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Animal Biotechnology

ISSN: 1049-5398 (Print) 1532-2378 (Online) Journal homepage: https://www.tandfonline.com/loi/labt20

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To cite this article: Luis Paulo Batista Sousa-Junior, Ariana Nascimento Meira, Hymerson Costa Azevedo, Evandro Neves Muniz, Luiz Lehmann Coutinho, Gerson Barreto Mourão, André Gustavo Leão, Victor Breno Pedrosa & Luís Fernando Batista Pinto (2020): Variants in myostatin and MyoD family genes are associated with meat quality traits in Santa Inês sheep, Animal Biotechnology, DOI: 10.1080/10495398.2020.1781651

To link to this article: https://doi.org/10.1080/10495398.2020.1781651

Published online: 07 Jul 2020.

ANIMAL BIOTECHNOLOGY
https://doi.org/10.1080/10495398.2020.1781651

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Variants in myostatin and MyoD family genes are associated with meat quality traits in Santa Inês sheep

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ABSTRACT

Myostatin and MyoD family genes play vital roles in myogenesis and this study aimed to identify association of variants in *MyoD1*, *MyoG*, *MyF5*, *MyF6*, and *MSTN* genes with meat traits in Santa Inês sheep. A dataset with 44 variants and records of seven meat traits in 192 lambs (pH0, pH24, a*, b*, L*, tenderness assessed by shear force, and water-holding capacity) was used. Single-locus and haplotype association analyses were performed, and the significance threshold was established according to Bonferroni's method. Single-locus analysis revealed two associations at a Bonferroni level, where the variant *c.935-185C > G* in *MyoD1* had an additive effect (−4.31 ± 1.08 N) on tenderness, while the variant *c.464 + 185G > A* in *MyoG* had an additive effect (−2.86 ± 0.64) on a*. Additionally, the haplotype replacement *GT>AC* in *MSTN* was associated with pH0 (1.26 ± 0.31), pH24 (1.07 ± 0.27), a* (−1.40 ± 0.51), and tenderness (3.83 ± 1.22 N), while the replacement *GT > AG* in *MyoD1* was associated with pH0 (1.43 ± 0.26), pH24 (1.25 ± 0.22), b* (−1.06 ± 0.39), and tenderness (−4.13 ± 1.16 N). Our results have demonstrated that some variants in *MyoG*, *MyF6*, *MyoD1*, and *MSTN* can be associated with physicochemical meat traits in Santa Inês sheep.

KEYWORDS

Lambs; meat; myogenesis; polymorphism; selection

Introduction

The Brazilian sheep population has about 18.9 million animals, and 66.7% of these are located in Northeast of Brazil.[1] This region has a large area characterized as semiarid weather, where the Caatinga is the main ecosystem. In this place, rusticity becomes an essential factor for sheep production. The Santa Inês is a hair sheep and the most numerous sheep breed in Northeast of Brazil, because of its higher tolerance to both endoparasites[2] and heat[3] than wool meat sheep breeds. Moreover, growth[4] and carcass[5] traits of Santa Inês qualify this breed for meat production.

The MyoD family genes (*MyoD1*, *MyoG*, *MyF5*, and *MyF6*) are myogenic regulatory factors with an effect on the determination and maturation of muscle fibers,[6] while the Myostatin gene (*MSTN*) is a negative regulator of myogenesis.[7] Thus, variants in these genes can be associated with growth, carcass, and meat traits in livestock. Notably, some MyoD family genes were associated with meat quality traits in pigs,[8] beef cattle,[9] and rabbits.[10] In sheep, variants in *MyoD1* were reported as being associated with body traits such as thoracic girth and loin width in Stavropol sheep.[11] Additionally, positive correlations between *MyoG* expression and body and carcass weights in Hu sheep were found.[12] Moreover, variants in the *MyF5* gene were associated with lean meat yield in both leg and loin cuts in New Zealand Romney sheep.[13] However, the effects of MyoD family genes on meat quality traits in sheep remain poor known.

On the other hand, the *MSTN* gene has been widely studied in sheep, since inhibiting this gene leads to an increase in muscle fiber.[14] Thus, alteration in the coding regions,[15] 3′UTR,[16] 5'UTR,[17] intron 1,[18] and intron 2[19] were associated with higher muscle development and less fat. However, the effect of *MSTN* on numerous meat quality traits in sheep also remains unknown. A haplotype in the *MSTN* gene was tested for association

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with the tenderness, color, and pH of meat in Texel sheep, with no associations identified;[20] In addition, no association between the myostatin variant g + 6723G > A (currently known as g + 6223G > A) and meat quality traits such as pH, color, and tenderness in sheep were found.[21] However, an association between myostatin variants and sensory measures of tenderness in sheep was reported.[19] Thus, this study aimed to identify association between variants in *MyoD1*, *MyoG*, *MyF5*, *MyF6*, and *MSTN* genes with longissimus lumborum (LL) traits (pH, a*, b*, L*, tenderness assessed by shear force, and water-holding capacity) in Santa Inês sheep.

Materials and methods

Population and phenotypes

The current study was performed with the approval of the Ethical Committee for Animal Use from the Veterinary Medicine and Animal Science School of Federal University of Bahia (protocol number 02/2010). A total of 192 Santa Inês lambs were used; of these, 106 were born between 2010 and 2012 at the Pedro Arle experimental farm of Embrapa Tabuleiros Costeiros in the municipality of Frei Paulo, Sergipe State, Brazil. The remaining 86 lambs were born in 2014 and raised on the experimental farm of the UFBA in the municipality of São Gonçalo dos Campos, Bahia State. The 106 lambs of the Embrapa farm are the progeny of 7 unrelated sires. The smallest sire-half-sib family had 9 lambs, while the largest had 17 lambs. However, no pedigree control was performed for the 86 animals in the UFBA group because the mating occurred at pasture. All animals were raised on pasture with access to areas containing Panicum maximum cv. Green Panic and cv. Aruana. Water and mineral salt were available *ad libitum*.

Lambs were slaughtered at approximately 8 months of age in two abattoirs after a 16-h fasting period, with an average live weight of 36.12 kg and a standard deviation of 4.4 kg. Lambs were slaughtered in four groups. The first three groups (68 lambs in 2010, 15 in 2011, and 17 in 2012) were slaughtered in an abattoir located in the municipality of Propriá, Sergipe State, while the remaining group (86 lambs in 2014) was slaughtered in an abattoir located in the municipality of Feira de Santana, Bahia State. Both abattoirs are under the control of the Federal Sanitary Inspection Service. The animals were slaughtered through cerebral concussion using a non-penetrative method, according to procedures followed by the Sanitary and Industrial Inspection Regulation for Animal Origin Products.[22] Thereafter, the carcasses were chilled to a final temperature of 3–4 °C for 24 h.

Table 1. Sample size (*N*), minimum, average, maximum, and standard deviation of the meat traits in Santa Ines sheep.

