., 2011). Myostatin (MSTN) is the only inhibitor of skeletal muscle growth and development. Loss of myostatin function increases the diameter and amount of muscle mass (Jang *et al*., 2021; Ayuti *et al.,* 2024). The potential for myostatin inhibition to increase muscle mass in KUB chickens has attracted interest in the poultry industry as a potential selection mechanism for increasing meat production. The effects of myostatin inhibition have also been investigated on egg production to determine whether myostatin could be used in the laying hen industry to increase egg production (Lee *et al*., 2021; Akintunde and Toye, 2024).

In KUB chickens, the expression of the myostatin (MSTN) gene is linked to body weight, growth rate and muscle development. Through the activation and renewal of satellite cells, myostatin expression controls the growth and development of muscle fibers (McCroskery *et al*., 2003). A study comparing Daweishan mini chickens (DMC) and commercial poultry broilers found that the DMC birds had a lower growth rate than the commercial poultry broilers (Dou *et al*., 2018). Variations in body weight in hens are linked to polymorphisms in the myostatin gene (Tanjung *et al*., 2019; Kannaki *et al*., 2017). To develop a



Superscripts (a, b, c) above the bars indicate significant differences (P<0.05) between groups (Two-way ANOVA followed by a Duncan posthoc test, P<0.05, n = 6).

Fig 4: MSTN expression profile in KUB chicken at different doses of protease enzyme under different dietary groups (mean±SEM).

Table 4: dCT, ddCT and dddCT values of protease enzyme treatment of MSTN genes.

Sample	Exon2	GAPDH	DCt	ddCt	2^ddCt	expression
PK	26.03	29.67	-3.63991	0	1	1^{c}±0.55
PP3	26.16	30.47	-4.3132	-0.67329	0.63	1.59^{a}±0.62
PP5	26.03	30.02	-3.99113	-0.35122	0.78	1.28^{b}±0.56
PP7	25.56	29.90	-4.34352	-0.70361	0.61	1.63^{a}±0.65

^{a,b,c} Mea without a common superscript were determined to be significantly different (P<0.05); PK (Feed without multienzymes), PP3 (feed using Protease enzyme dose of 0.03%), PP5 (feed using protease enzyme dose of 0.05%), PP7 (feed using protease enzyme dose of 0.07%).

tolerance to cold, chicks must control the expression of the myostatin gene in their leg muscles by the time they are seven days old (Ijiri *et al*., 2009). In freshly hatched chicks, myostatin is sensitive to the availability of nutrients and fasting lowers the amounts of MSTN mRNA in the muscle of older chickens (Saneyasu *et al*., 2015). The MSTN gene belongs to the superfamily of transforming growth factor beta (TGFβ), a class of proteins that aid in regulating the expansion and maturation of bodily tissues (Baig *et al*., 2022; Nihar *et al*., 2024).

CONCLUSION

The results of the study revealed significant differences ($P<0.05$) in the growth performance of KUB chickens between the treatments of phytase and protease enzymes with different doses. The expression of MSTN gene transcripts was detected in all enzyme doses investigated in this study.

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Disclaimers

The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

The ethical clearance committee of the Faculty of Veterinary Medicine at Universitas Airlangga, Indonesia provided consent for the use of animals with number: 1. KEH.039.03.2024.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

REFERENCES

Abd El Latif, M.A., Abdel-Wareth, A.A.A., Daley, M., Lohakare, J. (2023). Effect of dietary orange peel meal and multi-enzymes on productive, physiological and nutritional responses of broiler chickens. *Animals (Basel)*. **13:** 2473. doi: 10.3390/ani13152473.

Akintunde, A.O., Toye, A.A. (2024). Comparative study on egg characteristics of yoruba ecotype nigerian local chickens and Isa brown chickens fed graded levels of *Moringa oleifera* seed meal. *Agricultural Science Digest*. **43(8):** 877-882. doi: 10.18805/ag.DF-430.

