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The effect of fennel (*Foeniculum vulgare*) on MYOD1 gene expression in the muscle tissues of the thigh, shoulder, and loin in Kermani lambs

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Abstract Medicinal plants have gained attention as natural alternatives to antibiotic growth promoters in livestock nutrition due to their bioactive compounds. Among these, fennel (*Foeniculum vulgare*) shows promise, though its role in muscle development remains underexplored. The MYOD1 gene, a key member of the myogenic regulatory factors (MRFs) family, regulates muscle cell differentiation and growth. Increased expression of this gene is associated with enhanced muscle performance. This study examined the impact of fennel supplementation on MYOD1 gene expression in selected muscle tissues. A total of 48 muscle samples were collected from 16 lambs, with 8 animals assigned to each of two experimental groups. From each lamb, three muscle types (shoulder, thigh, and loin) were sampled, resulting in 24 samples per group. Fennel supplementation significantly increased MYOD1 expression in all muscle types ($P < 0.05$), with the highest expression in thigh and the lowest one in shoulder tissue. The results suggested that fennel may positively influence muscle development at the molecular level by upregulating the MYOD1 gene expression although its potential as a natural feed additive to support muscle growth and meat quality in livestock warrants further investigation. Given the MYOD1's central role in muscle growth regulation, exploring how medicinal plants affect its expression could advance functional livestock nutrition and genetic improvement strategies. Future studies should examine the underlying molecular mechanisms and interactions with other growth-related genes.

Keywords: functional nutrition, fennel, muscle development, MYOD1 gene, medicinal plants

Introduction

Sheep play a vital role in providing animal protein in many developing countries, including Iran, where over 27 indigenous breeds have evolved through centuries of natural and artificial selection to thrive in diverse ecological zones. Among these, the Kermani breed stands out for its dual-purpose utility, producing both high-quality meat and wool, and is extensively reared across Kerman Province (Mohammadabadi et al., 2023).

Despite the genetic potential of native sheep, Iran's

mutton production remains below optimal levels, largely attributed to inadequate nutritional management and limited implementation of scientifically validated fattening practices (Zamani et al., 2015). In response, recent strategies have turned to nutrigenomics and the use of medicinal plants as natural feed additives to improve livestock performance while reducing dependence on synthetic growth promoters and antibiotics (Hajalizadeh et al., 2019; Mohammadipour et al., 2023).

One such plant is fennel (*Foeniculum vulgare*), a member

of the Apiaceae family and known for its rich profile of bioactive compounds with antimicrobial, antioxidant, and anti-inflammatory properties (Amirteymoori et al., 2021). Fennel supplementation has demonstrated beneficial effects on rumen fermentation, nutrient absorption, and overall animal health (Saeedi et al., 2016; Hajalizadeh et al., 2019), making it a promising candidate for enhancing meat production naturally.

Meat yield in ruminants is closely tied to the growth and development of skeletal muscle, which constitutes the bulk of edible tissue. Muscle development is a complex process regulated by genetic, nutritional, epigenetic, and hormonal factors, with distinct mechanisms operating during prenatal and postnatal stages. Prenatal development determines the total number of muscle fibers, while postnatal growth is primarily driven by hypertrophy, characterized by satellite cell proliferation, protein accretion, and hormonal signaling (Costa et al., 2021; Gundersen and Anas, 2025).

Central to muscle formation are myogenic regulatory factors (MRFs), particularly *MYOD1*, which orchestrates myoblast differentiation and skeletal muscle development (Maridas et al., 2017; Cheng et al., 2020). *MYOD1*, along with *MYF5*, plays a pivotal role in satellite cell differentiation during muscle regeneration and growth (Gauvin et al., 2020). Its expression varies across breeds, muscle types, and physiological conditions, and has been linked to economically important traits such as carcass yield and fatty acid composition (Ropka-Molik et al., 2011; Lobo et al., 2012; Zhang et al., 2013, 2014). Moreover, *MYOD1* expression is associated with satellite cell activity and muscle repair processes (Campion et al., 1981; Gibson & Schultz, 1983; Ropka-Molik et al., 2015).

The complex nature of meat production traits in sheep involves a dynamic interplay between genetic and environmental factors, influencing muscle growth, carcass quality, fat deposition, and overall meat quantity and quality (Kaseja et al., 2024). Advances in molecular genetics and genomic technologies have opened new avenues for understanding the genetic architecture of these traits (Hosseini et al., 2023).

