Genes and proteins associated with ribeye area and meat tenderness in a commercial Nellore cattle population

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Abstract: Despite several studies on genetic markers and differently expressed genes related to ribeye area (REA) and tenderness traits in beef cattle, there is divergence in the results regarding the genes associated with these traits. Thirteen genes that had been associated or have biological functions that may influence such phenotypes were included in this study. A total of five genes for REA (*IGF-1*, *IGF-2*, *MSTN*, *NEDD4*, and *UBE4A*) and eight genes for meat tenderness (*CAPN1*, *CAPN2*, *CAST*, *HSPB1*, *DNAJA1*, *FABP4*, *SCD*, and *PRKAG3*) were selected from previously studies in beef cattle. Genes and its respective proteins expression were validated in a commercial population of Nellore cattle using quantitative real-time PCR (RT-qPCR) and advanced mass spectrometry (LC / MS-MS) techniques, respectively. *MSTN* gene was upregulated in animals with low REA. *CAPN1*, *CAPN2*, *CAST*, *HSPB1*, and *DNAJA1* genes were upregulated in animals with tougher meat. The proteins translated by these genes were not differentially expressed. Our results could confirm the potential of some studied genes as biomarkers for carcass and meat quality in Nellore cattle.

Keywords: beef cattle, biological processes, gene expression, meat quality, zebu.

1. Introduction

The Brazilian beef cattle expansion is directly associated with the competitiveness of the productive sector. Despite production and reproduction traits are crucial for the beef cattle operations, carcass and meat quality traits may play an important role in the success of the entire beef industry. Although Nellore cattle, the most important breed in Brazil, present adaptability and rusticity in tropical regions, they do not have the same carcass and meat quality when compared with European breeds (Crouse et al., 1989; Wheeler et al., 1994; Ferraz e Felício., 2010; Lage et al., 2012; Pereira et al., 2015). Thus, selection for increasing animal performance as well as to improve carcass and meat quality is necessary to meet consumer market demand.

Several studies have been conducted to identifying genetic markers and differentially expressed genes related to carcass and meat quality traits in different species (Pan, 2003; Lehnert et al., 2007; Fonseca et al., 2015; Fonseca et al., 2017; Li et al., 2016; Piórkowska et al., 2016; Vignato et al., 2017; Fang et al., 2018; Horodyska et al., 2018; Silva et al., 2020). In beef cattle, however, due to genetic and environmental factors, there is a wide divergence across breeds regarding the genes associated with such traits. Identify differently expressed genes, therefore, would provide a better understanding of muscle physiological processes and their influence on meat quality traits (Bernard et al., 2007; Carvalho et al., 2012; Fonseca et al., 2017; Malheiros et al., 2018, Silva et al., 2020).

Even though several large-scale gene expression tools have been developed, the real-time PCR technique (qRT-PCR) is known as the most effective and accurate method for identification of key genes. (Heid et al., 1996). Moreover, once proteins are the final molecules resulting from the central dogma of biology, studying the profile of differential gene expression at the mRNA and protein level may help to understand biological processes and allow scientists to find possible biomarkers that could contribute to the

selection of quality beef animals (Bendixen et al., 2005, Hollung et al., 2007). Thus, this study aimed to identify and validate differentially expressed genes for rib eye area (REA) and meat tenderness found in the literature and verify whether they are being translated into differentially expressed protein.

2. Materials and Methods

2.1 Sample collection

Meat samples were collected from 189 Nellore bulls belonged to São Marcelo farm, located in Tangará da Serra/MT – Brazil, which is part of the Cia de Melhoramento animal breeding program. Animals were raised on pasture, finished in a feedlot for about 90 days, and slaughtered in a commercial slaughterhouse with approximately 24 months of age. Slaughtering procedures followed the Sanitary and Industrial Inspection Regulation for Animal Origin Products from the Ministry of Agriculture, Livestock, and Supply. Tissue samples from Longissimus thoracis muscle were collected during slaughter and stored in 15 mL falcon tubes containing 5 mL of RNA holder (BioAgency, Sao Paulo, SP, Brazil). The samples were stored in an ultra-low freezer at -80 °C until RNA and proteins extraction was performed. After 24 to 48 hours of cooling in a cold chamber, samples from Longissimus thoracis muscle, between the 12th and 13th ribs of the left side of each carcass were collected and frozen at -20 °C. Steak samples of 2.54 cm thick were used to measure ribeye muscle area (REA), according to USDA Quality Grade (1989), and meat tenderness, through Warner-Bratzler shear force (WBSF) methodology proposed by Wheeler et al. (1995).

The phenotypes obtained were adjusted for the fixed effects of contemporary groups (CG) and age of animal at slaughter, as linear covariate. The CG were defined according to year and farm of birth, management group and farm at yearling, and slaughtered date. Fixed effect solutions were estimated through a single trait animal

model using airemlf90 software (Miszta et al., 2014). The dataset of 4.694 and 4.839 animals for rib eye area (REA) and meat tenderness, respectively, were considered in the analysis.

