Expression of genes related to quality of *Longissimus dorsi* muscle meat in Nellore (*Bos indicus*) and Canchim (*5/8 Bos taurus × 3/8 Bos indicus*) cattle

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This study was performed to compare CAPN1, CAPN2, CAST, TG, DGAT1 and LEP gene expressions and correlate them with meat quality traits in two genetic groups (Nellore and Canchim) in order to assess their expression profile and use their expression profile as genetic markers. We analyzed 30 young bulls (1 year old), 15 of each genetic group. Samples of the *Longissimus dorsi* muscle were collected for analysis of: total lipids (TL) and meat tenderness measured as Warner-Bratzler shear force (SF) and myofibrillar fragmentation (MFI) at day of slaughter and 7 days of aging. Gene expression profiles were obtained via RT-qPCR. TL and MFI showed differences between breeds, higher MFI in Canchim and higher TL in Nellore. Calpains showed no differential expression between groups, as did DGAT1, TG, and LEP. CAST was expressed more in the Nellore cattle. The only significant within-breed correlation (0.79) between gene expression and meat traits was found for DGAT1 and MFI in Canchim breed. Although the number of animals used in this study was small, the results indicate that the increased expression of CAST in Nellore may reflect tougher meat, but the lack of correlations with the meat traits indicates it is not a promising genetic marker.

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1. Introduction

Variability among breeds regarding meat tenderness has been attributed to different levels of proteolytic enzymes found in animal muscles (Whipple et al., 1990), especially enzymes of the calpain system. Wheeler, Savell, Cross, Lunt, and Smith (1990) found a lower μ-calpain protein expression and higher calpastatin protein expression in Brahman (*Bos indicus*) when compared to Hereford (*Bos taurus*), which resulted in lower meat tenderness in *B. indicus* animals, because calpastatin is a calpain inhibitor.

The evolution of beef cattle breeding in Brazil in recent years has been marked by increased herd size, greater productivity and improved meat quality. The intensification of production systems is probably responsible for these developments, and wider use of feedlot finishing is an important element in this scenario. The adaption of this system to European cattle breeds and Zebu cattle breeds (Connor et al., 1997; Shackelford, Koohmaraie, Miller, Crouse, & Reagan, 1991; Whipple et al., 1990). In general, as the percentage of *B. indicus* increases, the variability tends to decrease and tenderness tends to increase. The calpain system has been considered the main mechanism involved in meat tenderness (Koohmaraie, 1992, 1996; Wheeler, Cundiff, Koch, & Crouse, 1996). This system consists of two calcium dependent proteases, μ-calpain or calpain 1 (CAPN1) and m-calpain or calpain 2 (CAPN2), and a polypeptide whose function is to inhibit both calpain and calpastatin (CAST). Shackelford et al. (1991) indicated that a higher level of calpastatin was responsible
for lower meat tenderness from animals with some proportion of Zebu in their genetic composition, while Wheeler, Cundiff, and Koch (1994) also found lower levels of calpain 1 in B. indicus cattle.

Several factors influence proteolytic activity. Very rapid cooling of the carcass is one of these, as the shortening of muscle fibers (cold shortening) leads to concealment of enzyme recognition sites, reducing the access to substrates. Thus, even if there is availability of the proteolytic enzyme, its action will be compromised and ideal proteolysis of muscle fiber components will not occur (Koohmaraie, 1996). Another important factor in muscle proteolysis is cold shortening, this can be prevented with correct quantity of backfat cover, that protects the carcass against rapid cooling under normal conditions, avoiding the occurrence of shrinkage of fibers (Lesser, 1993). Zebus are slower-growing animals and tend to deposit fat sooner when fed diets with the high energy content of feedlots (Rubiano et al., 2009), being less influenced by this process.

Studies indicate the diacylglycerol acyltransferase 1 (DGAT1) gene, which encodes the enzyme diacylglycerol acyltransferase 1, can be associated with milk fat (Grisart et al., 2004). However, conflicting results regarding its role in fat deposition in beef cattle have been reported (Casas et al., 2005; Pannier, Mullen, Hamill, Stapleton, & Sweeney, 2010; Thaller et al., 2003).