Given the fennel's potential to modulate gene expression and the central role of *MYOD1* in muscle development, this study aimed to investigate the effect of fennel supplementation on *MYOD1* gene expression in three key muscle groups, thigh, shoulder, and loin of Kermani lambs. The objective was to identify tissue-specific transcriptional responses and evaluate fennel's efficacy as a natural growth promoter in sheep production systems.

Materials and methods

In this study, sixteen male Kermani lambs, approximately eight months old and with equal initial body weights, were randomly selected. The animals were housed individually in pens measuring 2 × 1 meters for a total of 110 days, comprising a 20-day adaptation period followed by a 90-day fattening phase. The lambs were randomly assigned to two experimental groups: the control group received a basal diet, while the treatment group was fed a diet supplemented with 1% fennel (*Foeniculum vulgare*) powder. Prior to the start of the experiment, all animals were shorn and received preventive treatments, including anti-parasitic administration and vaccination against enterotoxemia.

Feeding was carried out individually, with free access to a Total Mixed Ration (TMR), provided in two daily meals (8:00 AM and 6:00 PM). Approximately 5% of the feed was left in the troughs to ensure ad libitum intake. The diets of both groups were formulated to have identical metabolizable energy and crude protein levels. Additionally, clean drinking water was made available freely, with water troughs cleaned and refilled twice daily. At the end of the trial, all animals were slaughtered ethically for sample collection. Muscle tissue samples were collected from the shoulder, thigh, and loin of each lamb. In total, 48 samples were obtained for molecular analysis, based on 16 animals (8 per group) and 3 tissues per lamb. Primer design was based on gene sequences registered in the GenBank database. The *MYOD1* gene sequence was retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov>), and specific primers were designed using GeneRunner software (Table 1). The β -actin gene was used as the internal reference (housekeeping gene) in this study.

Table 1. Primer sequences used for *MYOD1* and β -actin genes

Gene name	Primer sequence	Amplicon length (bp)	Annealing temperature (°C)
β -actin	F: 5'cttccagccgtccttct3'	105	66
	R: 5'tgttggcatcacaggtccttct3'		
<i>MYOD1</i>	F: 5'gaacactacagcggcgactc3'	197	60
	R: 5'acgatgctggacaggcagt 3'		

Primers were synthesized by Takapo Zist Company, and working solutions were prepared according to the manufacturer's instructions and stored at -20°C. Total RNA was extracted from tissue samples using the RNXTM Plus kit (SinaClon, Iran). To assess RNA quality and quantity, two complementary methods were employed: (1) agarose gel electrophoresis (1%) to examine band integrity, and (2) spectrophotometric

absorbance measurement at 260 nm using a NanoDrop device to determine concentration and purity. To eliminate possible genomic DNA contamination, DNase I enzyme from Thermo Scientific was used. Subsequently, cDNA synthesis was performed using a kit from the same company.

Optimal conditions for conventional PCR reactions were first determined, after which Real-time PCR reacti-

ons were performed using a Biorad machine and a kit containing Syber Green dye. Relative gene expression levels were analyzed using the comparative CT method ($2^{-\Delta\Delta CT}$), considering reaction efficiency. PCR efficiency and Cq threshold values were calculated with LinReg software, and statistical analysis of gene expression changes was performed using REST software.

Results

RNA quality analysis and MYOD1 gene amplification in different tissues

Electrophoresis of RNA extracted from tissues on 1% agarose gel revealed distinct bands of 28S rRNA and 18S rRNA (Figure 1). The approximate 2:1 ratio in band intensity indicated high-quality and intact RNA. qRT-PCR results for the *MYOD1* in shoulder muscle samples showed amplification initiation between cycles 22 to 25, entering the exponential phase, progressing to the linear phase, and reaching the plateau phase around cycle 32. In samples treated with 1% fennel seed, amplification began at earlier cycles compared to the control group, indicating upregulated gene expression due to the treatment. Similarly, thigh muscle samples displayed amplification initiation between cycles 18 to 21, progressing through the exponential and linear phases, and stabilizing around cycle 28. Treated samples initiated amplification earlier than the control group in this tissue as well, suggesting elevated expression of *MYOD1*. In loin muscle samples, amplification started between cycles 23 to 25, followed the exponential and linear phases, and plateaued at cycle 32. Earlier amplification in fennel-treated samples compared to controls also indicated increased gene expression.

