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Influence of Dietary Protein Source on Muscle Structure and Myogenic Gene Expression in Broiler Chicken Pectoralis Major Muscle

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Abstract Broiler breast muscle development is strongly affected by nutritional quality, particularly the source and biological value of dietary protein. Dietary protein source may influence muscle fiber growth, connective tissue organization, glycogen deposition and the transcriptional activity of myogenic regulatory genes. The aim of the present study was to evaluate the effect of dietary protein source on muscle structure, glycogen content and myogenic gene expression in the pectoralis major muscle of broiler chickens. A total of 9000 one-day-old broiler chicks were raised under field conditions and divided into two dietary groups: an animal protein-based diet containing fish meal and a plant protein-based diet based on corn-soybean meal. Samples of pectoralis major muscle were taken at 42 days of age and analyzed using morphological, histological, histochemical and molecular techniques. Birds fed the animal protein diet had significantly larger muscle fiber area and muscle bundle area than birds fed the plant protein diet ($P < 0.05$), whereas birds fed the plant protein diet exhibited thicker connective tissue layers. Periodic acid-Schiff staining showed greater glycogen deposition in the animal protein group. RT-qPCR analysis showed that MYF6 (MRF4) and MYOG were significantly more expressed in the animal protein group than in the plant protein group ($P < 0.05$). In conclusion, animal protein enhanced muscle hypertrophy, glycogen storage and myogenic gene expression in the pectoralis major muscle, indicating the importance of protein quality in improving broiler production and meat quality.

Keywords: Broiler chickens; Pectoralis major; Dietary protein source; MYF6; MYOG; Glycogen

Introduction Broiler chickens are an important part of the global food chain, a necessary and inexpensive source of high-quality animal protein. Broiler meat is a popular food because it has high nutritional value, good protein content, and relatively low fat content compared with other animal foods (1-3). Also, broiler chickens have the advantage of fast growth and high efficiency in feed-to-meat conversion and are a major contributor to the expansion of the poultry industry worldwide (4, 5).

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In broilers, skeletal muscles are the most important tissue in meat production, with the pectoralis major muscle being the largest part of the edible muscle in chickens. Therefore, this muscle is of considerable economic and biological importance in the research of growth

performance and meat quality (5). Muscle development in broilers is a complex process regulated by both structural and molecular factors, such as muscle fiber formation, connective tissue organization, and metabolic activity.

Growth performance is one of the important traits in poultry production because it has direct impact on productivity and economic efficiency. Thus, the knowledge of biological mechanisms controlling muscle development has become a hot topic in poultry research (6). The analysis of gene expression has contributed to a good understanding of muscle fiber formation and development (7) and consequently to muscle characteristics and meat quality.

Myogenic regulatory factors (MRFs), including MYF6 (MRF4) and MYOG, are critical regulators of skeletal muscle growth. MYOG is mainly linked to myocyte terminal differentiation and MYF6 is involved in muscle fiber maturation and maintenance. These genes have been considered as important markers of muscle growth and potential markers for improvement of meat production traits in broilers (8, 9).

Besides genetic regulation, nutritional factors, particularly dietary protein source, play important roles in muscle growth and development. The quality of the protein influences amino acid availability, metabolic efficiency, and gene expression, and therefore muscle structure and function (10, 11, 12). The pectoralis major muscle is important in broiler meat production, but little is known about the effect of dietary protein source on its morphological and molecular properties. The present study was performed to assess the influence of animal protein compared to plant protein diets on muscle morphology, glycogen content, and the expression of the MYF6 and MYOG genes in the pectoralis major muscle of broiler chickens.

Materials and Methods

Ethical approval

The study was approved by the Ethics Committee of the College of Veterinary Medicine, University of Al-Qadisiyah, Iraq, under approval number 5642 dated 28/12/2025.

Collection of specimens

Broiler chickens of each diet group were randomly selected for sampling at 42 days of age. Birds were humanely killed according to current animal welfare guidelines. The pectoralis major muscle of each bird was carefully excised immediately after slaughter. Muscle samples were divided into parts for histological, histochemical and gene expression analyses. Histological and histochemical examination samples were fixed in 10% neutral buffered formalin. Samples for gene expression analysis were collected rapidly from the same anatomical region, rinsed with sterile saline and stored at -80°C until RNA extraction.