Traits	*N*	Minimum	Average	Maximum	Standard deviation
L*	185	27.66	44.59	59.9	5.51
a*	185	10.44	20.9	31.14	5.69
b*	185	3.59	8.38	13.57	2.42
Water holding capacity	99	0.19	0.24	0.31	0.02
Tenderness (N)	185	5.69	17.55	49.52	9.03
pH0	99	6.2	6.63	7.04	0.18
pH24	99	5.03	5.46	6.66	0.27

Lightness (L*), redness (a*), and yellowness (b*).

The pH of the LL was recorded after 45 min (pH0) and 24 h (pH24) postmortem on the left side of each carcass, between the 12th and 13th ribs, using a Testo 205 pH meter (Testo Instrument Co. LTD., Germany). The pH meter was calibrated before use to pH 7.0 and 4.0 using buffer solutions. Three sequential pH records were obtained at three different points in the LL of each carcass, and the average of this triplicate was utilized as a reference value (Table 1).

After 24 h of slaughter, the LL of each animal was removed from both sides of carcasses, packed, and frozen (stored for up to 1 week at −20 °C) until further analysis of color traits, tenderness, and water-holding capacity (Table 1). Three days after slaughter, the LL was defrosted at 4 °C for 12 h. LL samples were then allowed to bloom for 30 min at 4 °C, for chromatic characterization.[23] A Minolta chromameter (CR400, Minolta Inc., Osaka, Japan) was used to measure the color of each LL sample, which was expressed as CIE/Lab lightness (L*), redness (a*), and yellowness (b*). Black and white reference standards provided by the manufacturer were used to calibrate the chromameter. The light source was set at the D65 standard illuminant with the observer set to 10°. A measuring aperture area of 8 mm was used. Three readings were performed on the cranial end of each LL, utilizing the mean value.

After chromameter evaluation, the lumbar section of LL was sliced into steaks of 2.5 cm. A thermocouple probe was then inserted into the geometric center of each steak to monitor the cooking temperature. The steaks were then placed on an electric grill. When steak temperature reached 40 °C, they were turned over and the other side was grilled until it reached 71 °C, according to the methodology reported by Ramos and Gomide[24] After steak samples were cooled to room temperature, tenderness evaluations were performed using a shear force test with a Warner Bratzler Shear Force device. Using the cylindrical punch of the device, five cuboidal strips with a

Table 2. Forward (F) and reverse (R) primers, number of base pairs (bp) amplified, and its location in the sheep genome.

Gene	Primers	Bp	Location[a]
MyoD1	F: 5′CAGACCCTCAGTGCTTTGCT3′ R: 3′CCTGCCTGCCGTATAAACAT5′	2493	Chromosome 15 Positions from 34303414 to 34300922 (exons 1 to 3, including introns 1 and 2)
MyoG	F: 5′ACTACCTGCCTGTCCACCTC3′ R: 3′TCCCBTACTGTGATGCTGTC5′	1836	Chromosome 12 Positions from 198441 to 196606 (exons 1 to 3, including introns 1 and 2)
MyF5	F: 5′CTCCGGTTTCTCCCCTATCT3′ R: 3′CATCACCTTAACTCATGATTCCT5′	2813	Chromosome 3 Positions from 116459993 to 116462805 (exons 1 to 3, including introns 1 and 2)
MyF6	F: 5′CTTGGACGGGGAAAATGTTA3′ R: 3′GAGGAAATGCTGTCCACGAT5′	1126	Chromosome 3 Positions from 116444909 to 116446058 (exons 1 to 3, including introns 1 and 2)
MSTN	F: 5′AGAACAGCGAGCAGAAGGAA3′ R: 3′CAATGCTCTGCCAAATACCA5′	2380	Chromosome 2 Positions from 118140493 to 118142497 (exons 1 to 2, including intron 1)

[a]Location in sheep genome version 4.0 (NC_019468.2).

diameter of 1.27 cm were removed from the center of the steak samples, perpendicular to the meat fibers (with no fat or nerves). Peak shear force (*N*) was calculated as the mean of the five measurements. Meat samples were also placed on a filter paper to determine their water-holding capacity using the press method.[25] In this method, the samples were placed between two acrylic plates with a 10 kg weight placed on this structure for 5 min. Subsequently, the difference in weight was employed for calculating lost water.

Genotyping

Blood samples (5.0 mL) were collected from all 192 lambs and placed in vacutainer tubes containing EDTA. DNA extraction was performed using a salt precipitation method and proteinase K solutions following the Embrapa protocol.[26] The design of the primers for amplification of the DNA fragments was performed based on gene sequences in sheep genome version 4.0 (NC_019468.2) with the following access codes: *MyoD1* (ID: 443405), *MyoG* (ID: 443158), *MyF5* (ID: 443159), *MyF6* (ID: 100188930), and *MSTN* (ID: 443449). The forward and reverse primers of each gene are described in Table 2. For amplification of the target region, a 20 µL solution containing 1.2 µL of each primer, 10 µL of a mix of Taq Polymerase (Emeraldamp Max Hs – Takara Bio, USA), 2 µL of the DNA, and 6.8 µL of ultrapure water, was used. Further, the Thermal Cycler Veriti® (Applied Biosystems, USA) was applied for amplification, according to the protocol presented in Table 3. For *MSTN*, *MyoD1*, and *MyF6* genes, the denaturation, annealing, and extension phases were repeated for 40 cycles before a final extension. Touchdown PCR was performed for the *MyF5* and *MyoG* genes, in which the denaturation, annealing, and extension phases had 20 cycles in each phase. Libraries were obtained from PCR products, and the sequencing was performed in the MiSeq platform (Illumina, San Diego, USA). A full description of the genotyping methodology for these genes can be found in Sousa-Junior et al.[27]

Single-locus association analysis

Before association study, a principal component analysis[28] was performed to evaluate structuration in this Santa Ines population. A single-locus association analysis was conducted using Qxpak 5,[29] which performs a likelihood ratio test. The general model used can be illustrated as $\boldsymbol{y} = \boldsymbol{X\beta} + \sum_{i=1}^{n} \boldsymbol{Z\delta_k} + \boldsymbol{\varepsilon}$, where $\boldsymbol{y}$ is the vector containing the records of the traits, $\boldsymbol{\beta}$ is the vector of solutions for the fixed effects, $\boldsymbol{\delta_k}$ is the vector of solutions for the genetic effects for any of the $\boldsymbol{n}$ QTLs that affect the trait, and $\boldsymbol{\varepsilon}$ is the vector of the residuals. $\mathbf{X}$ and $\mathbf{Z}$ are the incidence matrices that associate observations in $\boldsymbol{y}$ to the solutions vectors in $\boldsymbol{\beta}$ and $\boldsymbol{\delta_k}$, respectively. The fixed effects included in the model were: (i) the farm (2 levels), (ii) year (4 levels), (iii) the month of birth (12 levels), and (iv) the covariate age of the animal. We used the PROC MIXED function of the Statistical Analysis System[30] to perform an analysis of variance and found that all fixed effects were significant at the 5% level. Residual values outside the interval of ±3 SD were considered outliers and deleted. Furthermore, the additive and dominance effects of the QTLs were calculated. Additive effect was calculated as the contrast between the genotypes (*AA*–*BB*), where the allelic variant (*A*) was found in the reference gene sequence, while (*B*) is the Santa Inês allelic variant. A positive dominance effect implies that the heterozygote showed a mean value closer to the *BB* genotype. Only variants with

Table 3. Polymerase chain reaction (PCR) protocols in the *MSTN* and *MyoD* family genes in Santa Ines sheep.