Al Arif, M.A., Warsito, S.H., Lamid, M., Lokapirnasari, W.P., Khairullah, A.R., Ayuti, S.R. *et al*. (2024). Phytochemical analysis of curry leaf extract (*Murraya koenigii* L.) as a potential animal feed and medicinal ingredient. *Pharmacognosy Journal*. **16:** 471-477. doi: 10.5530/pj.2024.16.75.

Alagawany, M., Ashour, E.A., El-Kholy, M.S., Mohamed, L.A., Abd El-Hack, M.E. (2021). Effect of dietary calcium and phosphorus levels on growth, carcass characteristics and liver and kidney functions of growing Egyptian geese. *Poultry Science*. **100:** 101244. doi: 10.1016/j.psj.2021.101244.

Alagawany, M., Elnesr, S.S., Farag, M.R. (2018). The role of exogenous enzymes in promoting growth and improving nutrient digestibility in poultry. *Iranian Journal of Veterinary Research*. **19:** 157-164.

Ayuti, S.R., Khairullah, A.R., Al-Arif, M.A., Lamid, M., Warsito, S.H., Moses, I.B., Hermawan, I.P., Silaen, O.S.M., Lokapirnasari, W.P., Aryaloka, S. and Ferasyi, T.R. (2024). Tackling salmonellosis: A comprehensive exploration of risks factors, impacts and solutions. *Open Veterinary Journal*. **14(6):** 1313-1329. doi: 10.5455/OVJ.2024.v14.i6.1.

Ayuti, S.R., Khairullah, A.R., Lamid, M., Al-Arif, M.A., Warsito, S.H., Martua Silaen, O.S., Moses, I.B., Hermawan, I.P., Yanestria, S.M., Delima, M. and Ferasyi, T.R. (2024). Avian influenza in birds: Insights from a comprehensive review. *Veterinary World*. **17(11):** 2544-2555. doi: 10.14202/vetworld.2024.2544-2555.

Ayuti, S.R., Lamid, M., Warsito, S.H., Al-Arif, M.A., Lokapirnasari, W.P., Rosyada, Z.N.A., Sugito, S., Akmal, M., Rimayanti, R., Gangil, R., Khairullah, A.R., Abuzahra, M., Moses, I.B. and Anggraini, L. (2024). A review of myostatin gene mutations: Enhancing meat production and potential in livestock genetic selection. *Open Vet. J*. **14(12):** 3189-3202. doi: 10.5455/OVJ.2024.v14.i12.4.

Azhar, A., Akmal, M., Hambal, M., Sabri, M., Rosa, T.S. (2020). Effects of polymorphism of myostatin and fatty acid-binding protein 4 genes on the chemical composition of meat in cull female Aceh cattle. *Veterinary World*. **13:** 1334-1343. doi: 10.14202/vetworld.2020.1334-1343.

Baig, M.H., Ahmad, K., Moon, J.S., Park, S.Y., Lim, J.H., Chun, H.J., Qadri, A.F., Hwang, Y.C., Jan, A.T., Ahmad, S.S., Ali, S., Shaikh, S., Lee, E.J., Choi, I. (2022). Myostatin and its regulation: A comprehensive review of myostatin inhibiting strategies. *Frontiers in Physiology*. **13:** 876078. doi: 10.3389/fphys.2022.876078.

Baloch, F.N., Baloch, H.N., Khan, A.U., Baloch, N.M., Baloch, I.A., Baloch, N.K., Qambrani, A.Q., Samejo, A.K. (2021). Effect of phytase enzyme on organs growth performance and blood profile of broiler. *Advances in Enzyme Research.* **9:** 37-49. doi: 10.4236/aer.2021.93004.

Bohn, L., Meyer, A.S., Rasmussen, S.K. (2008). Phytate: Impact on environment and human nutrition. A challenge for molecular breeding. *Journal of Zhejiang University-SCIENCE B*. **9(3):** 165-191. doi: 10.1631/jzus.B0710640.