After pre-adjust the phenotypes, 12 extreme animals were selected for each trait (REA and meat tenderness). To verify the statistical difference between the extreme groups, the Student's T test was performed using software R (Table 1).

Table 1: Number of animals (N), means, standard error (SE), minimum and maximum for extremes pre-adjusted of meat tenderness(kgf/cm²) and ribeye area (cm²) traits in Nellore cattle.

	N	Means	SE	Minimum	Maximum	p-value
REA* high	12	82.93	3.91	79.14	91.51	2.09E ⁻¹¹
REA* low	12	58.40	1.41	55.68	60.09	
Tender Meat	12	3.31	0.69	1.99	4.12	8.99E ⁻¹⁵
Tough Meat	12	9.16	0.54	8.66	10.05	

*Ribeye area.

2.2 Target genes

Thirteen genes that had been associated or that have biological functions that may influence rib eye area and meat tenderness, were chosen for validation (Table 2). Furthermore, differentially expressed genes in muscle tissue described in published papers or public database were considered in this study.

2.2.1 Extraction of total RNA and quantitative PCR real-time

Fifty mg of muscle tissue previously stored in RNAlater solution was used (Ambion, ThermoFisher Scientific, USA) to extract the total RNA using the extraction kit RNeasy Lipid Tissue Mini (Qiagen, Valencia, CA, EUA), according to instructions from manufacturers. RNA concentration and purity were evaluated using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Santa Clara, CA, EUA, 2007) by measuring absorbance ratio of 260, 280, and 230 nm. In addition, the Bioanalyzer determined the RIN (RNA integrity number) index, considering values equal or higher than 7 as satisfactory (Schroeder et al., 2006). The absence of contamination for each

sample with genomic DNA was confirmed in a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA, 2010) using Qubit brDNA Assay Kit (Invitrogen). The complementary DNA (cDNA) strand synthesis was performed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA), according to instructions from manufacturers.

Nucleotide sequences used to design the primers were obtained from mRNA sequences present in GeneBank Database (<http://www.ncbi.nlm.nih.gov>). Thirteen primer pairs were designed for the target genes studied here including: *IGF-1*, *IGF-2*, *MSTN*, *UBE4A*, and *NEDD4* for REA and *CAPN1*, *CAPN2*, *CAST*, *HSPB1*, *DNAJ1*, *FABP4*, *SCD*, and *PRKAG3* for meat tenderness (Table 3). Moreover, three reference genes were selected from literature to normalize the reactions: Beta Actin (*ACTB*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (Table 3). For oligonucleotide design, the Primer Express 3.0 program (Applied Biosystems, Foster, CA, USA, 2004) was used. Subsequently, the sequence specificity of the chosen oligonucleotides was verified using NCBI BLASTN algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>) related to the bovine genome.

The qRT-PCR reactions were performed on the Applied Biosystems™ 7500 Real-Time PCR System, using the SYBR Green Master Mix. Primer concentrations were determined by titration. For REA related genes the combinations of forward and reverse primers in nM were: *IGF-1* (100/100), *IGF-2* (100/100), *MSTN* (300/300), *NEDD4* (100/100) and *UBE4A* (300/300). For the tenderness related genes, the combinations of forward and reverse primers in nM were: *CAPN1* (600/600), *CAPN2* (100/100), *CAST* (300/300), *DNAJ1* (100/100), *HSPB1* (600/600), *FABP4* (300/300), *SCD* (300/300), and *PRKAG3* (600/600).

150 **Table 2:** Genes related to ribeye area and meat tenderness.