Experimental diets and rearing conditions

A total of 9000 one-day-old broiler chicks were reared under commercial field conditions in Al-Diwaniyah Province, Iraq. The birds were randomly divided into two dietary groups according to the dietary protein source. The animal protein group received a diet containing fish meal as the animal protein source, whereas the plant protein group received a corn-soybean meal-based diet. The feeding program consisted of two feeding phases: starter phase and finisher phase. The ingredients and nutritional composition of the experimental diets are presented in Table 1.

Birds were maintained under standard broiler rearing conditions throughout the experiment. The brooding temperature was maintained at 33-35°C during the first week of age and was gradually reduced by approximately 2-3°C weekly until reaching about 24°C during the later growing period. Ventilation, lighting, litter

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management, feed supply, and water availability entire experimental period.

Histochemical processing of tissue

Fixed specimens were kept in 10% neutral buffered formalin for 48 h and then washed with tap water for 2 h. Specimens were dehydrated in

Table 1. Ingredients and nutritional composition

Ingredient / value	Starter plant protein diet (g/kg)	Starter animal protein diet (g/kg)	Finisher plant protein diet (g/kg)	Finisher animal protein diet (g/kg)
Yellow corn	495	512	480	496
Soybean meal	376	345	333	300
Flour	50	50	75	75
Wheat bran	25	25	50	50
Oil	10	10	20	24
Limestone	16	16.5	13	13.5
Premix 3244	25	0	25	0
Capri premix	0	12.5	0	12.5
Fish meal	0	25	0	25
Salt	1	1	1	1
Enzyme	0.5	0.5	0.5	0.5
Lysine	1	1	1	1
Methionine	1	1	1	1
Threonine	0.5	0.5	0.5	0.5
Oil substitute	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000
Crude protein (%)	22.5	22.6	21.1	21.2
Metabolizable energy (kcal/kg)	2950	2945	3020	3010
Required quantity (ton)	4	4	2	2

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an ascending series of ethyl alcohol (60%, 70%, 80%, 90%, and 100%), cleared in xylene, infiltrated in paraffin wax, embedded in paraffin blocks, sectioned at 5-micron thickness, and mounted on glass slides. Sections were stained with hematoxylin and eosin (H&E) and with Masson's trichrome to show the general histological structure and the distribution of connective tissue. Glycogen was demonstrated in muscle tissue by Periodic Acid-Schiff (PAS) staining. The captured images were analyzed using the ImageJ software to obtain histometric measurements such as muscle fiber area, muscle bundle area, and connective tissue thickness (13).

Gene expression study

Gene expression analysis was performed on fresh pectoralis major muscle samples from 42-day-old broiler chickens from both dietary groups. About 100 mg of muscle tissue was taken from each sample and immediately put into sterile Eppendorf tubes with an appropriate volume of TRIzol reagent for total RNA extraction. Samples were snap frozen and stored at -80°C for RNA integrity until further processing.

Total RNA was extracted from pectoralis major muscle tissue with TRIzol reagent (Bioneer, Korea) following the manufacturer's instructions. The NanoDrop spectrophotometer was used to determine the concentration and purity of the extracted RNA by absorbance measurement at 260 and 280 nm. Residual genomic DNA contamination was eliminated by treating the extracted RNA with DNase I enzyme (Promega, USA). Complementary DNA (cDNA) was synthesized using the M-MLV Reverse Transcriptase kit following the manufacturer's protocol.

The expression levels of MYF6 (MRF4) and MYOG were determined by quantitative real-time PCR (RT-qPCR) and normalized to the internal housekeeping gene GAPDH. The primers used in this study are given in Table 2. Relative gene expression was calculated using the 2^-ΔΔCt method.

Table 2. Primers used for detection of target genes.