Ceccobelli, S., Perini, F., Trombetta, M.F., Tavoletti, S., Lasagna, E., Pasquini, M. (2022). Effect of myostatin gene mutation on slaughtering performance and meat quality in marchigiana bulls. *Animals (Basel).* **12:** 518. doi: 10.3390/ani12040518.

Chen, M.M., Zhao, Y.P., Zhao, Y., Deng, S.L., Yu, K. (2021). Regulation of myostatin on the growth and development of skeletal muscle. *Frontiers in Cell and Developmental Biology*. **9:** 785712. doi: 10.3389/fcell.2021.785712.

Collard, B.C., Mackill, D.J. (2008). Marker-assisted selection: An approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B - Journals*. **363:** 557-572. doi: 10.1098/rstb.2007.2170.

Dou, T., Li, Z., Wang, K., Liu, L., Rong, H., Xu, Z., Huang, Y., Gu, D., Chen, X., Hu, W., Zhang, J., Zhao, S., Jois, M., Li, Q., Ge, C., Pas, M.F.W.T., Jia, J. (2018). Regulation of myostatin expression is associated with growth and muscle development in commercial broiler and DMC muscle. *Molecular Biology Reports*. **45:** 511-522. doi: 10.1007/s11033-018-4187-7.

Gautier, A.E., Walk, C.L., Dilger, R.N. (2018). Effects of a high level of phytase on broiler performance, bone ash, phosphorus utilization and phytate dephosphorylation to inositol. *Poultry Science*. **97:** 211-218. doi: 10.3382/ps/pex291.

Gehring, C.K., Bedford, M.R., Dozier, W.A. (2013). Interactive effects of phytase and xylanase supplementation with extractable salt-soluble protein content of corn in diets with adequate calcium and nonphytate phosphorus fed to broilers. *Poultry Science*. **92:** 1858-1869. doi: 10.3382/ps.2013-03010.

Ghosh, A., Mandal, G.P., Roy, A., Patra, A.K. (2016). Effects of supplementation of manganese with or without phytase on growth performance, carcass traits, muscle and tibia composition and immunity in broiler chickens. *Livestock Science*. **191:** 80-85. doi: 10.1016/j.livsci.2016.07.014.

Hakami, Z., Al Sulaiman, A.R., Alharthi, A.S., Casserly, R., Bouwhuis, M.A., Abudabos, A.M. (2022). Growth performance, carcass and meat quality, bone strength and immune response of broilers fed low-calcium diets supplemented with marine mineral complex and phytase. *Poultry Science*. **101:** 101849. doi: 10.1016/j.psj.2022.101849.

Hao, X.Z., Yoo, J.S., Kim, I.H. (2018). Effect of phytase supplementation on growth performance, nutrient digestibility and meat quality in broilers. *Korean Journal of Agricultural Science*. **45:** 401-409. doi: 10.7744/kjoas.20170041.

Huyan, L., Kumar, A., Manafi, M., Soumeh, E.A. (2022). Effects of protease supplementation on growth performance, organ development, gut morphology and microbial profile of broiler chicken. *Acta Agriculturae Scandinavica, Section A-Animal Science*. **71:** 40-50. doi: 10.1080/09064702.2022.2113121.

Ijiri, D., Miura, M., Kanai, Y., Hirabayashi, M. (2009). Increased mass of slow-type skeletal muscles and depressed myostatin gene expression in cold-tolerant chicks. *Zoological Science*. **26:** 277-283. doi: 10.2108/zsj.26.277.

Jang, J., Park, S., Kim, Y., Jung, J., Lee, J., Chang, Y., Lee, S.P., Park, B.C., Wolfe, R.R., Choi, C.S., Kim, I.Y. (2021). Myostatin inhibition-induced increase in muscle mass and strength was amplified by resistance exercise training and dietary essential amino acids improved muscle quality in mice. *Nutrients*. **13:** 1508. doi: 10.3390/nu13051508.

Jiang, J., Wu, H., Zhu, D., Yang, J., Huang, J., Gao, S., Lv, G. (2020). Dietary supplementation with phytase and protease improves growth performance, serum metabolism status and intestinal digestive enzyme activities in meat ducks. *Animals (Basel)*. **10:** 268. doi: 10.3390/ani10020268.