	GENE	Author	Breed	Method
Ribeye area.	IGF-1	Guo <i>et al.</i> , (2015)	Brahman	Microarray
		Sadkowski <i>et al.</i> , (2009)	Limousin	Microarray
	IGF-2	Silva <i>et al.</i> , (2020)	Nellore	RNAseq ²
		Goodall and Schmutz <i>et al.</i> , (2007)	<i>Bos taurus</i>	Genotyping ³
	MSTN	Busato <i>et al.</i> , (2016)	Brahman	qRT-PCR ¹
		Silva <i>et al.</i> , (2020)	Nellore	RNAseq ²
		Zhang <i>et al.</i> , (2011)	Quinchuan	Microarray
	NEDD4	Vignato <i>et al.</i> , (2017)	Nellore	RNAseq ²
		Júnior <i>et al.</i> , (2015)	Nellore	GWAS ⁴
	UBE4A	Vignato <i>et al.</i> , (2017)	Nellore	RNAseq ²
Meat tenderness.	CAPN1	Pinto <i>et al.</i> , (2010)	Nellore	Genotyping ³
		Page <i>et al.</i> , (2002)	Simmental x Angus	Genotyping ³
		Castro <i>et al.</i> , (2017)	Nellore	GWAS ⁴
		White <i>et al.</i> , (2005)	Brahman	Genotyping ³
		Curi <i>et al.</i> , (2009)	Several Breeds	ARMS – PCR ⁵
		Corazzin <i>et al.</i> , (2013)	Simmental	qRT-PCR ¹
		Tizioto <i>et al.</i> , (2013)	Nellore	GWAS ⁴
		Natras <i>et al.</i> , (2014)	Brahman/Angus	qRT-PCR ¹
	CAPN2	Moreno <i>et al.</i> , (2009)	Colombian Creole	PCR-RFLP ⁶
		Tizioto <i>et al.</i> , (2013)	Nellore	GWAS ²
		Castro <i>et al.</i> , (2017)	Nellore	GWAS ²
	CAST	Schenckel <i>et al.</i> , (2006)	Several breeds	PCR-RFLP ³
		Pinto <i>et al.</i> , (2010)	Nellore	Genotyping ³
		Natras <i>et al.</i> , (2014)	Brahman/Angus	qRT-PCR ¹
		Curi <i>et al.</i> , (2009)	Several Breeds	ARMS – PCR ⁵
		Magalhães <i>et al.</i> , (2016)	Nellore	GWAS ⁴
		Bernard <i>et al.</i> , (2007)	Charolais	Microarray
		Castro <i>et al.</i> , (2017)	Nellore	GWAS ⁴
		Malheiros <i>et al.</i> , (2018)	Nellore	qRT-PCR ¹
		Hocquette <i>et al.</i> , (2012)	Charolais	Microarray
		Bernard <i>et al.</i> , (2007)	Charolais	Microarray
	DNAJ1	Corazzin <i>et al.</i> , (2013)	Simmental	qRT-PCR ¹
		Bernard <i>et al.</i> , (2007)	Charolais	Microarray
		Malheiros <i>et al.</i> , (2018)	Nellore	qRT-PCR ¹
	FABP4	Hocquette <i>et al.</i> , (2012)	Charolais	Microarray
		Zhao <i>et al.</i> , (2012)	Angus	Microarray
	SCD	Vignato <i>et al.</i> , (2017)	Nellore	RNAseq ²
	PRKAG3	Bongiorni <i>et al.</i> ,	Several Breeds	RNAseq ²

¹ Real time PCR, ² RNA sequencing, ³ Specific SNP genotyping, ⁴ Genome wide association study ⁵ Amplification- refractory mutation system, ⁶ Restriction fragment length polymorphism.

Table 3: Forward (F) and reverse (R) primer oligonucleotides sequences used in qRT - PCR to obtain amplicons for ribeye area, meat tenderness and reference genes.

GENE	Number of Access (GENBANK)	Sequence (5' – 3')	MT ¹ of Amplicon (°C)	Size of Amplicon (pb ²)
<i>IGF-1 F</i>		TCAGCAGTCTTCCAACCCAAT		
<i>IGF-1 R</i>	NM_001077828.1	GCACAGGGCCAGATAGAAGAGA	81	72
<i>IGF-2 F</i>		TGTGCAGCTGAGACCGAATT		
<i>IGF-2 R</i>	NM_174087.3	GGAGCACGGAACAGGAAAAC	82	77
<i>MSTN-F</i>		CGGCTCCTTGGAAGACGAT		
<i>MSTN-R</i>	NM_001001525.3	AGACTCCGTGGGCATGGTAAT	83	67
<i>NEDD4-F</i>		CCCTTCAGGACAACCTAACAGATG		
<i>NEDD4-R</i>	NM_010812047.2	TCGGTTGTCAACACTTTCTGTTTC	79	113
<i>UBE4A-F</i>		GACTGAGCCACAGATGCTACAAA		
<i>UBE4A-R</i>	NM_001098999	TGCCTATGGCCAGCTGAAC	81	80
<i>CAPN1 –F</i>		CTCTCAGGAGGCAGCACATCT		
<i>CAPN1-R</i>	NM_174259.2	CTTGCGCAGCTCGTACCA	85	75
<i>CAPN2 –F</i>		TCTTTCAACGACTTCCTGAGACACTA		
<i>CAPN2-R</i>	NM_001103086.1	ACTTCTTGTAAGCTGTCAGTGGTGAGT	80	88
<i>CAST-F</i>		TGCCTTGTCATCCGACTTCAC		
<i>CAST-R</i>	NM_001025569.1	CCCCAGCTGACTGAGCTTTT	79	104
<i>HSPB1-F</i>		GAACAGCCGGAAACAAGTAAAGA		
<i>HSPB1-R</i>	NM_001025569.1	GGGTGAGGATGTCCAGTGATG	83	76
<i>DNAJAI-F</i>		GGAGGAGAACAGGCAATTAAAGAA		
<i>DNAJAI-R</i>	NM_001015637.1	ACAACGTTTTTACCTCTCCTCTCTCT	80	125
<i>SCD – F</i>		TGGTCAGCTCCCCTCATAGTAAG		
<i>SCD – R</i>	NM_173959.4	GCTCACCACCATACCACCAAT	79	58
<i>FABP4 – F</i>		CTGCCACCAGAGTTTATGAGAGA		
<i>FABP4 – R</i>	NM_174314.2	ACCAACATAACCATATCAAAATCAG	78	64
<i>PRKAG3 – F</i>		CCCTTTTGTAGGTTCCGCTCTT		
<i>PRKAG3-R</i>	NM_001162421	GCTTCTGGGAGCACAAGCA	84	80
<i>GAPDH-F</i>		CCGTTTCGACAGATAGCCGTAA		
<i>GAPDH-R</i>	NM_001034034	CGACCTTCACCATCTTGTCTCA	83	69
<i>BACTIN-F</i>		CAGCAAGCAGGAGTACGATGAG		
<i>BACTIN-R</i>	NM_177945	AAGGGTGTAACGCAGCTAACAGT	83	85
<i>HPRT1-F</i>		TGATGAAGGAGATGGGTGGC		
<i>HPRT1-R</i>	NM_001034035	CCAACAGGTCGGCAAAGAAC	83	81