Gene	Initial denaturation	Denaturing	Annealing	Extension	Final extension
MyoD1	98 °C/5 min	98 °C/10 s	63 °C/30s	72 °C/3 min	72 °C/5 min
MyoG (Step 1)	98 °C/5 min	98 °C/10 s	65 °C–55 °C Δ–0.5 °C/30 s	72 °C/2 min	
MyoG (Step 2)		98 °C/10 s	55 °C/30 s	72 °C/2 min	72 °C/5 min
MyF5 (Step 1)	96 °C/30 s	94 °C/15 s	59 °C–54 °C Δ–0.5 °C/30 s	68 °C/4 min	
MyF5 (Step 2)		94 °C/15 s	54 °C/30 s	68 °C/4 min	68 °C/5 min
MyF6	98 °C/5 min	98 °C/10 s	56 °C/30 s	72 °C/2 min	72 °C/5 min
MSTN	98 °C/5 min	98 °C/10 s	59 °C/30 s	72 °C/3 min	72 °C/5 min

Touchdown PCR was performed for *MyoG* and *MyF5* genes.

minor allele frequency (MAF) ≥2% and in Hardy–Weinberg equilibrium (HWE) ($p > 0.05$) were used in association analysis.

Haplotype association analysis

Haploview software[31] was used to identify the haplotype linkage disequilibrium blocks. Posteriorly, the haplotype association analysis was performed using the subroutine Haplo.GLM of Haplo.Stat version 1.7.7.[32] Only haplotypes with a frequency greater than 4% were used in this analysis.

Significance threshold

The significance threshold was established according to Bonferroni's method, considering a global type I error equal to 5%. This correction considers the number of variants in the analysis. For the single-locus analysis, 44 variants were tested. Then, a nominal significance level of 0.0011 was used. A total of five linkage disequilibrium blocks were tested in the haplotype association analysis. Consequently, the nominal threshold was 0.01. Additionally, significant results for the uncorrected probability of 5% were considered as suggestive effects.

Binding site research

When intronic variants were found to be in association with some traits, then binding site research was carried out to identify either Transcription Factor Binding Site (TFBS) changes or microRNA Binding Site. The AnimalTFDB v.3[33] was used to predict TFBS; while the miRBase v.22[34] was used to identify microRNA binding site.

Results

Variants and haplotypes

The sequencing of the *MyoD1*, *MyoG*, *MyF5*, *MyF6*, and *MSTN* genes in Santa Inês revealed 44 single nucleotide polymorphisms (SNP) with MAF >2% and in HWE (Table 4). For the *MyoD1* gene, one SNP was found in 3′UTR, seven in intron-2, and two in exon-3, which showed MAF values ranging from 2 to 22.1% and observed heterozygosity between 2.9 and 32.6%. In the *MyoG* gene, three SNPs were found in intron-1, eight in intron-2, and one in exon-3. These variants had MAF ranging from 3.1 to 44.8% and observed heterozygosity between 5.8 and 56.0%. For the *MyF5* gene, two variants were found in exon-1, four in intron-1, and two in 3′UTR, which showed MAF ranging from 2.1 to 3.9% and observed heterozygosity between 4.2 and 7.9%. For the *MyF6* gene, three variants were found in intron-2 and one in exon-3. These variants showed MAF ranging from 6.1 to 26.3% and observed heterozygosity between 11.1 and 38.4%. For the *MSTN*, 10 variants in intron-1 showing MAF between 4.5 and 45.9% and observed heterozygosity from 9 to 54.1%.

No linkage disequilibrium block was found in *MyF5* gene (Figure 1). On the other hand, haplotype analysis revealed five linkage disequilibrium blocks in other genes. One block was found in *MyoD1* gene (Figure 2), being both variants ($c.935\text{-}206G > A$ and $c.935\text{-}185C > G$) located intron 2. One block was found with two variants in intron 1 ($c.464 + 289T > C$ and $c.464 + 185G > A$) of *MyoG* gene (Figure 3). Two blocks were found in *MyF6* gene (Figure 4), with the first block with two variants in intron 2 ($c.653 + 66G > A$ and $c.653 + 67T > G$), and the second block with a variant in intron 2 ($c.654\text{-}104A > C$) and another in exon 3 ($c.697T > C$). Moreover, one block with two variants ($c.373 + 243G > A$ and $c.373 + 249T > C$) in intron 1 of *MSTN* gene was found (Figure 5).

Association analysis

The principal component analysis, with the 44 SNPs used in this study, did not indicate structuration (Figure 6). The single-locus analysis revealed 10 suggestive ($p < 0.05$) and two significant ($p < 0.0011$)

Table 4. Heterozygosities observed (HO) and predict (HP), Hardy–Weinberg Equilibrium (HWE) *p*-value, minor allele frequency (MAF), and location of variants found in Santa Inês sheep.