Junior, D.T.V., Genova, J.L., Kim, S.W., Saraiva, A., Rocha, G.C. (2024). Carbohydrases and phytase in poultry and pig nutrition: A review beyond the nutrients and energy matrix. *Animals (Basel)*. **14:** 226. doi: 10.3390/ani14020226.

Kaimkhani, A.Z., Mughal, G.A., Manzoor, M.N., Khan, Z.A., Urooj, M. (2025). Effect of supplementation of different levels of protease enzyme on growth performance and protein digestibility of broiler. *Pure and Applied Biology (PAB)*. **14:** 43-52. doi: 10.19045/bspab.2025.140006.

Kannaki, R.T., Reddy, M.R., Raja, K.S.R., Chatterjee, R.N. (2017). Genetic diversity analysis of the major histocompatibility complex (MHC) region in Indian native chicken breeds and pureline chickens using the LEI0258 microsatellite marker. *Indian Journal of Animal Research*. **51:** 998-1001. doi: 10. 18805/ijar.v0iOF.6989.

Khalil, K., Elayat, M., Khalifa, E., Daghash, S., Elaswad, A., Miller, M., Abdelrahman, H., Ye, Z., Odin, R., Drescher, D., Vo, K., Gosh, K., Bugg, W., Robinson, D., Dunham, R. (2017). Generation of myostatin gene-edited channel catfish (*Ictalurus punctatus*) *via* zygote injection of CRISPR/Cas9 System. *Scientific Reports*. **7:** 7301. doi: 10.1038/s41598-017-07223-7.

Lee, J., Kim, D.H., Brower, A.M., Schlachter, I., Lee, K. (2021). Effects of myostatin mutation on onset of laying, egg production, fertility and hatchability. *Animals (Basel)*. **11:** 1935. doi: 10.3390/ani11071935.

Ma, F., Xu, S., Tang, Z., Li, Z., Zhang, L. (2021). Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health*. **3:** 32-38. doi: 10.1016/j.bsheal.2020.09.004.

Maynard, C.J., Maynard, C.W., Mullenix, G.J., Ramser, A., Greene, E.S., Bedford, M.R., Dridi, S. (2023). Impact of phytase supplementation on meat quality of heat-stressed broilers. *Animals*. **13:** 2043. doi: 10.3390/ani13122043.

McCroskery, S., Thomas, M., Maxwell, L., Sharma, M., Kambadur, R. (2003). Myostatin negatively regulates satellite cell activation and self-renewal. *Journal of Cell Biology*. **162:** 1135-1147. doi: 10.1083/jcb.200207056.

McFarlane, C., Hui, G.Z., Amanda, W.Z., Lau, H.Y., Lokireddy, S., Xiaojia, G., Mouly, V., Butler-Browne, G., Gluckman, P.D., Sharma, M., Kambadur, R. (2011). Human myostatin negatively regulates human myoblast growth and differentiation. *American Journal of Physiology-Cell Physiology*. **301:** C195-203. doi: 10.1152/ajpcell.00012.2011.

Mierziak, J., Kostyn, K., Boba, A., Czemplik, M., Kulma, A., Wojtasik, W. (2021). Influence of the bioactive diet components on the gene expression regulation. *Nutrients*. **13:** 3673. doi: 10.3390/nu13113673.

Miladinovic, D.D., Storebakken, T., Lekang, O.I., Salas-Bringas, C. (2021). The effect of feed enzymes phytase, protease and xylanase on pelleting of microalgal biomass. *Heliyon*. **7:** e08598. doi: 10.1016/j.heliyon.2021.e08598.

Nihar, S.., Kumar, N.S., Ruban, W., Yathish, H.M., Nagaraja R., Nagaraja C.S. (2024). Genetic polymorphisms of chicken antiviral *Mx1* and *TVB* genes in indigenous and giriraja chicken. *Indian Journal of Animal Research*. **58(12):** 2052-2056. doi: 10.18805/IJAR.B-4710.