¹ Melting temperature; ²: base pairs

For the amplification of the reference genes, the selected concentrations in nM were: GAPDH (300/300), BETA ACTIN (300/300), and HPRT1 (100/100). Reactions by titration and standard curve were performed as described by Fonseca et al. (2015).

The generated threshold cycle (Ct) data were analyzed using SAS University Edition statistical software (<https://www.sas.com/software/university-edition/download-software.html>). For data normalization, a geometric mean of Ct value obtained from the three reference genes (GAPDH, BETA ACTIN, and HRPT1) was established as suggested by Vandesompele et al. (2002). The relative expression of the target genes (*IGF-1*, *IGF-2*, *MSTN*, *NEDD4*, *UBE4A*, *CAPN1*, *CAPN2*, *CAST*, *DNAJ1*, *HSPB1*, *ASAH1* and *PRKAG3*) was calculated as proposed by Steibel et al. (2009), according to the following model:

$$y_{gikr} = t_{ig} + d_{ik} + e_{gikr},$$

where y_{gikr} is the Ct obtained from the thermocycler for gene g (geometric mean of the reference genes and target gene) in the r the well of the plate (referring to the technical replicate) in a sample obtained from animal k of treatment i; t_{ig} is the effect of treatment i on gene expression g; d_{ik} is the random sampling specific effect which captures the differences, such as differential extraction or amplification efficiencies between samples; and e_{gikr} is the residual effect.

2.3 Proteomic analyses

2.3.1 Sample preparation

The samples were initially washed with phosphate buffered saline (PBS). The tissue was weighed, frozen in liquid nitrogen and pressed in a mortar. Protein extraction was performed by adding a buffer containing 8M urea, 0.15 M Tris-HCl buffer (pH 8.8), O.G detergent 0.5% (1-S-Octyl-β-D-Thioglucopyranoside) and 10 μL of Protease Inhibitor Cocktail (Sigma-Aldrich, USA). Samples were sonicated in 3 cycles of 1 minute, followed by centrifugation at 12,000xg for 5 min at 4 °C. The protein quantification was performed using Bradford method with Protein Assay Dye Reagent Concentrate (Bio-Rad, USA) (Bradford, 1976) and the standard curve was performed using different

dilutions of BSA (bovine serum albumin). The samples were distributed in duplicate in microplates and the absorbance at 595nm was read in a spectrophotometer (Molecular Devices, SpectraMax Plus 384).

Samples preparation (50 µg) for advanced mass spectrometry was conducted following three main steps: protein reduction and alkylation, enzymatic digestion of proteins with trypsin, and clean up/desalting of the samples. Each sample was submitted to disulfide bonds reduction of the protein through the addition of DTT (dithiothreitol) in a proportion of 1 mg of DTT/mg protein and incubated for 2 h at room temperature. Then, alkylating IA (iodoacetamide) was added in a proportion of 3 mg IA/mg of protein and incubated for 1 h at room temperature in the dark.

The volume of the sample was diluted five times in 0.1 M solution of ammonium bicarbonate (pH ≥ 8.0) resulting in a final volume of 500 µl. The sample was incubated with 1 µg trypsin (Promega, Madison, Wisconsin, USA) at 37 ° C overnight. Before the application of samples into mass spectrometer, clean-up / desalting was performed using the OASIS HLB cartridge 1cc column, according to recommendation of the manufacturer. Then, the samples were dried in speed vac and applied to a mass spectrometer.

2.3.2 Data Processing

From each sample, 1 µg of each sample was injected into an OrbiTrap Fusion mass spectrometer and the data obtained were automatically processed by the Computational Proteomics Analysis System (CPAS) (Rauch et al., 2006). The identified peptides were grouped into proteins using the “Protein Prophet” algorithm. A list of tags with an error rate of less than 2.0% was created. For reference, we used the database: Bovine Proteome Uniprot (<https://www.uniprot.org/proteomes/UP000009136>). The comparative and quantitative analysis was performed using the number of peptide-spectrum matches

observed for each peptide, which provides an estimation of abundance, also known as "spectral counting".