NCBI number	HGVS names	Gene	HO	HP	HWE (*p*-value)	MAF	Location
rs1135847320	*c.1293G > T*	*MyoD1*	0.105	0.120	0.28	0.064	3′UTR
rs1135847343	*c.934 + 29G > C*	*MyoD1*	0.326	0.344	0.59	0.221	Intron-2
rs1135847345	*c.934 + 135T > G*	*MyoD1*	0.029	0.040	0.12	0.020	Intron-2
rs1135847350	*c.935-224C > G*	*MyoD1*	0.297	0.299	1.00	0.183	Intron-2
rs1135847351	*c.935-206G > A*	*MyoD1*	0.099	0.125	0.06	0.067	Intron-2
rs1135847353	*c.935-185C > G*	*MyoD1*	0.105	0.130	0.07	0.070	Intron-2
rs1135847355	*c.935-82A > G*	*MyoD1*	0.267	0.240	0.24	0.140	Intron-2
rs1135847358	*c.935-78G > A*	*MyoD1*	0.140	0.130	0.84	0.070	Intron-2
rs599663516	*c.546T > C*	*MyoD1*	0.308	0.307	1.00	0.189	Exon-3
rs1086681542	*c.668G > A*	*MyoD1*	0.134	0.125	0.91	0.067	Exon-3
rs1135847312	*c.465-157G > A*	*MyoG*	0.141	0.140	1.00	0.076	Intron-1
rs417690032	*c.464 + 289T > C*	*MyoG*	0.445	0.389	0.07	0.264	Intron-1
rs426956376	*c.464 + 185G > A*	*MyoG*	0.236	0.231	1.00	0.134	Intron-1
rs410212255	*c.383-26C > T*	*MyoG*	0.403	0.357	0.12	0.233	Intron-2
rs599563675	*c.383-92A > C*	*MyoG*	0.063	0.061	1.00	0.031	Intron-2
rs419534498	*c.383-306C > T*	*MyoG*	0.230	0.243	0.63	0.141	Intron-2
rs400160301	*c.383-356T > C*	*MyoG*	0.560	0.495	0.10	0.448	Intron-2
rs412989269	*c.382 + 365G > A*	*MyoG*	0.058	0.075	0.06	0.039	Intron-2
rs422285781	*c.382 + 276G > C*	*MyoG*	0.298	0.322	0.41	0.202	Intron-2
rs405981477	*c.382 + 256A > G*	*MyoG*	0.084	0.080	1.00	0.042	Intron-2
rs412105535	*c.382 + 41C > T*	*MyoG*	0.398	0.401	1.00	0.277	Intron-2
rs410772203	*c.109C > A*	*MyoG*	0.084	0.090	0.66	0.047	Exon-3
rs1135847279	*c.390G > C*	*MyF5*	0.058	0.056	1.00	0.029	Exon 1
rs1135847290	*c.441T > A*	*MyF5*	0.058	0.056	1.00	0.029	Exon 1
rs416158998	*c.600 + 32T > C*	*MyF5*	0.058	0.056	1.00	0.029	Intron-1
rs421299802	*c.600 + 222G > A*	*MyF5*	0.042	0.041	1.00	0.021	Intron-1
rs1135847302	*c.600 + 271T > C*	*MyF5*	0.053	0.051	1.00	0.026	Intron-1
rs399775445	*c.600 + 399G > C*	*MyF5*	0.079	0.076	1.00	0.039	Intron-1
rs401351612	*c.880G > C*	*MyF5*	0.058	0.056	1.00	0.029	3′UTR
rs412427068	*c.1059C > T*	*MyF5*	0.047	0.046	1.00	0.024	3′UTR
rs595997498	*c.653 + 66G > A*	*MyF6*	0.384	0.385	1.00	0.261	Intron-2
rs591524187	*c.653 + 67T > G*	*MyF6*	0.379	0.388	0.86	0.263	Intron-2
rs409632361	*c.654-104A > C*	*MyF6*	0.111	0.114	1.00	0.061	Intron-2
rs399504900	*c.697T > C*	*MyF6*	0.111	0.132	0.11	0.071	Exon-3
rs119102825	*c.373 + 18G > T*	*MSTN*	0.541	0.497	0.45	0.459	Intron-1
rs119102826	*c.373 + 241T > C*	*MSTN*	0.287	0.269	0.75	0.160	Intron-1
rs427811339	*c.373 + 243G > A*	*MSTN*	0.484	0.413	0.10	0.291	Intron-1
rs417602601	*c.373 + 249T > C*	*MSTN*	0.180	0.190	0.80	0.107	Intron-1
rs119102828	*c.373 + 259G > T*	*MSTN*	0.377	0.466	0.05	0.369	Intron-1
rs407388367	*c.373 + 323C > T*	*MSTN*	0.254	0.246	1.00	0.143	Intron-1
rs408710650	*c.373 + 564G > A*	*MSTN*	0.295	0.274	0.67	0.164	Intron-1
rs413881846	*c.373 + 914A > G*	*MSTN*	0.090	0.086	1.00	0.045	Intron-1
rs420853334	*c.374-667A > G*	*MSTN*	0.254	0.246	1.00	0.143	Intron-1
rs1135847247	*c.374-123T > C*	*MSTN*	0.230	0.216	0.87	0.123	Intron-1

HGVS: Human Genome Variation Society; NCBI: National Center for Biotechnology Information.

additive effects (Table 5). The variants *c.935-185C > G* in intron 2 of *MyoD1* and *c.464 + 185G > A* in intron 1 of *MyoG* were found to be significantly associated with tenderness and a*, respectively. The genotype *TT* showed higher tenderness than *GG*, being a difference of 8.62 N; while the difference between *GG* and *AA* for a* was 5.72. The variant *c.935-185C > G* showed the frequencies 87.9% (TT), 10.4% (TG), and 1.7% (GG), while the variant *c.464 + 185G > A* had genotype frequencies of 75.0% (GG), 23.4% (GA), and 1.6% (AA). Moreover, four suggestive ($p < 0.05$) and eight significant ($p < 0.01$) associations were found when haplotype association analysis was performed (Table 6). The haplotype replacement *GT > AG* in intron 2 of *MyoD1* was significantly associated with higher values of pH0 and pH24, and lower mean values of b* and tenderness; while the replacement *GT > AC* in intron 1 of *MSTN* was significantly associated with higher mean values of pH0, pH24, and tenderness, and the lower mean value of a*.

Changes of the TFBS

The SNP *c.464 + 185G > A* in intron 1 of *MyoG* gene was found to be in association with a* (Table 5). Consequently, a TFBS prediction was performed for this variant. The allele *c.464 + 185G* was a binding site for the transcription factors EBF1, GLI1, GLIS1, GLIS2, NR1D2, RARA, TCF12, TCF3, and VDR, while the allele *c.464 + 185A* was a binding site for the transcription factors CREBBP, EBF1, ESR1, HNF4A, MYC, NR2F2, RARA, SP2, SRF, TCF12, TCF3, TCF7, TP53, and VDR.

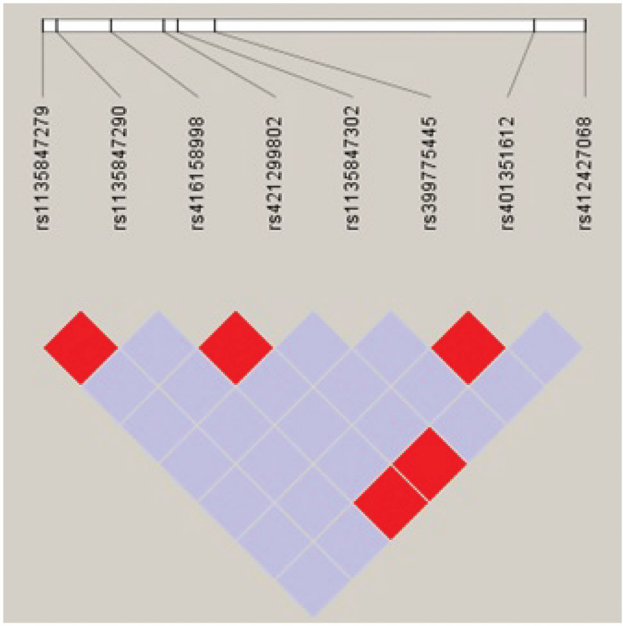

Figure 1. No haplotype blocks in Santa Inês *MyF5* gene.