Gene	Primer sequence (5′-3′)	Annealing temp. (°C)	Product size (bp)
MRF4 (MYF6)	F: CAGTGGAGGAC GAGGAGAA R: GGTGATGGTGAT GGTGATG	60	120
MYOG	F: GAGACATCCCCC TATTTCTACCA R: GCTCAGTCCGCT CATAGCC	60	110
GAPDH	F: TGCACCACCAAC TGCTTAGC R: GGCATGGACTGT GGTCATGAG	60	150

Statistical analysis

All data were analyzed using IBM SPSS Statistics version 26.0. Data normality was assessed using the Shapiro-Wilk test ($P > 0.05$), and homogeneity of variances was evaluated using Levene's test. For normally distributed variables, including muscle fiber area, muscle bundle area, connective tissue thickness, and MYF6 (MRF4) and MYOG expression levels, differences between the animal protein and plant protein groups were analyzed using an independent samples t-test. Data are presented as mean ± standard error of the mean (SEM), with five samples per group ($n = 5$). Statistical significance was considered at $P < 0.05$. Effect sizes were calculated using Cohen's d to evaluate the magnitude of differences between groups.

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Results

The present study was aimed at investigating the histological structure, glycogen content, and mRNA expression of MYF6 (MRF4) and MYOG genes in the pectoralis major muscle of broiler chickens fed on different dietary protein sources. Microscopically, the pectoralis major muscle consisted of elongated, cylindrical, multinucleated muscle fibers with oval nuclei located peripherally beneath the sarcolemma and distinct cross-striations (Figure 1).

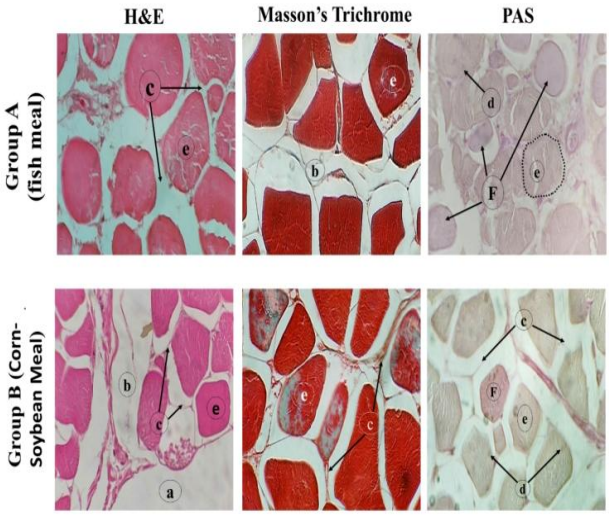

Figure 1. Histological and histochemical structure of the pectoralis major muscle of broiler chickens stained with H&E, Masson's trichrome, and PAS. a- Epimysium. b- Perimysium. c- Endomysium. d- Nucleus. e- Muscle fiber. f- Glycogen granules. Magnification: 400×.

The pectoralis major muscle fiber area was significantly different between the diet groups. The largest mean fiber area was seen in birds on the animal protein diet (4800.89 ± 312.65 µm²) and the lowest in the plant protein group (3332.35 ± 149.96 µm²). Statistical analysis revealed a significant difference between the two dietary groups ($P < 0.05$) (Table 3, Figure 3).

The area of muscle bundle of the pectoralis major muscle was also significantly higher in the animal protein group than in the plant protein group. The mean muscle bundle area was highest in birds fed on the animal protein diet (92,988.29 ± 2,345.08 µm2) and lowest in the plant protein group (71,116.90 ± 2,708.15 µm2). This difference was statistically significant between the two dietary groups ($P < 0.05$), as presented in Table 3 and Figure 3.

Table 3. Histometric parameters of the pectoralis major muscle in broiler chickens fed different dietary protein sources.

Parameter	Animal protein	Plant protein
Epimysium thickness (µm)	22.54 ± 1.49	24.19 ± 1.29
Perimysium thickness (µm)	12.84 ± 0.98	17.58 ± 0.70
Endomysium thickness (µm)	3.07 ± 0.39	4.51 ± 0.44
Muscle fiber area (µm²)	4800.89 ± 312.65	3332.35 ± 149.96
Muscle bundle area (µm²)	92,988.29 ± 2,345.08	71,116.90 ± 2,708.15