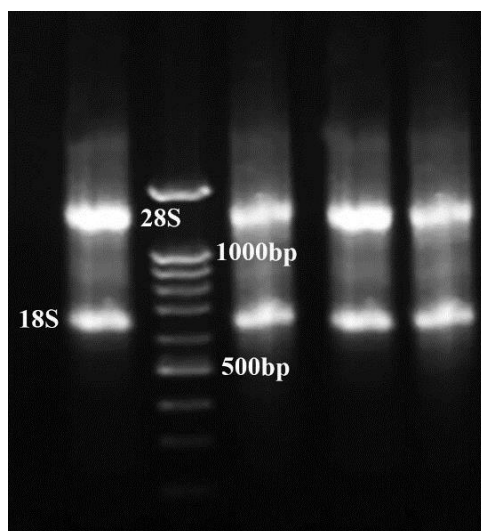


Figure 1. Electrophoresis of RNA samples extracted from **muscle** tissues using 1% agarose gel, validated with a 100 bp size marker

β -actin gene amplification results

Amplification curves from PCR demonstrated that the β -actin was amplified from early cycles in all examined tissues (shoulder, thigh, and loin), showing consistent progression. Amplification in shoulder muscle started at cycle 12, promptly entered the exponential phase, reached the linear phase, and stabilized by cycle 20. The similar amplification onset in both treated and control groups suggested stable expression of this gene across conditions. In thigh muscle, amplification began at cycle 11 and followed similar progression to the plateau phase at cycle 20. Loin muscle also showed amplification initiation at cycle 11 with uniform progression. The simultaneous onset across treatment and control groups confirmed β -actin's stability and suitability as a reference gene under experimental conditions.

Melting curve analysis of MYOD1 gene in different tissues

Melting curve analysis from Real-Time PCR revealed a distinct peak at 87°C for *MYOD1* in all three muscle tissues (shoulder, thigh, and loin), indicating the formation of specific amplification products and confirming reaction fidelity. In shoulder muscle, a clear peak at this temperature signified high purity and effectiveness of the PCR reaction. The identical peak pattern in thigh and loin muscles reflected reproducibility and consistency across laboratory conditions. These findings confirm precise and specific *MYOD1* amplification, supported by accurate primer design.

Melting curve analysis of β -actin gene in different tissues

Real-Time PCR melting curve analysis showed that the β -actin produced a distinct and unique peak at 87°C in all muscle tissues examined. This peak confirmed the specific and pure amplification of the target, validating the accuracy of the PCR process and primer selection. Each tissue shoulder, thigh, and loin exhibited consistent melting curve peaks, indicating high fidelity and specificity of β -actin amplification. Collectively, the results highlight β -actin's reliability as a reference gene for gene expression studies under varied experimental conditions.

Electrophoresis of PCR products for β -actin and MYOD1 genes

To verify specificity and fidelity of Real-Time PCR reactions, the amplified products of β -actin and *MYOD1* were subjected to electrophoresis on 1% agarose gel (Figure 2). Each gene produced a single distinct band, with no extra bands observed, demonstrating specific and pure PCR amplification. These results support findings from melting curve analyses and affirm the efficacy of the designed primers and overall reaction precision. Accordingly, amplified products of β -actin and

MYOD1 possessed appropriate molecular purity and can

be reliably used for further gene expression analysis.