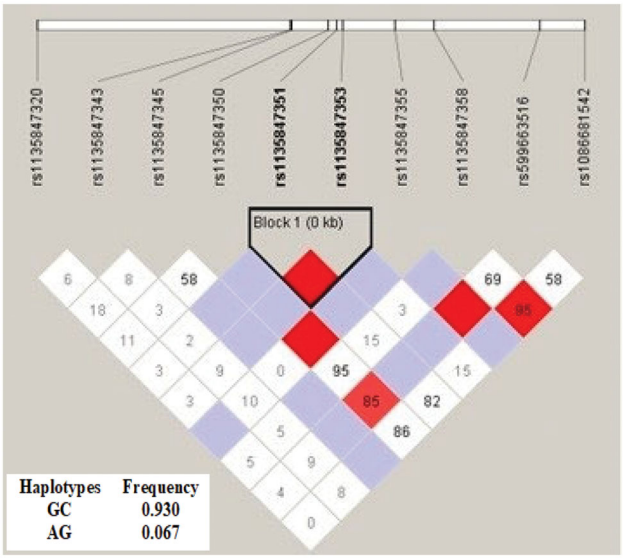

Figure 2. Haplotype block in Santa Inês *MyoD1* gene and the haplotype copy frequencies.

In *MSTN* gene, single-locus analysis revealed an association between the variant $c.373+243G>A$ with both tenderness and a* (Table 5); while haplotype association analysis revealed an association of LDB in intron 1, formed with the variants $c.373+243G>A$ and $c.373+249T>C$, with pH0, pH24, a*, and tenderness (Table 6). So, a TFBS prediction was performed for the haplotypes *GT*, *AC*, and *AT*. TFBS for the haplotype GT were ESR1 and TRIM28. The TFBS for haplotype *AC* were DMRT1, ESR1, GTF2B, POUZ6F1, SMAD4, SOX21, and SOX7; while the TFBS for haplotype *AT* were ESR1, IRX4, SMAD4, SOX21, SOX7, and TRIM28.

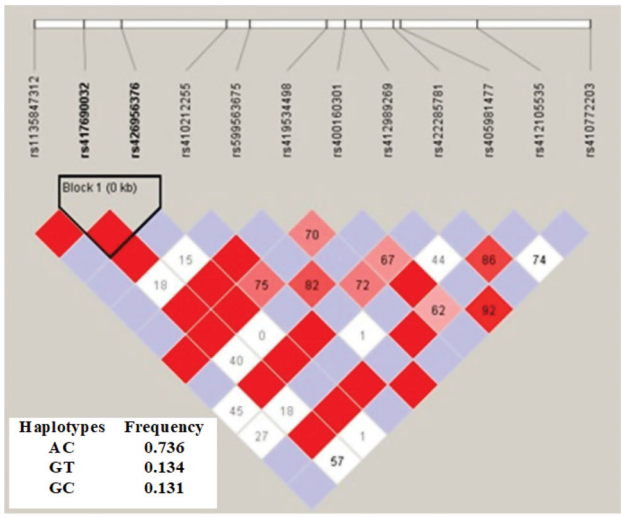

Figure 3. Haplotype block in Santa Inês *MyoG* gene and the haplotype copy frequencies.

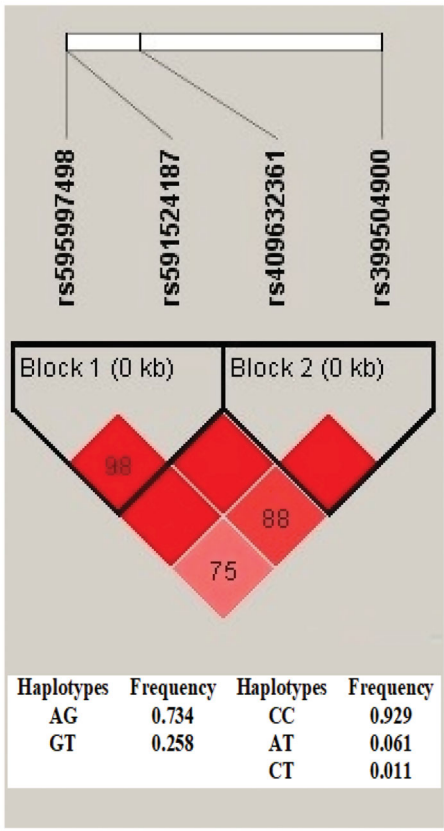

Figure 4. Haplotype blocks in Santa Inês *MyF6* gene and the haplotype copy frequencies.

microRNA binding site

The variants ($c.935\text{-}206G>A$, $c.935\text{-}185C>G$) in intron 2 of *MyoD1* gene were associated with meat quality traits in Santa Inês sheep (Tables 5 and 6). A binding site prediction for miRNA was carried out for *GC* and *AG* haplotype copies, which revealed eight

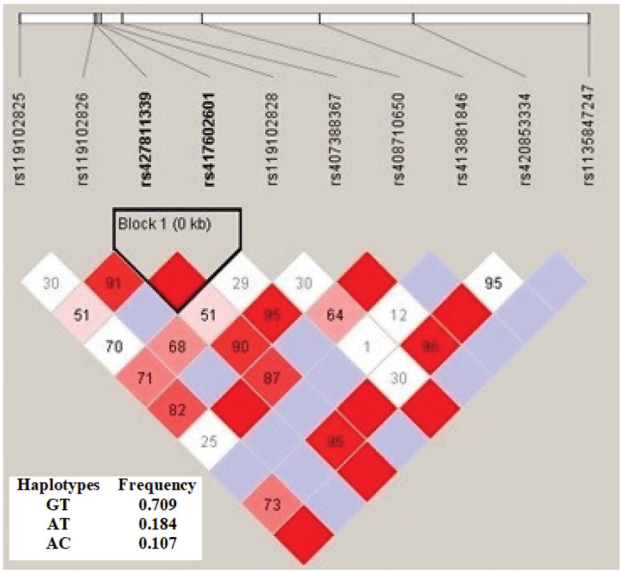

Figure 5. Haplotype block in Santa Inês *MSTN* gene and the haplotype copy frequencies.

and 12 miRNA binds the sequences containing the *GC* and *AG*, respectively. Four and eight miRNAs were specifics for *GC* and *AG*, respectively (Table 7).