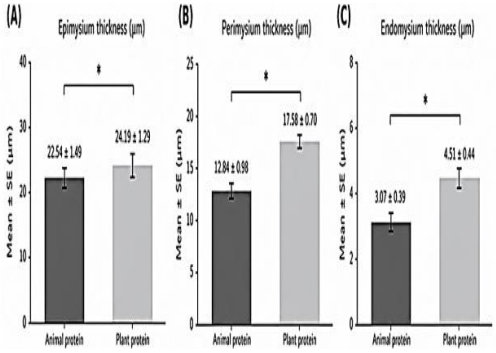

Figure 2. Thickness of connective tissue layers in the pectoralis major muscle of broiler

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chickens fed animal and plant protein diets. A- Epimysium thickness. B- Perimysium thickness. C- Endomysium thickness. Data are presented as mean ± standard error (n = 5). Asterisks (*) indicate significant differences between dietary groups (P < 0.05).

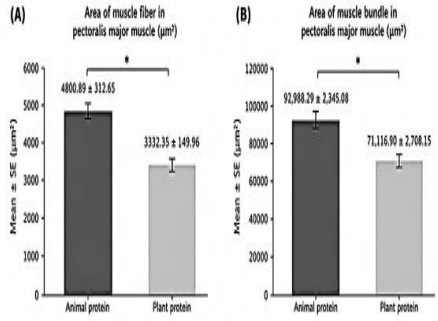

Figure 3. Histometric area measurements of the pectoralis major muscle of broiler chickens fed animal and plant protein diets. A- Muscle fiber area. B- Muscle bundle area. Data are presented as mean ± standard error (n = 5). Asterisks (*) indicate significant differences between dietary groups (P < 0.05).

The pectoralis major muscle was externally enveloped by a dense irregular connective tissue layer, the epimysium, which surrounded the entire muscle mass. Connective tissue septa ran inward from this layer, forming the perimysium that surrounded the muscle fascicles and contained small blood vessels. Each muscle fiber was covered by a thin layer of endomysium, a loose connective tissue associated with capillaries and nerve fibers that supply the muscle tissue (Figure 1).

The thickness of connective tissue layers differed significantly between the dietary groups. The epimysium was thicker in the plant protein group (24.19±1.29 µm) than in the animal protein group (22.54±1.49 µm).

The thickness of the perimysium was also higher in the plant protein group (17.58 ± 0.70 µm) than the animal protein group (12.84 ± 0.98 µm). The endomysium was also thicker in the plant protein group (4.51 ± 0.44 µm) than in the animal protein group (3.07 ± 0.39 µm). These parameters were significantly different between the two groups (P < 0.05), as presented in Table 3 and Figure 2.

PAS staining showed glycogen as magenta granules between myofibrils in muscle fibers. The animal protein group had a higher PAS staining intensity in the pectoralis major muscle compared to the plant protein group, indicating more glycogen accumulation.

etabolic activity in these muscle fibers.

The transcript expression of MYF6 (MRF4) and MYOG was analyzed in the pectoralis major muscle of broiler chickens fed different dietary protein sources using real-time PCR (RT-qPCR). RT-qPCR analysis showed consistent amplification curves and distinct melting peaks, suggesting the amplification was highly specific and accurate (Figures 4 and 5).

MYF6 (MRF4) expression differed significantly between dietary groups.

The highest average expression was recorded in birds receiving an animal protein diet (6.48 ± 1.29), while the lowest expression was found in the plant protein group (1.00 ± 0.19), as shown in Table 5. Statistical analysis showed a significant difference between the two groups (P < 0.05).

Likewise, MYOG expression was significantly different among dietary treatments. The animal protein group had the highest average expression (5.38 ± 1.38) and the plant protein group had the lowest value (1.00 ± 0.13) in Table 5. Statistical analysis showed significant difference between the two groups (P < 0.05). Moreover, MYF6 and MYOG were significantly higher expressed in the animal protein group compared with the plant protein group.