The gene that encodes thyroglobulin protein (TG), a glycoprotein synthesized in thyroid follicular cells that is a precursor molecule for the thyroid hormones, thyroxine and triiodothyronine. Some studies have shown associations of this gene with fat thickness (Casas et al., 2005), and with marbling score (Gan et al., 2008).

The gene encoding leptin (LEP), a cytokine secreted predominantly from adipose tissue, plays an important role in the regulation of body energy balance. Leptin is involved in food intake, energy balance, reproductive efficiency, fat deposition (Houseknecht, Baile, Materri, & Spurlock, 1998; Lagonigro, Wiener, Pilla, Woolliams, & Williams, 2003; Pannier et al., 2009; Schenkel et al., 2005), and possibly formation of marbling fat (Taniguchi, Itoh, Yamada, & Sasaki, 2002).

The aim of this work was to study gene expression of proteins related to meat tenderness like CAPN1, CAPN2 and CAST and intramuscular fat deposition DGAT1, TG, and LEP in Nellore (Zebu) and Canchim (3/8 Zebu x 5/8 Charolais) cattle, and to estimate the within-breed correlations of the gene expression profiles and the traits studied.

2. Material and methods

In this study we used 30 beef bulls, reared under creep feeding and weaned at seven months, with an average weight of 209.4 kg (±23.3 kg). The animals were evenly divided between the two genetic groups, with 15 Nellore (Zebu) and 15 Canchim (3/8 Zebu x 5/8 Charolais).

2.1. Management, feeding and care of animals

After weaning, the animals were kept in experimental feedlot facilities at the School of Veterinary Medicine/UNESP-Botucatu. All animals were given the same diet (ad libitum), housing and management. They were weighed and subjected to a period of 21 days for diet adaptation.

The growth and fat deposition were monitored by ultrasound every weighing period (every 28 days). The diets had high quality nutrition, formulated according to the Cornell Net Carbohydrate and Protein System 5.0.26.

When the animals reached the pre-established slaughter weight of approximately 370 kg and finishing fat cover of at least 4 mm, they were submitted to the creation of a very early model, and were slaughtered in a commercial abattoir before mature.

2.2. Collection and processing of samples

The samples for RNA extraction, used in the gene expression analysis, were collected immediately after slaughter, taken from the Longissimus dorsi muscle in the region of the 11th and 13th rib of each animal, and immediately frozen in liquid nitrogen and subsequently kept at −80 °C in freezers. The samples used in the analysis of shear force (SF), myofibrillar fragmentation index (MFI) and total lipids (TL) were collected 24 h after carcass cooling in the same region of the Longissimus dorsi muscle and maintained at 4 °C. Half of the samples collected 24 h after carcass cooling was used to measure the shear force (SF0), myofibrillar fragmentation index (MFI0) and total lipids extraction without the influence of aging. The remaining of the samples were vacuum packed and kept at 4 °C for seven days (aging period) before analysis of shear force (SF7) and myofibrillar fragmentation index (MFI7).

2.3. Shear force analysis

Longissimus dorsi samples, approximately 2.54 cm thick, not aged and aged for seven days, were subjected to shear force analysis, following the method described by Wheeler, Koohmaraie, and Shackelford (1995) as adapted by Hadlich (2007).

2.4. Myofibrillar fragmentation index (MFI) analysis

The determination of the myofibrillar fragmentation index (MFI) was based on the method proposed by Culler, Farrish, and Smith (1978).

2.5. Total lipids extraction

The determination of total lipids in subcutaneous fat-free samples was performed according to the protocol described by Bligh and Dyer (1959).