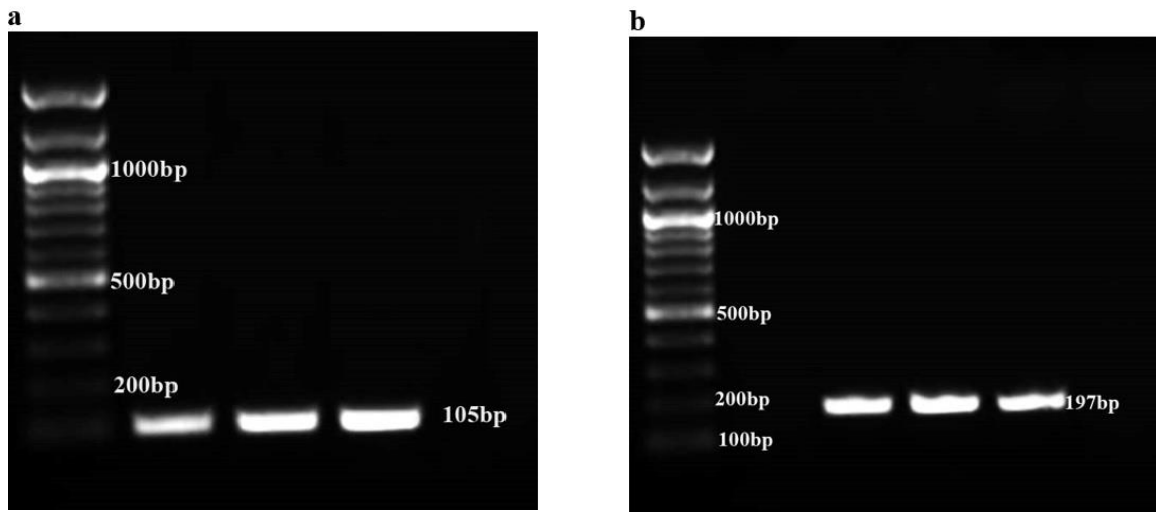


Figure 2. PCR amplification of target gene fragments visualized alongside a 100 bp DNA ladder. **a)** Amplified β -Actin gene fragment, with a 105 base pairs in length, **b)** Amplified *MYOD1* gene fragment, with a 197 base pairs in length

Relative expression of MYOD1 gene in different muscle tissues

A statistical summary of *MYOD1* expression in shoulder, thigh, and loin muscle samples is presented in Table 2. The cycle threshold (Ct) values provide insight into the relative expression levels of *MYOD1*, normalized against the housekeeping gene β -actin, across the different muscle tissues. Consistently low Δ Ct values for *MYOD1*, particularly in the thigh and loin, suggest strong expression relative to β -actin. Among the tissues analyzed, the thigh muscle exhibited the highest $2^{-\Delta\Delta Ct}$ values (~5.28), indicating significantly elevated

MYOD1 expression. This finding aligns with the thigh's known role in muscle differentiation and regeneration. In contrast, the shoulder muscle showed the lowest *MYOD1* expression, which may reflect differences in fiber type composition or developmental stage.

Ct values for β -actin remained relatively stable across all tissue types, supporting its suitability as a reliable reference gene for normalization. Minor variations, such as slightly higher Ct values in the shoulder, may be attributed to sample handling or inherent tissue-specific differences but remained within acceptable limits. Lower coefficients of variation (CV), particularly for *MYOD1* in the loin, indicate high consistency in Ct measurements and suggest stable gene expression.

Table 2. Descriptive statistics of *MYOD1* Ct values in skeletal muscle samples

Tissue	Gene	Mean Ct	Std Dev	CV (%)
Shoulder	<i>MYOD1</i>	26.37	0.36	1.37
Shoulder	B-actin	22.63	0.62	2.74
Thigh	<i>MYOD1</i>	25.30	0.52	2.06
Thigh	B-actin	23.70	0.26	1.10
Loin	<i>MYOD1</i>	25.00	0.16	0.64
Loin	B-actin	23.48	0.34	1.45

This study also assessed the impact of fennel (*Foeniculum vulgare*) supplementation on *MYOD1* expression in shoulder, thigh, and loin muscles. Figure 3 illustrates the comparative expression levels of *MYOD1* in muscle tissues between treatment and control groups. Treatments marked with an asterisk (*) denote statistically significant differences from the control group ($P < 0.05$), highlighting fennel's positive effect on gene expression under the experimental conditions. Further statistical analysis of *MYOD1* relative expression, as shown in Figure 3 (sections a, b, and c), revealed that fennel supplementation significantly increased *MYOD1* transcript levels in all evaluated muscle tissues ($P < 0.05$). In the shoulder muscle (Figure 3-a), fennel feeding led to

a marked increase in *MYOD1* expression compared to the control. Similarly, the thigh muscle (Figure 3-b) showed significantly elevated expression following fennel treatment. The loin muscle (Figure 3-c) also demonstrated enhanced transcript levels in the treated group. These findings suggested that fennel may promote *MYOD1* expression, potentially through its modulatory effects on muscle growth and differentiation pathways.