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Table 4. Relative gene expression of MYF6 (MRF4) and GAPDH in the pectoralis major muscle of broiler chickens fed different dietary

Group	Ct: MYF6	Ct: GAPDH	ΔCt	Relative expression	Mean ± SE
Animal protein	23.186	20.196	-2.990	5.8486267	6.48 ± 1.29
Animal protein	23.127	20.141	-2.986	5.8648650	
Animal protein	23.324	19.576	-3.748	3.4583836	
Animal protein	22.449	20.410	-2.039	11.306636	
Animal protein	22.812	19.842	-2.970	5.9302704	
Plant protein	25.665	19.823	-5.842	0.8100584	1.00 ± 0.19
Plant protein	25.247	20.434	-4.813	1.6530128	
Plant protein	25.949	20.377	-5.572	0.9767748	
Plant protein	26.140	19.617	-6.523	0.5052600	
Plant protein	25.878	20.417	-5.461	1.0548939	

Values are presented as mean ± standard error (n = 5). Relative expression was calculated after normalization to GAPDH. Differences were considered significant at $P < 0.05$.

Table 5. Relative gene expression of MYOG and GAPDH in the pectoralis major muscle of broiler chickens fed different dietary protein sources.

Goup	Ct: MYOG	Ct: GAPDH	ΔCt	Relative expression	Mean ± SE
Animal protein	24.111	20.196	-3.915	3.940771238	5.38 ± 1.38
Animal protein	24.298	20.141	-4.157	3.332206949	
Animal protein	24.261	19.576	-4.685	2.310937117	
Animal protein	23.082	20.410	-2.672	9.327419495	
Animal protein	22.733	19.842	-2.891	8.013752775	
Plant protein	26.460	19.823	-6.637	0.597279455	1.00 ± 0.13
Plant protein	26.551	20.434	-6.117	0.856472009	
Plant protein	26.227	20.377	-5.850	1.030595361	
Plant protein	25.128	19.617	-5.511	1.303578988	
Plant protein	26.033	20.417	-5.616	1.212074186	

Values are presented as mean ± standard error (n = 5). Relative expression was calculated after normalization to GAPDH. Differences were considered significant at $P < 0.05$.

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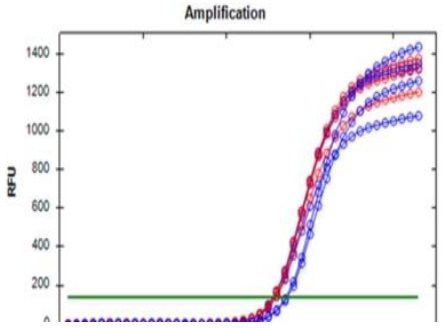

Figure 4. qPCR amplification plots of MYOG expression in the pectoralis major muscle. a- Animal protein group. b- Plant protein group.

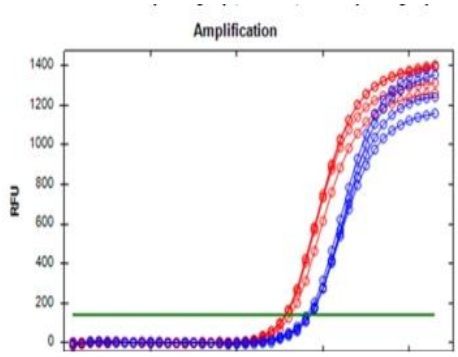

Figure 5. qPCR amplification plots of MRF4 (MYF6) expression in the pectoralis major muscle. a- Animal protein group. b- Plant protein group.

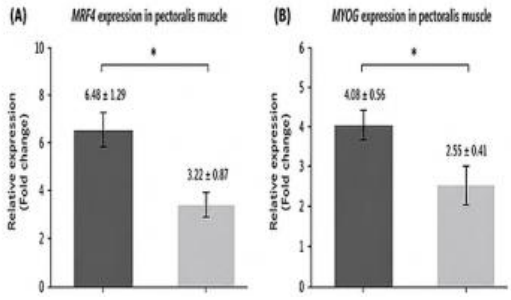

Figure 6. Relative expression of MYF6 (MRF4) and MYOG in the pectoralis major muscle. A- MRF4 expression. B- MYOG expression. Data are presented as mean ± standard error (n = 5). Asterisks (*) indicate significant differences between dietary groups ($P < 0.05$).