2.3.3 Statistical analysis of mass spectrometry data

Statistical analysis of differentially expressed proteins (DEP) was performed using the msmsTests package in R (Gregory et al., 2019), where the negative binomial of the edgeR package is available. The package uses statistical tests for label-free LC-MS/MS data by spectral counts, to discover differentially expressed proteins between two biological conditions. The multiple testing correction false discovery rate ($FDR < 0.05$) estimation was used.

3. Results

For REA, the expression level of the MSTN gene between the high and low REA group was significantly different ($p\text{-value} < 0.05$). The analysis of relative expression of the genes in muscle tissue showed that expression of the MSTN gene was 1.76 times higher in low REA animals (Table 4). For meat tenderness, *CAPN1*, *CAPN2*, *CAST*, *HSPB1*, and *DNAJAI* genes showed significantly distinct expression between the analyzed groups for tender and tough meat ($p\text{-value} < 0.05$) (Table 5). The expression level of the *CAPN1*, *CAPN2*, *CAST*, *HSPB1*, and *DNAJAI* genes were respectively 1.47, 1.53, 1.49 and 1.50 times higher in tough meat when compared with group of tender meat. Thus, all differentially expressed genes were upregulated in the tough meat group (Figure 1).

Table 4: Expression of *IGF-1*, *IGF-2*, *MSTN*, *NEDD4* and *UBE4A* genes in *Longissimus Thoracis* muscle of Nellore cattle from extreme groups for ribeye area trait.

Gene	Group (REA)	Fold Change ¹	Standard error	p-value
<i>IGF-1</i>	High	1,00	0.29	0,8745
	Low	0.93	0.29	
<i>IGF-2</i>	High	1,00	0.23	0,3971
	Low	0.71	0.23	
<i>MSTN</i>	High	1,00	0.24	0.0132*
	Low	1.76	0.24	
<i>NEDD4</i>	High	1,00	0.19	0.3429
	Low	1.14	0.19	
<i>UBE4A</i>	High	1,00	0.22	0.4224
	Low	1.15	0.22	

* significant at 5%, ¹Fold change calculated based on highest ribeye area (REA) group.

Table 5: Expression of *CAPN1*, *CAPN2*, *CAST*, *HSPB1*, *DNAJ1*, *FABP4*, *SCD* and *PRKAG3* genes in *Longissimus Thoracis* muscle of Nellore cattle from extreme groups for meat tenderness.

Gene	Group	Fold Change ¹	Standard error	p-value
<i>CAPN1</i>	Tender	1,00	0.15	0.0143*
	Tough	1.47	0.15	
<i>CAPN2</i>	Tender	1,00	0.2	0.0009**
	Tough	1.53	0.2	
<i>CAST</i>	Tender	1,00	0.15	0.0183*
	Tough	1.33	0.15	
<i>HSBP1</i>	Tender	1,00	0.17	0.0225*
	Tough	1.43	0.17	
<i>DNAJ1</i>	Tender	1,00	0.21	0.001**
	Tough	1.50	0.21	
<i>FABP4</i>	Tender	1,00	0.4	0.3439
	Tough	0.51	0.4	
<i>SCD</i>	Tender	1,00	0.17	0.8859
	Tough	0.72	0.17	
<i>PRKAG3</i>	Tender	1,00	0.31	0.4754
	Tough	0.54	0.31	

* significant at 5%, ** significant at 1%, ¹Fold change calculated based on tender meat group.

Figure 1: Relative expression of genes in *Longissimus thoracis* muscle of Nellore animals belonging of groups extremes for meat tenderness (*CAPN1*, *CAPN2*, *CAST*, *HSPB1*, and *DNAJA1*) and ribeye area (*MSTN*). (*significant at 5%).

Considering only the differentially expressed genes (*CAPN1*, *CAPN2*, *CAST*, *HSPB1*, *DNAJA1* and *MSTN*), just the proteins traduced by *CAPN1*, *CAPN2*, *CAST* and *HSPB1* were detected, but all of them, showed no significant differences in expression between groups (Table 6). The proteins traduced by *DNAJA1* and *MSTN* were not identified in the analysis.

Table 6: Protein name, uniprot accession number, gene symbol, number of peptides identified, log2 fold change, p-value and q-value, for proteins whose genes were differentially expressed.