Statistical analysis of MYOD1 relative expression in muscles of lambs

To assess *MYOD1* gene expression changes following dietary supplementation with 1% fennel, mean values

from three experimental replicates were compared using the Duncan's multiple range test ($P < 0.05$). The means sharing at least one common letter were considered statistically non-significant. The findings showed that 1% fennel treatment significantly increased *MYOD1* expression in thigh muscle (Figure 4). Comparison across thigh, loin, and shoulder muscles revealed that

expression in the thigh was significantly higher than in the other two muscles ($P < 0.05$). No significant difference was observed between loin and shoulder muscles. Notably, shoulder muscle displayed the lowest *MYOD1* expression level (Figure 4). These findings suggested distinct tissue-specific responses to fennel supplementation.

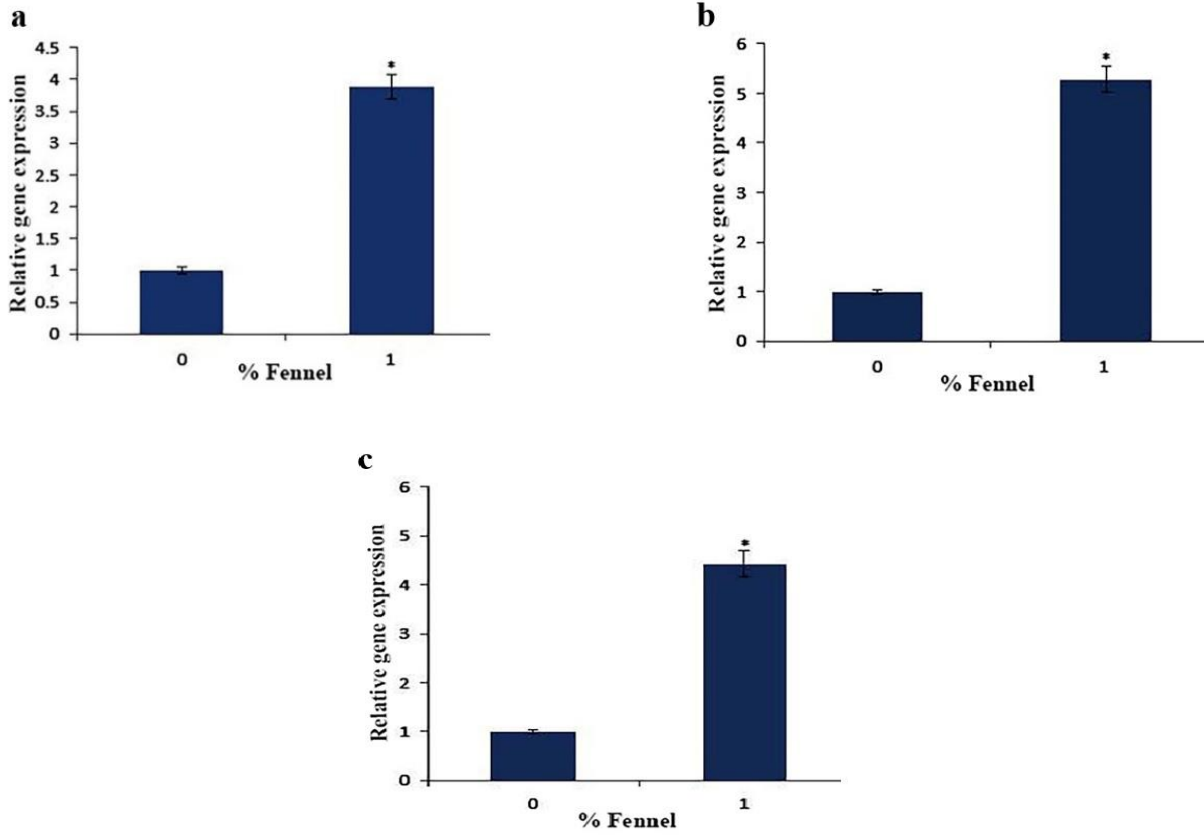


Figure 3. Analysis of *MYOD1* gene expression levels in muscle tissues following dietary supplementation with 1% fennel. Gene expression was assessed in the **shoulder (a)**, **thigh (b)**, and **loin (c)** muscles