2.6. RNA extraction and reverse transcription

Total RNA extraction of skeletal muscle was performed using the TRIzol (Life Technologies, USA) protocol. Total RNA was eluted in distilled and autoclaved water, treated with diethylpyrocarbonate (Sigma − DEPC, 0.01%) and stored at −80 °C. To check the quality and quantity of total RNA, a NanoVue spectrophotometer was used (GE Healthcare Life Sciences, USA).

After quantification of the extracted RNA, the integrity of the material was analyzed. This process was accomplished through the presence of bands corresponding to 18S and 28S ribosomal RNAs after capillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, USA). The RNA integrity was verified by calculating the RNA integrity number, with the mean value of all samples (Nellore and Canchim) being 8.0 ± 0.3 (range 1–10), indicating high-quality RNA and minimum degradation.

Total RNA was treated with the enzyme DNase to remove possible contaminating genomic DNA, as indicated by the protocol DNase I − Amplification Grade (Life Technologies, USA), and was then used for the reverse transcription reaction.

The reverse transcription reaction was performed using the High Capacity Archive cDNA kit (Life Technologies, USA) following the manufacturer’s protocol. The specimens were stored at −20 °C.

2.6.1. Selection of reference genes

The stability of the reference genes was tested using the Assist v2.0 software (Life Technologies, USA), an algorithm to determine the most stable reference genes from a set of tested candidate reference genes in a sample panel given. Five reference genes were tested in RT-qPCR analysis 18S ribosomal RNA, β-actin,
glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine fosfornoribosil transferase (HPRT) and TATA-box binding protein (TBP). Only three proved stable genes were used (GAPDH, HPRT and TBP). All RT-qPCR laboratory procedures were performed according to the MIQE guidelines (Bustin et al., 2009).

The RT reactions were performed in the qPCR-ABI 7300 platform according to the protocol of Life Technologies.

We used primers and hydrolysis probes (TaqMan® assays) for the CAPN1, CAPN2, CAST, TG, LEP and TBP genes (Table 1) expressed in B. taurus (Life Technologies, USA). For the DGAT1, GAPDH and HPRT genes, the primers and hydrolysis probes were designed based on the sequences available in the GenBank database of the National Center for Biotechnology Information (NCBI), using the Primer Express® 3.0 software (Life Technologies, USA) (Tables 1 and 2).

Tagman gene expression reactions were performed following the manufacturer’s recommendations.

2.6.2. Determination of the parameters of the qPCR

The reactions were performed using the Real Time PCR system 7300 (Life Technologies, USA) under the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The reactions were performed in triplicate for each sample, generating an average expression of each animal, to minimize technical errors. After the run, the melting curve was analyzed to confirm the specific amplification product of each qPCR gene.

The results were presented as fold changes relative to a Canchim animal, using the method described by Livak and Schmittgen (2001). All mathematical procedures were performed by using the Data Assist v2.0 program (Life Technologies, USA).

2.7. Statistical analysis

All statistical analyses were performed using the Statistical Analysis System V.9.1 program (SAS). The data for total lipids were subjected to analysis of variance using the GLM procedure. The System V.9.1 program (SAS). The data for total lipids were subjected to analysis of variance using the GLM procedure. The System V.9.1 program (SAS).

All mathematical procedures were performed by using the Data Assist v2.0 program (Life Technologies, USA).

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay sequence</th>
</tr>
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<tbody>
<tr>
<td>CAPN1</td>
<td>BSF03223357.m1</td>
</tr>
<tr>
<td>CAPN2</td>
<td>BSF03817738.m1</td>
</tr>
<tr>
<td>CAST</td>
<td>BSF03252008.m1</td>
</tr>
<tr>
<td>TG</td>
<td>BSF03211731.m1</td>
</tr>
<tr>
<td>LEP</td>
<td>BSF03211905.m1</td>
</tr>
<tr>
<td>TBP</td>
<td>BSF03241947.m1</td>
</tr>
</tbody>
</table>

The results are in agreement with those reported in many studies, where B. taurus and their crosses produced more tender meat than pure B. indicus animals, even after aging (Crouse, Cundiff, Koch, Koohmaraie, & Seideman, 1989; Wheeler et al., 1996). However, they do not agree with the findings of Hadlich (2007). Studying meat tenderness of purebred B. indicus and B. taurus cattle, they found no difference in meat tenderness between groups after aging. These show that the aging process is efficient in reducing the difference between tenderness of B. indicus and B. taurus meat.