Protein name*	UniProt	Gene	Peptides	Log2FC**	P-value	q-value
Calpain-1 catalytic subunit	Q27970	CAPN1	57	0,199	0,69	0,73
Calpain-2 catalytic subunit	Q27971	CAPN2	4	0,217	0,81	0,85
Calpastatin	P20811	CAST	10	-0,162	0,88	0,91
Heat shock protein beta-1	Q3T149	HSPB1	2462	-0,054	0,59	0,64

*Proteins traduced by *DNAJA1* and *MSTN* were not detected

**The log2 fold-change estimates (relative expression) refer to the tender meat group

4. Discussion

4.1 REA-related genes

The gene that encodes myostatin (MSTN) was downregulated in the group with the highest ribeye area (p-value <0.05) (Table 4). Myostatin, also referred as growth and differentiation factor-8, is a protein secreted in muscle tissues that is essential for regulation of skeletal muscle. The highest gene concentrations cause a decrease in normal muscle development and muscle weight loss (Mcpherron and Lee, 1997). Similar results had been reported by Silva et al. (2020), who also observed MSTN was downregulated in the highest ribeye area group in Nellore animals. Likewise, Zhang et al. (2011) found significant differences in gene expression between male and female Quichuan cattle. MSTN was downregulated in the male group compared with the female group, indicating that male cattle had greater muscle protein deposition than females. Since our results showed that MSTN was downregulated in the highest ribeye area group, this gene may contribute to the increased muscle growth in these animals.

The Insulin-like growth factors (IGFs) were not differentially expressed in our study although an association between ribeye area and IGFs genes has been reported in cattle (Goodall and Schmutz et al., 2007; Sadkowski et al., 2009; Guo et al., 2015). The IGF's contribute to cell regulation and stimulate protein synthesis, acting on embryonic and postnatal growth and development (Schmid, 1995). Once the ribeye area is considered a polygenic trait and is highly influenced by several environmental factors, many genes and mechanisms that induce differences in the growth of the Nellore breed are still unknown. Despite the crossing between the taurine and zebu breeds leads to higher carcass yields and ribeye area, as the proportion of zebu genes increases (Mendonça et al., 2021), our results suggest that the expression levels of IGF-1 and IGF-2 genes do not exert a direct effect on REA in Nellore animals of this study and other genes

are related with the highest ribeye area (Vignato et al., 2017, Silva et al., 2020). No significant differences were found in the expression of *NEDD4* (E3 ubiquitin protein ligase) and *UBE4A* (Ubiquitin conjugation factor E4) genes between the two groups (Table 4). The *NEDD4* gene encodes the ubiquitin-ligase protein belonging to the *NEDD4* family, while the *UBE4A* gene plays a fundamental role in the ubiquitination process (Contino et al., 2004). Among the functions of ubiquitin-ligase proteins stand out the support in the protein turnover and in the internationalization of other proteins in the cell. (Cao et al., 2004; Monami et al., 2008; Goel et al., 2015.) Proteins are continually being synthesized and degraded by cells, with growth occurring when the rate of synthesis exceeds the rate of degradation.

Gomes et al. (2013) have already reported that genes related to protein turnover were associated with growth and carcass traits in Nellore cattle. However, Heiderich et al. (2014), using non castrated Nellore bulls, observed that protein turnover decreases as body weight increases (480 kg, weight similar to the animals used in this study). This could explain why these genes were not differentially expressed. The maturity or increase in body weight gradually decreases the synthesis of muscle protein, changing the body composition. Thus, this process leads to a decrease in the percentage of muscle in the body, which could change the turnover rate (Heiderich et al., 2014).

Furthermore, the differences in gene expression observed by Silva et al. (2020) highlight the complexity of the transcriptome in Nellore cattle with divergent ribeye area phenotypes, in addition to the different biological pathways and genes involved with the trait.

4.2 Genes related to meat tenderness

The μ -calpain (*CAPN1*) and m-calpain (*CAPN2*) genes showed higher expression in animals presenting tough meat (p-value <0.05) (Table 5). The calpain system in the

skeletal muscle include at least three calpains (μ - and m-calpains and calpain 3) and a third polypeptide, calpastatin, which acts as specific endogenous inhibitor (Bhat et al., 2018). Calpains act on the degradation of functionally relevant structural proteins such as the myofibrillar proteins (Lana and Zolla., 2016) and are generally identified as the main responsible for the proteolysis and tenderization processes (Bhat et al., 2018) mainly in Bos Taurus cattle (Casas et al., 2006; Corva et al., 2007). There is evidence that the action of CAPN1 occurs during maturation of meat. Carvalho et al. (2012) using qRT-PCR technique found a negative association between CAPN1 gene expression and tenderness of meat without maturation, as well as our study. However, these authors observed that at 14 days of maturation there was a positive association between gene expression and meat tenderness.

One possible explanation is that immediately after slaughter the action of μ -calpain is not expected on postmortem proteolysis, but at 14th day of maturation this effect would be evident. Thus, greater expression of these genes in matured meat may be expected. The lack of association between *CAPN1* and *CAPN2* gene expression levels and meat tenderness shows the complexity of the mechanisms that occur during the tenderization phase. In addition, transcriptome analysis (Gonçalves et al., 2018) and genome-wide associations studies (GWAS) (Tizioto et al., 2013) identified transcripts and metabolic pathways that could be responsible for variation in beef tenderness in Nellore cattle, which differ from those previously identified in *Bos Taurus* cattle.