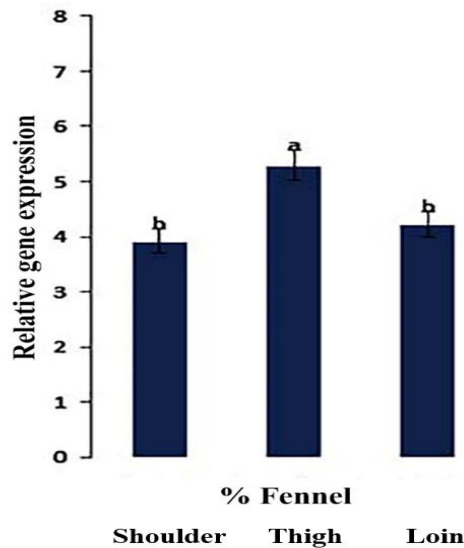


Figure 4. Effect of dietary treatment with 1% fennel on the relative expression of the *MYOD1* gene in shoulder, thigh, and loin muscles

Discussion

Quantifying mRNA levels in muscle tissues is a reliable method for identifying genes involved in muscle growth and development in sheep. These expression profiles reflect the functional activity of individual genes and the dynamics of protein synthesis, which vary across breeds and muscle types. Hamill et al. (2012) demonstrated that structural changes in highly expressed genes can significantly influence muscle development.

Among the myogenic regulatory factors (MRFs), *MYOD1* is a principal regulator of myogenesis. It activates transcriptional pathways that guide precursor cells toward differentiation into mature muscle fibers by binding to specific DNA sequences and promoting the expression of structural proteins essential for skeletal muscle formation (Maridas et al., 2017; Cheng et al., 2020). Recent genomic studies have revealed that polymorphisms in *MYOD1* can shift allele frequencies over time, influencing meat productivity traits in breeds such as Manych Merino sheep (Krivoruchko et al., 2025).

In the present study, the observed differential expression of *MYOD1* across muscle types suggested the involvement of tissue-specific regulatory mechanisms. The highest expression was observed in the thigh muscle, as indicated by the lowest ΔC_t and highest $2^{-\Delta\Delta C_t}$ values (mean: 5.28), reflecting active myogenesis likely driven by its role in locomotion and mechanical load-bearing. These findings supported the hypothesis that anatomical location and functional demands influence gene expression patterns. The coefficient of variation (CV) for *MYOD1* Ct values further supported the tissue-specific consistency. The loin muscle exhibited the lowest CV (0.64%), followed by the thigh (2.06%) and shoulder (1.37%), indicating that the thigh and loin are more stable tissues for gene expression analysis. The reference gene β -actin also showed stable expression, with CVs of 1.10% in the thigh and 1.45% in the loin, validating its use for normalization. The shoulder muscle, however, showed higher variability (CV=2.74%), which may reflect heterogeneity in cellular composition or technical variation during RNA extraction and cDNA synthesis. According to Bustin (2025), rigorous validation of reference genes, along with strict adherence to the updated Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE 2.0) guidelines, is essential for achieving reproducible qPCR results, particularly in tissues exhibiting variable transcriptional activity.

Breed, age, and muscle type are known to influence satellite cell activity, which is a key driver of postnatal muscle growth and regeneration (Campion et al., 1981; Gibson and Schultz, 1983; Ropka-Molik et al., 2015). Among the transcriptional regulators of these cells, *MYOD1* plays a central role in directing myogenic commitment and differentiation. Its expression has been positively correlated with satellite cell abundance and

myogenic potential in livestock species (Ropka-Molik et al., 2011; Zhang et al., 2013, 2014). Evaluating *MYOD1* together with related myogenic regulatory factors such as MYF5, MYF6, and MYOG can provide valuable insights for identifying the genetic candidates that may enhance meat production efficiency. Recent findings also highlight the importance of large MAF transcription factors, including MAFA, MAFB, and MAF, in modulating muscle fiber composition. These factors appear to reawaken dormant fast-glycolytic type IIb fibers by upregulating *MYH4* expression and stimulating glycolytic metabolic pathways. This activity contributes to a phenotypic shift that favors fast-twitch fiber dominance, which is associated with improved growth performance and desirable meat quality traits. Their potential interaction with *MYOD1* suggests the presence of a coordinated transcriptional network that regulates fiber-type specification and plasticity in response to physiological and environmental cues (Sadaki et al., 2025).