Okafor, O.L., Okoro, V.M.O, Mbajiorgu, C.A., Okoli, I.C., Ogbuewu, I.P., Ogundu, U.E. (2019). Influence of chicken growth hormone (cGH) SNP genotypes on morphometric and growth traits of three chicken breeds in Nigeria. *Indian Journal of Animal Research*. **53(12):** 1559-1565. doi: 10.18805/ijar.B-1077.

Radhi, K.S., Arif, M., Rehman, A.U., Faizan, M., Almohmadi, N.H., Youssef, I.M., Swelum, A.A., Suliman, G.M., Tharwat, M., Ebrahim, A., Abd El-Hack, M.E., Mahrose, K.M. (2023). Growth performance of broiler chickens fed diets supplemented with amylase and protease enzymes individually or combined. *Open Veterinary Journal*. **13:** 1425-1435. doi: 10.5455/OVJ.2023.v13.i11.5.

Ramasamy, K.T., Reddy M.R., Raja K.S.R., Chatterjee, R.N. (2017). Chicken *Mx* gene polymorphisms in Indian native chicken breeds and White Leghorn by real time multiplex allele specific PCR. *Indian Journal of Animal Research*. **52:** 649-651. doi: 10.18805/ijar.B-3285.

Ren, L., Zuo, J., Li, G., Zheng, L., Zhang, Z., Ye, H., Xia W.I., Feng, D. (2015). Effects of the combination of non-phytate phosphorus, phytase and 25-hydroxycholecalciferol on the performance and meat quality of broiler chickens. *Revista Brasileira de Cıkncia Avícola*. **17:** 371-380. doi: 10.1590/1516-635×1703371-380.

Rizwanuddin, S., Kumar, V., Singh, P., Naik, B., Mishra, S., Chauhan, M., Saris, P.E.J., Verma, A., Kumar, V. (2023). Insight into phytase-producing microorganisms for phytate solubilization and soil sustainability. *Frontiers in Microbiology*. **14:** 1127249. doi: 10.3389/fmicb.2023.1127249.

Salazar-Villanea, S., Herrera-Muñoz, J.I., Salas-Durán, C. (2023). Effect of phytase and protease supplementation on growth performance, protein digestibility and gizzard and pancreas development of broilers fed with low-protein diets. *Animal Feed Science and Technology*. **304:** 115717. doi: 10.1016/j.anifeedsci.2023.115717.

Saleh, A.A., El-Naggar, K., El Sayed El Bayoumi, M., Amber, K., MA, K., Alhotan, R.A., Osman Hussein, E., Galik, B., Kirrella, A.A. (2025). Effects of supplementing low-energy and/or protein diets with xylanase and protease on broiler chicken growth performance, nutritional utilization, blood biochemical parameters and gene expression associated with growth. *Cogent Food and Agriculture*. **11:** 2520959. doi: 10.1080/23311932.2025.2520959.

Saneyasu, T., Kimura, S., Inui, M., Yoshimoto, Y., Honda, K., Kamisoyama, H. (2015). Differences in the expression of genes involved in skeletal muscle proteolysis between broiler and layer chicks during food deprivation. *Comparative Biochemistry and Physiology Part B*. **186:** 36-42. doi: 10.1016/j.cbpb.2015.04.007.

Solanki, P., Putatunda, C., Kumar, A., Bhatia, R., Walia, A. (2021). Microbial proteases: Ubiquitous enzymes with innumerable uses. *3 Biotech*. **11:** 428. doi: 10.1007/s13205-021-02928-z.

Sozcu, A. (2019). Growth performance, pH value of gizzard, hepatic enzyme activity, immunologic indicators, intestinal histomorphology and cecal microflora of broilers fed diets supplemented with processed lignocellulose. *Poultry Science*. **98:** 6880-6887. doi: 10.3382/ps/pez449.

Sprigg, C., Whitfield, H., Burton, E., Scholey, D., Bedford, M.R., Brearley, C.A. (2022). Phytase dose-dependent response of kidney inositol phosphate levels in poultry. *Plos One*. **17:** e0275742. doi: 10.1371/journal.pone.0275742.