The variants (*c.383-306C > T*, *c.383-356T > C*, *c.382 + 276G > C*) in intron 2 of *MyoG* gene were found to be in association with some trait (Table 5). For these variants, the miRNA biding site research revealed differences in both the number and type of miRNA that can binding the sequences (Table 7). For the SNP *c.383-306C > T* were found 28 and 27 possible miRNAs to bind the sequences when the *c.383-306C* and *c.383-306T* allele were used, respectively. Six and five specific miRNAs for *c.383-306C* and *c.383-306T* alleles were found. For the SNP *c.383-356T > C* were found seven miRNA for both *c.383-356T* and *c.383-356C* alleles, but only one specific miRNA for each allele was detected. Regarding SNP *c.382 + 276G > C*, 38 and 11 miRNAs were found with *c.382 + 276G* and *c.382 + 276C* allele, respectively. Thirty miRNA were

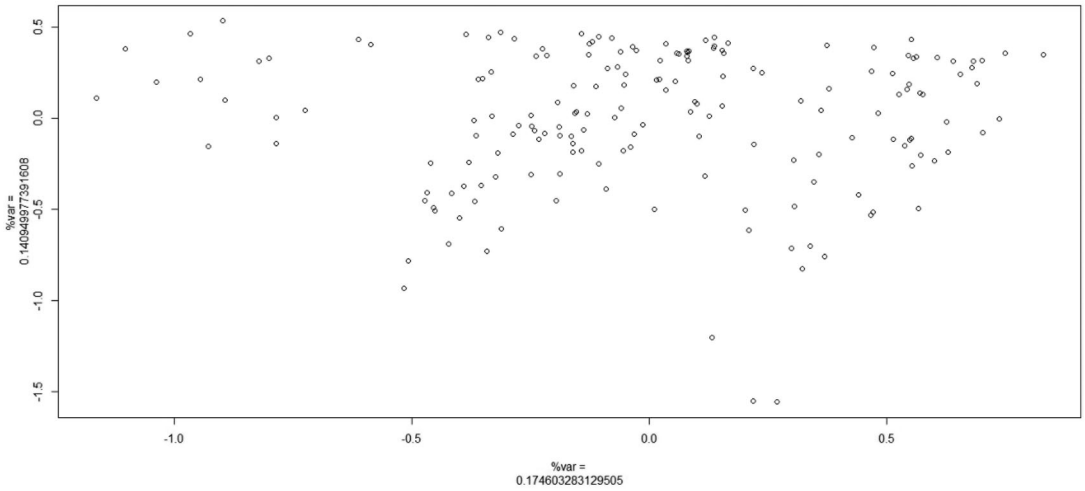

Figure 6. First (horizontal axis) and second (vertical axis) principal components obtained with variants found in this Santa Inês population and their eigenvalues.

Table 5. Additive (a) and dominance (d) effects, with their respective standard errors (SE), estimated with single locus analysis of polymorphisms in the *MyoD* family and *MSTN* genes in Santa Ines sheep.

Trait	Gene (variant)	Location	a (SE)	d (SE)	LRT[a]	*p*-Value
a*	*MyoD1* (*c.935-185C > G*)	Intron 2	1.14 ± 0.48	—	5.65	0.0174
b*	*MyoD1* (*c.935-185C > G*)	Intron 2	1.23 ± 0.37	—	10.71	0.0047
L*	*MyoD1* (*c.935-185C > G*)	Intron 2	2.53 ± 0.87	—	8.28	0.0040
Tenderness (*N*)	*MyoD1* (*c.935-185C > G*)	Intron 2	−4.31 ± 1.08	—	15.54	<0.0001†
a*	*MyoG* (*c.464 + 185G > A*)	Intron 1	−2.86 ± 0.64	−2.87 (0.72)	19.18	<0.0001†
pH0	*MyoG* (*c.383-306C > T*)	Intron 2	−0.08 ± 0.03	—	4.95	0.0260
L*	*MyoG* (*c.383-356T > C*)	Intron 2	0.97 ± 0.47	—	4.26	0.0390
Tenderness (*N*)	*MyoG* (*c.382 + 276G > C*)	Intron 2	1.57 ± 0.78	—	3.99	0.0457
pH24	*MyoG* (*c.382 + 276G > C*)	Intron 2	0.09 ± 0.04	—	6.20	0.0128
Tenderness (*N*)	*MyF6* (*c.653 + 67T > G*)	Intron 2	1.37 ± 0.68	—	4.58	0.0324
Tenderness (*N*)	*MSTN* (*c.373 + 243G > A*)	Intron 1	−2.84 ± 0.95	—	10.03	0.0015
a*	*MSTN* (*c.373 + 243G > A*)	Intron 1	0.85 ± 0.37	—	5.12	0.0236

[a]LRT: Likelihood ratio test.
†Significant association on Bonferroni threshold ($p < 0.0011$).

found to be specific for the sequence with *c.382 + 276G* allele, while only three miRNA were found to be specific for *c.382 + 276C* allele.

Single-locus analysis revealed an association of the variant *c.653 + 67T > G* in intron 2 of *MyF6* gene with Tenderness (Table 5). Moreover, a haplotype of this variant with the variant *c.653 + 66G > A* was also associated with tenderness (Table 6). Then, the sequences around the haplotype *GT* and *AG* were used in miRbase to find miRNA binding sites. For GT were found 12 miRNAs, but only one specific, while for the haplotype AG were found 15 miRNAs, being four specifics (Table 7).

Discussion

Variants and haplotypes

The candidate genes used in the present study were sequenced in Santa Ines sheep by Sousa-Junior et al.[27] which reported 59 variants in *MyoD1*, 24 in *MyoG*, 63 in *MyF5*, four in *MyF6*, and 10 in *MSTN*. The allele and genotype frequencies of almost all these variants were similar to those reported for other sheep populations.[27] Of the 160 variants reported by Sousa-Junior et al.[27] 116 were either in Hardy–Weinberg disequilibrium ($p < 0.05$) or showed MAF <2%. Then, we selected the 44 variants (Table 4) in HWE ($p > 0.05$) and with MAF >2% to perform an association study with physical meat traits in Santa Inês sheep. Of the 44 variants selected, 35 were located in intron (Table 4). Therefore, we expected to find more non-coding than coding variants associated with physical meat trait in Santa Ines sheep. Moreover, in the present study five haplotypes were found and can be used to perform association analysis. Haplotypes in *MyoD1*[35] and *MyoG*[36] were associated with muscle fibers traits in pigs, but no previous study reported association of haplotypes in these genes with meat traits in sheep.

Table 6. Regression coefficients (β) and standard errors (SE) estimated with the haplotype association analysis in *MyoD* family *and MSTN* gene in Santa Ines sheep.