Lobo et al. (2012) reported a negative correlation between *MYOD1* expression and daily weight gain ($r = -0.45$), and a positive correlation with cold carcass yield ($r = 0.66$), highlighting its role in regulating muscle mass and myoblast numbers. Additionally, *MYOD1* expression influences fatty acid composition, with higher expression linked to reduced polyunsaturated fatty acids (PUFAs) and increased monounsaturated fatty acids (MUFAs), underscoring its relevance in genetic selection for meat quality. Tomczyk (2024) and Therdyothin et al. (2025) further support the role of omega-3 fatty acids in enhancing muscle strength and protein synthesis, suggesting that *MYOD1*-driven muscle development may also impact nutritional value.

Arsalan et al. (2024) evaluated *MYOD1*, *MYF5*, *MYF6*, and *MYOG* expression in Longissimus dorsi (LD) and gluteal (GL) muscles of four Turkish sheep breeds. Significant breed-specific differences were observed ($P < 0.05$), with *MYOD1* overexpressed in the GL muscle of the fat-tailed Akkaraman breed (15.41-fold) and underexpressed in the LD muscle of the fatless Karayaka breed (-0.22-fold), reinforcing its role in breed-specific muscle development. Banerjee et al. (2025) emphasize that structural variants and epigenetic regulation contribute to breed-specific gene expression, reinforcing the importance of integrating genomic data with expression profiles.

Taken together, the findings of the current study emphasize the importance of tissue selection in gene expression analysis. The thigh muscle, characterized by high *MYOD1* expression and low β -actin variability, emerges as the most biologically and statistically robust tissue for investigating myogenic activity. Although the loin muscle shows slightly lower *MYOD1* expression, it demonstrates exceptional consistency and serves as a reliable secondary tissue. In contrast, the shoulder muscle, despite moderate *MYOD1* expression, presents challenges due to greater variability in reference gene expression. These results deepen our understanding of

muscle-specific gene regulation and support the use of β -actin as a reference gene in tissues with low transcriptional variability. They also underscore the interplay between biological function and gene expression stability, offering a foundation for future studies on muscle development, regeneration, and pathology.

Fennel (*Foeniculum vulgare*) possesses a wide range of pharmacological properties, including antioxidant, anti-inflammatory, anti-cancer, antifungal, antibacterial, and estrogenic effects, largely attributed to its aromatic constituents such as anethole, estragole, and fenchone (Kooti et al., 2015). These bioactive compounds may contribute to a cellular environment that supports muscle development. Although the precise role of *MYOD1* in muscle differentiation is complex and context-dependent, its expression is known to be a key marker of myogenic commitment. The upregulation of *MYOD1* observed in this study may be partially explained by the antioxidant and anti-inflammatory actions of fennel's constituents, which could reduce cellular stress and promote myoblast proliferation and differentiation.

Future research should prioritize the thigh and loin muscles in qPCR-based studies that require high reproducibility and minimal biological noise. Investigating the molecular mechanisms underlying *MYOD1* activity including fiber type composition, mechanical loading, and satellite cell dynamics could further clarify its role in muscle plasticity. Moreover, integrating qPCR with transcriptomics, proteomics, and epigenetic profiling, as proposed by Banerjee et al. (2025), may provide a more comprehensive view of gene regulation and position *MYOD1* within broader biological networks.

Conclusions

Thigh muscle, based on its high transcriptional activity and low variability, was the most reliable tissue for analyzing the *MYOD1* gene expression. The β -actin gene showed stable expression in both the thigh and loin tissues, confirming its suitability as a reference gene. Supplementing the lamb diets with 1% fennel seed significantly increased the *MYOD1* expression, particularly in the thigh muscle, indicating the fennel's potential role in promoting muscle growth and differentiation. These findings supported the use of fennel as a natural feed additive to enhance meat quality and muscle development in livestock. Additionally, *MYOD1* is reaffirmed as a candidate gene for molecular selection in sheep breeding programs. Further studies across diverse species are recommended to validate the long-term efficacy and practical application of fennel in animal production.

Conflict of interest

The authors affirm that no conflicts of interest are associated with the conduct or publication of this research.

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