Based on studies of meat quality and aging in Nellore and Taurine breeds, already cited in this paper, we expected the pure Nellore cattle to present lower MFI and higher SF than the cross-bred Canchim animals, both in the meat with and without aging, since these have only 3/8 Zebu genes in their composition. Nevertheless, the MFI values (MF0 and MF7) were greater than 60 and the SF was below 4.5 kg, indicating tender meat in Nellore (Culler et al., 1978; McKee, 1985 and Shackelford et al., 1991), even before the aging process.

Although significant, the difference between the MFI and SF parameters was small, which can be explained by the production system. According to Hadlich (2007), although animals differ in their growth,
they can have similar meat quality with appropriate handling and similar feeding conditions from birth.

3.1.2. Indices of total lipids

With respect to total lipids, there were differences between the breeds (P<0.05). The Nellore animals had more deposition of marbling fat (1.3%) than the Canchim animals (0.9%). The higher rate of lipids in the Longissimus dorsi muscle of the Nellore animals indicates greater deposition of marbling fat, as noted in the literature (Fortes et al., 2009). Thus, Nellore cattle have better marbling fat deposition characteristics than the Charolais (Taurus Continental) and Canchim breeds, which were selected to have higher muscle deposition (Zadra, 2005).

3.1.3. Gene expression

3.1.3.1. Real-time polymerase chain reaction after reverse transcription (RT-qPCR). The defaults for carrying out reactions via qPCR allowed checking the linearity and efficiency of the reaction from the slope of the standard curve generated by the 7300 SDS System software (Life Technologies, USA) for each of the genes analyzed. Normalization by the correction factor (generated by the expression of the three reference genes) is the most accepted method to prevent these disparities (Vandesompele et al., 2002).

The normalization of target genes by gene reference data was done for each group. Gene expression of calpains showed no differences (P>0.05) for CAPN1 or CAPN2 between the Nellore and Canchim genetic groups (P>0.05) (Fig. 1). The results corroborate Ferraz’s (2009) work, who compared the expression of CAPN2 in Nellore (B. indicus) and Angus (B. taurus), finding no difference.

The results reveal that the slightly lower tenderness of meat from Zebu animals is probably not a result of lower expression of genes encoding proteases (CAPN1 and CAPN2), but rather is due to the increased expression of CAST, an inhibitor of CAPNs, whose expression was higher in the Nellore animals (P<0.05). This assumption is reinforced by the results obtained by Whipple et al. (1990), who in analyzing the activity of enzymes encoded by these genes for Taurine and Zebu cattle found higher enzymatic activity of calpastatin in Zebu animals, but no differences in activity of the enzyme calpain in the two genetic groups.

According to Geesink and Koohmaraie (1999) and Geesink, Kuchay, Chishti, and Koohmaraie (2006), the increased level of calpastatin activity results in reduction of calpain-mediated proteolysis and consequently decreases meat tenderness. The increase in enzyme activity associated with this process works by increasing the amount of enzyme that is synthesized. Therefore, the higher the expression of the gene encoding the enzyme, the greater its activity will be. Other factors are also involved in intracellular control of enzyme activity, but were not addressed in this work.

Several other authors have related increased enzymatic activity of calpastatin to higher SF values in the muscle of B. indicus animals compared with B. taurus (Ibrahim et al., 2008; Morgan, Wheeler, Koohmaraie, Savell, & Crouse, 1993; O’Connor et al., 1997; Pringle, Williams, Lamb, Johnson, & West, 1997; Shackelford et al., 1991; Wheeler et al., 1990; Whipple et al., 1990). However, the quantification of messenger RNAs (mRNA) involved in the process has not been widely studied, so further investigation in this respect is fundamental to provide information on the process of the calpain–calpastatin proteolytic system.