Discussion

The present study demonstrated that dietary protein source significantly influenced the structural, metabolic, and molecular characteristics of the pectoralis major muscle in broiler chickens. These results demonstrate the importance of dietary protein quality in the regulation of muscle development and meat related traits. Histological examination revealed the typical organization of skeletal muscle with elongated multinucleated fibers with peripheral nuclei and layers of connective tissue formed by epimysium, perimysium and endomysium. Such observations are consistent with previous descriptions of skeletal muscle structure and its relationship to meat quality (12,14). There were however clear differences between diet groups. The animal protein group presented bigger and more homogeneous muscle fibers, while the plant protein group showed smaller fibers and higher interstitial spaces, indicating less efficient muscle organization (14).

Plant protein group showed increased thickness of connective tissue layers (perimysium and endomysium) indicating increased deposition of connective tissues. The elevated content of the connective tissue might have a negative effect on the meat quality due to the contribution of the intramuscular connective tissue to the texture and tenderness characteristics (15). The lower connective tissue thickness in the animal protein group may therefore reflect better muscle organization and structural development.

The PAS staining showed that birds fed the animal protein diet had more glycogen accumulation in the pectoralis major muscle. Glycogen, a major energy store in skeletal muscle, is closely linked to postmortem metabolism and meat quality. The nutritional composition and amino acid balance can affect the body composition and meat quality of broilers (16) and crude protein level was shown to influence glycogen storage and processing ability of breast meat in chickens (17). The increased PAS staining in the animal protein

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group in this study indicates a better capacity to utilize nutrients and to store energy when compared to the plant protein group.

Histometric analysis revealed that birds fed animal protein diet had significantly higher muscle fiber area and muscle bundle area than the birds fed plant protein diet. The increase in fiber size suggests an increased hypertrophic growth which is one of the major mechanisms of post-hatch muscle development in broilers (18). The improvement observed in the animal protein group might be attributed to the fact that animal protein has a higher biological value and a more balanced amino acid profile that facilitates protein synthesis and muscle growth. Similar nutritional effects on breast muscle growth and myogenic gene expression have been reported in broilers, supporting the link between amino acid supply and muscle hypertrophy (11, 21).

The expression of MYF6 (MRF4) was significantly increased at the molecular level in the pectoralis major muscle of birds fed the animal protein diet. MYF6 belongs to the myogenic regulatory factor family and is involved in the maturation and maintenance of muscle fibers. Earlier studies indicated that myogenic regulatory genes participate in the development of postnatal muscle and growth traits in chicken (8, 9). The increased MYF6 expression in the animal protein group suggests a more pronounced activation of myogenic regulatory pathways (19). In addition, the reactivation of myogenic factors in chicken breast muscle has been reported during muscle remodeling, supporting the regulatory role of MYF6-related pathways in avian muscle tissue (18).

The expression of MYOG was significantly higher in the animal protein group as well. MYOG plays a critical role in myoblast differentiation and muscle fiber maturation. In the present study we found increased MYOG expression, which correlates to greater muscle fiber size and better histological organization in the animal protein group. This coordinated response indicates that quality of dietary protein influences muscle growth not only by structural changes but also by transcriptional regulation of myogenic genes. This interpretation is supported by previous observations showing that myogenin participates in chicken myoblast differentiation and myotube formation (20).

Conversely, the plant protein group had reduced expression of MYF6 and MYOG, smaller muscle fibers, thicker connective tissue and lower glycogen accumulation. The results indicate lesser muscle growth efficiency and imply that plant-based protein diets may have limited myogenic activity and metabolic performance due to possible amino acid availability or digestibility limitations. Overall, the results show that the origin of dietary protein affects the pectoralis major muscle through integrated structural, metabolic and molecular mechanisms. These differences may also reflect variation in the biological value and digestibility of specialized protein ingredients used in broiler nutrition (22, 23).

Conclusion

The present study concluded that dietary protein source exerted a significant effect on the morphological, metabolic and molecular characteristics of the pectoralis major muscle in broiler chickens. Birds fed the animal protein diet showed improved muscle development, as evidenced by increased muscle fiber and muscle bundle areas, reduced connective tissue thickness, greater glycogen deposition and higher expression of MYF6 (MRF4) and MYOG compared with birds fed the plant protein diet. These findings indicate that animal protein improved muscle hypertrophy and activated myogenic regulatory pathways related to muscle differentiation and maturation. Therefore, dietary protein quality is an important nutritional factor for improving broiler breast muscle growth, structural organization, and meat-related traits.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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