Regarding the calpastatin (*CAST*) gene, the expression was significantly upregulated in tough meat when compared to the tender meat (p-value <0.05). Calpastatin acts inhibiting calpain by preventing the calpain proteolytic activation and the expression of catalytic activity (Lonergan et al., 2010). These results suggest that despite the highest expression of calpains in tough meat, the inhibitory activity of calpastatin was responsible

for blocking the active site of calpains and inhibiting its activity (Kawasaki and Kawashima, 1996; Lonergan et al., 2010). Several studies have shown that the lowest tenderness of zebu beef could be due to the greatest calpastatin activity, inhibiting the proteolytic activity of calpain (Whipple et al., 1990; Wheeler et al., 1994; Nattrass et al., 2014). Moreover, the highest calpastatin activity has been considered one of the main factors related to lower zebu beef tenderness compared to taurine (Whipple et al., 1990). Thus, the antagonistic action between the components of the proteolytic system suggests that the greater the activity of calpastatin in the muscle, the lower the action of calpain and therefore the tenderness of the meat.

The results found in the present study for calpain / calpastatin complex suggest that low meat tenderness could be attributed in part to the high expression of calpastatin gene. However, other metabolic pathways that involve different genes are possibly involved in the meat tenderness process (Nagai, 2015; Fonseca et al., 2017).

For genes (*HSPB1* and *DNAJ1*) that encode heat shock proteins (HSPs), the higher expression was observed in tough meat (Table 5), showing a negative association with meat tenderness (p-value <0.05). Studies have proposed that HSPs in muscles mediate the progression of apoptotic cell death and therefore they are involved in meat tenderization process (Bernard et al., 2007; Lomiwes et al., 2014; Malheiros et al., 2018). The negative association of *HSPB1* and beef tenderness has been reported in other studies of gene and protein expression (Kim et al., 2008; Malheiros et al., 2018). Probably the function of HSP proteins to protect against the myofibrillar proteolysis decrease the degradation of muscle proteins such as actin and myosin reducing the meat tenderness.

The *DNAJ1* gene, also downregulated for meat tenderness, encodes a member of the 40 kDa heat shock protein family (HSP40), which is a co-chaperone of the 70 kDa heat shock protein family (HSP70). The HSP40/HSP70 complex directly inhibits

apoptosis retarding cellular death during the conversion of muscle into meat and consequently to lowering meat tenderization (Bernard et al., 2007; Picard et al., 2010). Our results corroborate with Malheiros et al. (2018) and Bernard et al. (2007) who observed negative association of this gene with meat tenderness in Nellore and Charolais cattle, respectively.

The *FABP4* (Fatty acid-binding protein) and *SCD* genes (Acyl-CoA desaturase), related to lipidic metabolism, were not differentially expressed in the present study (Table 5). In contrast, using microarray on muscle tissue of divergent Angus animals for tenderness, Zhao et al. (2012) found that both genes (*FABP4* and *SCD*) were upregulated to meat tenderness. These authors worked with Angus animals (*Bos taurus*) which naturally have more tender and marbling meat when compared to *Bos indicus* animals (Whipple et al., 1990; Cunduff et al., 1992; Wheeler et al., 1994). Furthermore, Rodrigues et al. (2014) using proteomic approach found that proteins involved in contraction, muscle organization and heat shock proteins might be associated with differences in beef quality between Angus (*Bos Taurus*) and Nellore (*Bos indicus*). Crouse et al. (1989) studying crossbred animals with different ratios of zebu / taurine genes concluded that as the proportion of zebu genes increased, there was an increase in shear force and a decrease in meat marbling. In addition, Koohmaraie et al. (2002) suggested that only 15% of the variation in beef tenderness is due to differences in marbling and collagen levels and remaining 85% may be attributed to the postmortem enzymatic actions.

For *PRKAG3* gene, no significant difference in expression was observed between groups (Table 5). Previous studies have indicated that the *PRKAG3* gene affects the glycogen content in muscle and hence, meat quality traits such the tenderness (Milan et al., 2000; Ciobanu et al., 2001; Bongiorno et al., 2016). In contrast to our results, Bongiorno et al. (2016) found four isoforms of the *PRKAG3* gene that were differentially

expressed in a transcriptome study of meat tenderness in two Italian cattle breeds. In muscle, the glycogen content affects the ultimate pH and consequently the postmortem maturation process. Thus, this distinct pattern of *PRKAG3* gene expression could indicate impaired glycogen storage in skeletal muscle, and consequently influence meat tenderness. However, our results suggest that regardless the animal group, there seems to be no deficiency in glycogen storage that influences meat tenderness. Thus, probably the greater or lesser tenderness would be due other metabolic pathways that involve other different genes (Fonseca et al., 2017).