Trait	Gene	Haplotype replacement	β±SE	*p*-Value
pH0	*MyoD1*	*GC > AG*	1.43 ± 0.26	<0.001+
pH24	*MyoD1*	*GC > AG*	1.25 ± 0.22	<0.001+
L*	*MyoD1*	*GC > AG*	−2.30 ± 0.91	0.013
b*	*MyoD1*	*GC > AG*	−1.06 ± 0.39	0.006+
Tenderness (N)	*MyoD1*	*GC > AG*	−4.13 ± 1.16	0.001+
Tenderness (N)	*MyF6*	*AG > GT*	1.92 ± 0.85	0.025
pH0	*MSTN*	*GT>AC*	1.26 ± 0.31	<0.001+
pH24	*MSTN*	*GT>AC*	1.07 ± 0.27	<0.001+
a*	*MSTN*	*GT>AC*	−1.39 ± 0.50	0.007+
a*	*MSTN*	*GT > AT*	−0.89 ± 0.44	0.047
Tenderness (N)	*MSTN*	*GT>AC*	3.83 ± 1.22	0.002+
Tenderness (N)	*MSTN*	*GT > AT*	2.43 ± 1.07	0.024

+Significant association on Bonferroni threshold ($p < 0.01$).

Association analysis

Our results on *MyoD1*, although novel for sheep, are supported by previous studies conducted on other species. The variant *g.1264C > A* in intron 1 was associated with L* in pork,[35] while some studies reported an association of the variant *g.489C > T* in *MyoD1* with the pH of the LL muscle 48 hours post-slaughter[8] and with the pH of semi-membranous muscle of pigs 45 minutes and 24 hours post-slaughter.[37] Moreover, an effect of variant *g.1406G > A* in intron 1 of *MyoD1* on pH in pork was reported.[38] The effects on pH, meat color parameters, and tenderness observed in this study may be related to composition and density of fibers in the LL. In pigs, two haplotypes in *MyoD1*

Table 7. Specific miRNAs bind the sequences with different alleles in intronic variants of Santa Inês sheep.

Variants	Alleles	Number of miRNAs binding the sequences: Total	Specifics	Specific miRNA
c.383-306C > T	*C*	28	6	ppy-miR-1914, hsa-miR-1587, hsa-miR-3620-5p, hsa-miR-6848-5p, hsa-miR-7113-3p, aga-miR-10363-3p
	T	27	5	hsa-miR-6824-5p, mdo-miR-7377-3p, efu-miR-9362, cja-miR-506, mmr-miR-152
c.383-356T > C	*T*	7	1	gmo-miR-33b-2-3p
	C	7	1	bma-miR-9529
c.382 + 276G > C	*G*	38	30	dme-miR-13a-3p,hsa-miR-147a, dps-miR-13a, ptr-miR-147a, ppy-miR-147a, sla-miR-147, mne-miR-147, ppa-miR-147, ame-miR-13a-3p, hsa-miR-146b-3p, mml-miR-147a, dan-miR-13a, der-miR-13a, dgr-miR-13a, dmo-miR-13a, dpe-miR-13a, dse-miR-13a, dsi-miR-13a, dvi-miR-13a-3p, dwi-miR-13a, dya-miR-13a, nvi-miR-13a, ppy-miR-146b-3p, ngi-miR-13a, nlo-miR-13a, pma-miR-147, aca-miR-212-3p, aga-miR-2c-3p, dqu-miR-13a-3p, pte-miR-2c-3p
	C	11	3	osa-miR5158, csi-miR530b-3p, sfr-miR-10475-3p
c.653 + 66G > A	*GT*	12	1	ocu-miR-342-5p
and c.653 + 67T > G	*AG*	15	4	mmu-miR-1966-5p, hsa-miR-3175, mmu-miR-6946-5p, pal-miR-9287-3p
c.935-206G > A and	*GC*	8	4	bmo-miR-2807a, cin-miR-15-5p, cfa-miR-8871, eca-miR-8969
c.935-185C > G	*AG*	12	8	hsa-miR-300, ptr-miR-300, ppy-miR-300, aca-miR-5433, hsa-miR-5699-3p, hsa-miR-7157-3p, cfa-miR-8906, mmu-miR-9768-3p

were associated with the composition and density of muscle fibers,[35] which also impacted the total rib eye area. Similarly, the effects of variants in *MyoD1* on the diameter and density of muscle fibers in chicken[39] and rainbow trout[40] were found. Although no study has associated variants in the *MyoD1* gene with the type or density of muscle fibers in sheep, the association between variants in this gene and muscle growth in sheep has been reported. An association of the rs412308724 variant in *MyoD1* with loin width and chest girth was reported for Stavropol sheep,[11] while a positive correlation between *MyoD1* expression and cold carcass yield in Brazilian hair sheep (including Santa Inês sheep) was reported.[41] These results revealed the vital role of the *MyoD1* gene in muscle mass increase, which can affect physical meat traits, as observed for Santa Inês sheep. Fat composition is another factor that may explain the effects on physical meat traits. *MyoD1* can initiate the myogenic program in mature adipocytes *in vivo*,[42] while a negative correlation between *MyoD1* expression and total proportion of polyunsaturated fatty acids, n6, and essential fatty acids, as well as a positive relationship with monounsaturated fatty acids, were reported.[41] Therefore, changes in muscle/fat ratio caused by *MyoD1* would explain the effects on physical meat traits observed in the present study.

The myogenin is an embryonic protein that acts on the differentiation of myoblasts in multinucleated myofibrils.[43] In the postnatal phase, myogenin is found to be in association with the repair of damage to muscle fibers and to hypertrophic growth. Therefore, myogenin is directly related to the muscular mass and the total amount of meat in the carcass.[44] We have conducted, for the first time, an association study with variants in the *MyoG* gene and physical meat traits in sheep and found some additive effects on meat color traits, pH, and tenderness. Early studies in other livestock species reported effects on physical meat traits. Variants in *MyoG* were associated with a* and L* in swine,[8] while the effects on the water content in some muscles of swine were also reported.[45] A haplotype in *MyoG* was associated with muscular fiber type, the total number of fibers, and the rib eye area in pigs,[36] while effects on the diameter of muscle fibers were reported in chicken.[39] No previous studies have reported any effect of *MyoG* on physical meat traits in sheep; however, variants in this gene were associated with body weight, height, and length in some Tibetan sheep breeds,[46] while changes in muscle development can affect physical meat traits.

The results of the single-locus association analysis found for *MyF5* were probably a consequence of the distribution of genotypic and allele frequencies of the variants evaluated, since more than 95% of the animals were found to be homozygous for the reference allele.[27] Although significant effects were not observed, previous studies of swine,[47] bovine[48] and rabbits[10] revealed associations between variants in *MyF5* and physical meat traits. The effect of *MyF5* variants on lean meat yield in both the leg and loin cuts of sheep were reported.[13] Moreover, significant correlations between *MyF5* expression in the longissimus muscle of Wuzhumuqin sheep and the type of fibers in this muscle was reported.[49] Therefore, there is evidence of the effects of *MyF5* variants on meat quality traits and an increase in the Santa Inês sample size may be able to identify these effects.