The relationships between MFI and lower levels of CAST expression reported in the literature are consistent with the results observed in this work, where the MFI parameter was lower in the Nellore animals and the CAST gene expression was higher. However, gene expression alone does not explain the small difference in MFI between the breeds, suggesting that this influence can be lower in young animals because they have high protein turnover and thus do not require large amounts of proteolytic enzymes to produce tender meat.

Given the results of gene expression, shear force and MFI, we can affirm that the meat of Nellore cattle is less tender than that of Canchim animals, although it is still within the acceptable standards of tenderness. These animals, because they are young or less mature, have benefited from a favorable cell environment where there is high protein turnover, high concentrations of muscle glycogen and consequent reduced pH, which makes the meat tenderer.

Analysis of the expression of genes related to fat deposition showed no differential expression between breeds (P>0.05). This equality of gene expression can be the result of similar development of the breeds in the feedlot period, promoting the deposition of body tissues in the same period. Thus the Canchim animals, which naturally have little marbling fat deposition because of their genetic makeup (5/8 Charolais–B. taurus continental) (Nardon, Razook, & Sampao, 2001), showed the expected expression of genes related to fat deposition. In contrast, the Nellore animals likely have higher expression, since their meat showed higher amounts of total lipids (marbling fat), as found in this study. These results indicate that other factors mediate the process of gene transcription for effective translation into protein, and possibly there are other genes involved in the formation of fat. The results could be more helpful if the expression of genes for fat deposition had been measured in adipocytes and muscle tissue, but these analyses were not performed in this study. However, one should look at the results carefully, since the analysis was performed on small numbers of animals.

3.2. Correlations between meat quality and gene expression

None of the correlations between the traits measured in the animals and the gene expression in the Nellore cattle (Table 3) were significant after applying correction by the Bonferroni method, which ensures maintenance of the error rate of statistical tests. The lack of correlation between measurements of tenderness and gene expression of CAPNs and CAST was not expected, since a greater amount of CAST has been associated with lower tenderness of meat after the aging period (Geesink et al., 2006). Moreover, calpain acts to increase tenderness, so one could expect animals with higher CAPNs expression to have more tender meat, although the action of calpain is only significant 150 h after the onset of rigor mortis. Possible explanations for the results found in this study are the fact that the animals were young and therefore had higher expression of enzymes involved in the process of protein turnover, including calpain and calpastatin. The correlation between the expression of CAPNs and CAST in this breed is greater than 0.63 (data not shown), which seems to indicate that the more calpastatin available, the more calpain also is available in the muscle. Thus, some interference between the “antagonistic” expressions of these genes may have affected the results. Moreover, other factors
inherent to young animals may have acted to decrease the variability in meat tenderness. The present results do not agree with those of Ferraz (2009), who studied the gene expression of Bos indicus and Bos taurus inheritance to young animals may have acted to decrease the variability in meat quality against the effects of rapid cooling and thus mean variations in gene expression were accompanied by variations in meat tenderness. However, that work could not establish a direct relationship between tenderness and polymorphism. In the present study, we did not measure the thickness of the fat layer, but rather only the TL muscle tissue, which was not significantly correlated with the expression of DGAT1.

4. Conclusions

Although the number of animals used in this study was small, and so the results must be taken with care, they indicate that gene expression cannot be used as a genetic marker for screening individuals with tender meat, since no evidence was found indicating that variations in gene expression were accompanied by variations in meat tenderness within breeds, even though there was, as expected, higher expression of the gene encoding the enzyme for calpastatin in Nelore (B. indicus) animals than in Canchim (B. taurus x 3/8 B. indicus) ones. Compared to Canchim animals, the Nelore animals had higher percentages of marbling fat. However, perhaps due to the small sample size, we could not find differences between breeds with respect to the expression of the genes under study or establish a cause and effect relationship between the expression of these genes and the proportion of lipids in the muscle tissue.

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References