Tanjung, A., Saragih, H.T.S.S.G., Trijoko, Soenarwan, H.P., Widianto, S., Mahardhika, I.W.S., Daryono, B.S. (2019). Polymorphism of myostatin gene and its association with body weight traits in a hybrid of GAMA chicken (*Gallus gallus domesticus* Linn. 1758). *Biodiversitas*. **20:** 3207-3212. doi: 10.13057/biodiv/d201113.

Tirajoh, S., Dominanto, G.P., Usman, Soplanit, A., Bakrie, B. (2021). Production performance of KUB chicken with the inclusion of Lamtoro (*Leucaena leucocephala*) leaf flour in the feed. *IOP Conference Series: Earth and Environmental Science*. **788:** 012035. doi: 10.1088/1755-1315/788/1/012035.

Tona, K., Voemesse, K., N'nanlé, O., Oke, O.E., Kouame, Y.A.E., Bilalissi, A., Meteyake, H., Oso, O.M. (2022). Chicken incubation conditions: Role in embryo development, physiology and adaptation to the post-hatch environment. *Frontiers in Physiology*. **13:** 895854. doi: 10.3389/fphys.2022.895854.

Tüzün, A.E., Olgun, O., Yıldız, A.Ö., Şentürk, E.T. (2020). Effect of different dietary inclusion levels of sunflower meal and multi-enzyme supplementation on performance, meat yield, ileum histomorphology and pancreatic enzyme activities in growing quails. *Animals (Basel)*. **10:** 680. doi: 10.3390/ani10040680.

Wahyono, N.D., Utami, M.M.D. (2018). A review of the poultry meat production industry for food safety in Indonesia. *IOP Conference Series: Journal of Physics*. **953:** 012125. doi: 10.1088/1742-6596/953/1/012125.

Walk, C.L., Bedford, M.R., Olukosi, O.A. (2018). Effect of phytase on growth performance, phytate degradation and gene expression of myo-inositol transporters in the small intestine, liver and kidney of 21 day old broilers. *Poultry Science*. **97:** 1155-1162. doi: 10.3382/ps/pex392.

Warsito, S.H., Lamid, M., Arif, M.A.A., Lokapirnasari, W.P., Ayuti, S.R., Khairullah, A.R., Delima, M., Hermawan, I.P., Gangil, R., Ahmad, Z. (2024). Analysis of quercetin levels in the ethanol extract of curry leaves (*Murraya koenigii* L.) as a potential animal feed using high-performance liquid chromatography. *BIO Integration*. **5:** 974-978. doi: 10.15212/bioi-2024-0031.

Yulianto, A.B., Lamid, M., Lokapirnasari, W.P., Al-Arif, M.A., Sherasiya, A., Marbun, T.D., Khairullah, A.R., Kurniawan, S.C., Hasib, A., Silaen, O.S.M., Rehman, S. (2024). Hematological and performance variables of male broiler chickens fed with *Moringa oleifera* extract and probiotic in drinking water. *Tropical Animal Science Journal*. **47:** 215-224. doi: 10.5398/tasj.2024.47.2.215.

Zhang, P. (2022). Influence of foods and nutrition on the gut microbiome and implications for intestinal health. *International Journal of Molecular Sciences*. **23:** 9588. doi: 10.3390/ijms23179588.

Zheng, M., Bai, Y., Sun, Y., An, J., Chen, Q., Zhang, T. (2023). Effects of different proteases on protein digestion *In vitro* and *In vivo* and growth performance of broilers fed corn-soybean meal diets. *Animals (Basel)*. **13:** 1746. doi: 10.3390/ani13111746.

Zulfan, Yaman, M.A., Ariga, M.F., Ammar, M. (2024). The ability of KUB rooster to produce Indonesian super native chickens. *Indonesian Journal of Animal Science*. **34:** 108-117. doi: 10.21776/ub.jiip.2024 .034.01.12.