4.3 Protein expression for genes differentially expressed

Despite being identified, the proteins encoded by the genes calpain and calpastatin were not differentially expressed between contrasting groups for meat tenderness (table 6). As discussed, calpains compound a family of proteolytic proteins involved in myofibrillar degradation and meat tenderization during aging whereas calpastatin acts as a natural inhibitor of the protease calpain (Bhat et al., 2018). Dias et al. (2016) aiming to quantify the enzymes μ -calpain, m-calpain and calpastatin within contrasting meat tenderness groups, found no significant difference on the amount of enzyme μ and m calpain. However, there was a higher calpastatin activity in animals of tough meat group. Additionally, Rodrigues et al. (2017) comparing the muscle proteomic profile of Angus and Nellore also have not detected differences in the abundance of calpastatin and calpain. However, in a parallel study with the same animals used by Rodrigues et al. (2017), there was a higher calpastatin activity in Nellore beef (Duarte et al., 2013).

Although the genes encoding these proteins have been differentially expressed, the post-transcriptional and translational gene regulatory mechanisms directly influence the final protein production. Moreover, a proteome is a highly dynamic entity and protein expression in a biological system constantly changes. In view of the above, we cannot

exclude the role of the enzyme calpastatin in the lesser tenderness of the meat, since the enzyme was more expressed in tough meat in Real-Time PCR analysis.

The protein encoded by HSPB1 gene was not differentially expressed between the groups for meat tenderness, while the protein encoded by DNAJA1 gene was not identified in the samples studied. Contrasting results from proteomic studies are found in the literature. Guillemin et al. (2011) observed that both heat shock proteins encoding by HSPB1 and DNAJA1 genes, were positively correlated with highest meat tenderness in Charolais cattle. Probably in this case, the structural proteins protection was a positive factor in the tenderization process by inhibiting the formation of aggregates and favoring proteolysis (Guillemin et al., 2011). Nonetheless, Kim et al. (2008) observed an increase of HSP27 protein, encoding by HSPB1 gene, in native Korean cattle with tough meat. Similarly, Morzel et al. (2008) observed a decrease in the amount of HSP27 after 14 days of aging in young bulls, which was correlated with meat tenderness. Probably the negative regulation of HSP27 may increase the degradation of muscle proteins and consequently improve meat tenderness, since one of the functions that HSPB1 is maintain the protein integrity and difficult to proteolytic degradation of myofibrillar proteins.

Although the genes encoding the heat shock proteins (HSPB1 and DNAJA1) were differentially expressed between the groups for meat tenderness, the same was not observed for the expression of the proteins encoded by them. These results suggest once again that gene regulation mechanisms may act influencing protein production. Moreover, the proteome of the cell is constantly changing reflecting the immediate environment of the protein.

Myostatin, a protein synthesized and secreted by skeletal muscle that negatively regulates muscle mass, was not identified in proteomic analyses. Loss-of-function

mutations in the MSTN gene are known to cause an extreme ‘double muscling’ phenotype in cattle. (Mcpherron and Lee, 1997)

The central dogma of molecular biology deals with the transfer of information from DNA via mRNA to proteins. It is mechanically well known how genes are transcribed, and the mRNA processed and sequentially translated into proteins. However, most reports of mRNA and protein abundance find only a weak correlation between the respective abundances of these two classes of biological molecules (Greenbaum et al., 2003; Maier et al., 2009).

As showed by Lehninger et al. (2010), when the messenger RNA leaves the cell nucleus, it may or may not is translated to proteins, being susceptible to various post-transcriptional and post-translational regulatory factors that mediate protein production. Moreover, stability is a big factor to explain the divergence between molecules. The proteins are in a constant process of turnover which makes half-lives highly variable. (Glickman and Ciechanover, 2002; Maier et al., 2009). Concomitantly, there is a significant amount of error and noise in both protein and mRNA trials that limit the possibility to get a totally clear result (Greenbaum et al., 2003). However, despite proteomics and transcriptomics data have almost no overlap, the integrative analysis of biological pathways influencing meat quality traits can help to elucidate the biological processes and gene networks involved in the phenotype expression. Moreover, a single gene or protein is not directly responsible for meat tenderness or ribeye area, but rather a group of genes and proteins that play a role together in their pathways to favor the meat quality process.

5. Conclusion

Our results could confirm the potential of some studied genes as biomarkers for carcass and meat quality in Nellore cattle. The highest expression of calpain gene in tough

meat indicate inconsistencies regarding calpain mRNA activity and meat tenderness trait in Nellore. The selected genes did not show differential protein expression, suggesting that several factors may influence in the transcription and translation processes.

Ethics statement

All experimental procedures involving animals were approved by Ethics Committee of the São Paulo State University (UNESP), School of Agricultural and Veterinarian Sciences, Jaboticabal, SP, Brazil (CEUA protocol:005622/17).

Consent for publication

Not applicable.

Availability of data and materials.

The dataset utilized in this study belong to the Cia do Melhoramento animal breeding program company and could be available on request. The author does not have authorization to share the data.

Competing interests

The authors declare no competing interests.

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