No previous studies have described an association between alteration in *MyF6* (also known as *MRF4*) and phenotypic traits in sheep. However, variants in this gene were associated with growth[50,51] and carcass[52,53] traits in pigs. In cattle, effects on growth[54] and carcass[55] traits were also reported. Additionally, the effects of variants in *MyF6* on the length, depth, and weight of Nile tilapia have also been reported.[56] In the context of meat traits, Yang et al.[39] reported an effect on the diameter of muscular fibers in chicken, while an association with drip loss in pork[39] and insignificant negative correlation between *MyF6* expression in longissimus muscle and type I (−0.47) and type I/IIB ratio (−0.49) were also reported.[49]

Of the candidate genes in the present study, *MSTN* have been the most studied in sheep, since alteration in this gene has been associated with greater muscular development and lower fat deposition. Variants in the coding region[15,57,58] in 3′ UTR[16,17,57–65] and 5′UTR[19] were associated with increased muscle development and lower fat deposition in sheep. In this study, we sequenced 47.7% of the *MSTN* gene, including part of exons 1 and 2, and all intron 1, with variants only being found in intron 1. A PCR-SSCP in intron 1 was associated with birth weight in Makoei sheep,[66] while a PCR-SSCP in intron 1 of *MSTN* was associated with slaughter weight and primal cuts yields such as shoulder, leg, and loin yield in New Zealand Romney sheep.[18] Therefore, it is possible that variants in intron 1 influence phenotypic traits in sheep. In addition, an association between variant *g-41C > A* in the 5′UTR region and sensorial tenderness was reported,[19] though no significant association between the variant $g + 6723G > A$ (currently known as $g + 6223G > A$) and meat quality traits in sheep was found,[21] which is

potentially a consequence of the small sample size used (22 animals).

Single-locus and haplotype association analyzes revealed some effects of variants in *MSNT* and MyoD family genes on physical meat traits in Santa Inês sheep. Many of these results were reported for the first time in sheep, but similar results were found in other livestock species. However, the mechanism by which these genes affect physical meat traits remains unknown. Some previous studies reported these genes as being differentially expressed in the longissimus muscle of sheep. *MyoD1* was differentially expressed in the longissimus muscle and may be responsible for the different muscle growth rates of Dorset and Han sheep breeds.[67] *MyoG* and *MyoD1* were up-regulated, while *MyF6* was down-regulated in the longissimus muscles of Qianhua Mutton Merino sheep compared to Small-tail Han sheep.[68] In addition, these genes are known to be involved in several biological processes, with some of these processes being related to muscle development.[6,7] Thus, the activity of these five genes changes the development of several muscles. As a result, modifications in physical meat traits can occur.

Causal hypotheses

In this study, only intron variants were associated with the traits. Intronic variants contributes to the variability of gene expression and splicing in human populations.[69] Thus, we investigate the potential causal effect of the intronic variants on the meat quality traits in the current study. This study revealed that the effects of variants located in intron 1 of the *MyoG* ($c.464+185G>A$) and *MSTN* ($c.373+243G>A$ and $c.373+249T>C$) genes on meat quality traits could be a consequence of the differences in number and type of TFBS. For the $464+185\,G$ and $464+185\,A$ alleles, were found 9 and 14 TFBS, respectively. In addition, a group of four specific TFBS for $464+185\,G$ allele (GLI1, GLIS1, GLIS2, NR1D2) and nine particular TFBS for $464+185\,A$ (CREBBP, ESR1, HNF4A, MYC, NR2F2, SP2, SRF, TCF7, TP53) were found. Therefore, the SNP $c.464+185G>A$ may have an important key role in *MyoG* expression.

Regarding *MSTN* variants, TFBS prediction also revealed differences between the *GT*, *AC*, and *AT* haplotypes. The most frequent haplotype (*GT*) in Santa Ines population had only two TFBS (ESR1 and TRIM28), while alternative haplotypes *AC* and *AT* showed seven (DMRT1, ESR1, GTF2B, POUZ6F1, SMAD4, SOX21, SOX7) and six (ESR1, IRX4, SMAD4, SOX21, SOX7, and TRIM28) TFBS, respectively. Thus, higher *MSTN* expression is expected with *AC* and *AT* than *GT* haplotype. According to Bagatoli et al.[70] high levels of *MSTN* expression are associated with lower values of meat tenderness in Santa Inês sheep. Our result also indicated that both replacements $GT>AC$ and $GT>AT$ increased the shear force in LL samples and consequently reduced the meat tenderness (Table 6). Hickford et al.[71] reported a *B* SSCP standard in intron 1 of ovine *MSTN* gene associated with increased leg, loin, and total yields in New Zealand Romney sheep, where the *B* haplotype copy carries the $c.373+243A$ and $c.373+249T$ alleles. Therefore, *AT* haplotype may be associated with both increased muscle mass and reduced meat tenderness in sheep.

The variants located in intron 2 of the *MyoG* ($c.383\text{-}306C>T$, $c.383\text{-}356T>C$, $c.382+276G>C$), *MyF6* ($c.653+66G>A$, $c.653+67T>G$), and *MyoD1* ($c.935\text{-}206G>A$, $c.935\text{-}185C>G$) genes were also associated with meat quality traits in the current study (Tables 5 and 6). In this case, a miRNA binding site prediction was carried out, which revealed a different number and type of miRNA binds the sequences evaluated (Table 7). miRNAs are small single-stranded molecules that suppress the expression of protein-coding genes by translational repression, messenger RNA degradation, or both.[72]

Future gene expression studies about MyoD family and *MSTN* genes can reveal if intronic variants in these genes are related to their expression in Santa Inês sheep. However, the results of the current study confirmed the hypothesis of an association between variants in these genes and physical-chemical meat quality-related traits in Santa Inês sheep. These traits are known to have a complex genetic inheritance, and this explains the small additive effects found in the current study. Therefore, a marker-assisted selection scheme, only with these variants, probably will result in a small genetic gain. Moreover, nowadays, the slaughterhouses in Brazil do not pay a premium for sheep meat quality, which reduces the chance of practical application of these variants in selection schemes. Despite this, some variants, especially in *MSTN* gene, are known to have a substantial effect on both increasing muscle mass and reducing fat-related traits in sheep, which generated a considerable interest of worldwide ovine industry. Our results serve as an alert to the sheep industry since there are no long-term negative consequences for meat quality.

Acknowledgments

To Embrapa Tabuleiros Costeiros for the infrastructure of the experimental farm; and To Dr. Luiz Lehmann Coutinho

for the infrastructure of Functional Genomics Core facility of ESALQ/USP. LL Coutinho, LFB Pinto, GB Mourão, and VB Pedrosa are recipient of a CNPq Productivity Scholarship.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the [FAPESB] under Grant [APP0116/2009]; [CNPq] under Grant [562551/2010-7 and 474494/2010-1]. This study was financed in part by the CAPES – Finance Code 001.

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