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Expression of calpain system transcripts responds inversely to beef tenderization after vitamin D_3 supplementation in Nellore cattle

Expressão de transcritos do sistema calpaína responde de forma inversa ao amaciamento após suplementação com vitamina D_3 em bovinos Nelore

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Resumo

Uma vez que o sistema calpaína é central para o amaciamento da carne e dada a importância da atividade de calpastatina na determinação da maciez de bifes de bovinos *Bos taurus indicus*, o objetivo do presente estudo foi determinar se a expressão do gene de μ-calpaína (CAPN1), calpastatina total (CAST T), e suas variantes (CAST I e II) foi induzido pela inclusão de vitamina D_3 na dieta. Os animais receberam nenhuma ou 2×10^6 UI dose de vitamina D_3 por 2 ou 8 dias antes do abate, e foram submetidos a diferentes condições durante o confinamento: exposição solar ou sombreamento artificial. Bifes do *Longissimus lumborum* foram fabricados e submetidos a maturação por 1, 7, e 21 dias *post-mortem* e posteriormente usados para determinação da força de cisalhamento e do índice de fragmentação miofibrilar. Vitamina D_3 não influenciou a abundância de RNAm, exceto para maior abundância de

transcritos de CAST II em animais que foram suplementados por 8 dias antes do abate. Foi encontrada associação negativa entre a abundância de CAST II e a força de cisalhamento. Essa contradição revela uma importante modulação da expressão do sistema calpaína resultado da suplementação com vitamina D que pode ser importante na determinação de estratégias para melhorar a maciez da carne.
Palavras-chave: *Bos taurus indicus*, cálcio, calpastatina, carne bovina, maciez

ABSTRACT

The calpain system is the central player for meat tenderization and the calpastatin activity plays an important role in beef tenderness of *Bos taurus indicus* cattle. This study investigated whether dietary vitamin D_3 induced gene expression of μ-calpain (CAPN1), total calpastatin (CAST T), and their variants (CAST I and II). Animals received none or 2×10^6 IU of vitamin D_3 for either 2 or 8 days before slaughter and were submitted to different conditions during feedlot: sun exposure or artificial shade. Steaks from *Longissimus lumborum* were fabricated, aged for 1, 7, and 21 days *post-mortem*, and later used for the analyses of shear force and the myofibrillar fragmentation index. Vitamin D_3 did not influence mRNA abundance; however, it induced a greater CAST II transcript in animals supplemented 8 days before slaughter. There was a negative association between CAST II abundance and the shear force, which revealed an important modulation of the calpain system expression due to vitamin D supplementation. This result is an important tool for strategies to improve beef tenderness.
Keywords: beef, *Bos taurus indicus*, calcium, calpastatin, tenderness

INTRODUCTION

Myofibrillar proteolysis by calpain, mainly μ-calpain, is one of the factors that influence meat tenderness and is responsible for the natural tenderization process (Geesink et al. 2006; Morgan et al., 1991). The inhibitory effect of calpastatin is used to predict beef quality by the calpastatin activity (Geesink et al., 2006; Grant et al., 2005); however, it is necessary to consider the significant heterogeneity between calpastatin variants generated by alternative splicing (Raynaud et al., 2005). This variability is associated to the calpastatin inhibitory activity (Nattrass et al., 2014) and can be identified by their mRNAs and by the transcriptional activity of calpastatin (CAST) gene promoters. Different responses to stimuli among species may be partly responsible for variations in CAST expression (species, breeds, and individuals). Responses to CAST expression cause variations in meat tenderness, which can be influenced by calcium fluxes and changes promoted by vitamin D.

The active form of vitamin D stimulates greater calcium transport and absorption in skeletal muscle. Supplementation with vitamin D_3 [25(OH)D_3] and metabolites in animal diets has a positive influence on meat tenderness (Foote et al., 2001; Karges et al., 2001; Montgomery et al., 2002; Swanek et al., 1999). However, *Bos taurus indicus* animals do not seem to respond to tenderization caused by vitamin

D (Lobo-Jr. et al., 2012; Pedreira et al., 2003; Tipton et al., 2007).

Data on association between gene expression and the tenderization process could be used to explore how the calpain system responds to vitamin D. This study investigated changes in the expression of μ-calpain/calpain-1 (CAPN1) gene and CAST, as well as their variants (CAST I and II), in beef from indicine cattle fed a diet supplemented with vitamin D_3 and exposed to sunlight using different systems.

MATERIALS AND METHODS

Animals and treatments

Crossbred animals with great genetic contribution from *Bos taurus indicus* cattle, mainly Nellore breed, with an initial average body weight of 435 kg and 30 months of age were fed a diet with concentrates for 45 days. Animals were allocated in an experimental feedlot (20°53′45″ South and 51°22′44″ West and altitude 405 m in the municipality of Andradina, São Paulo State, Brazil), and under two distinct exposure systems to sunlight: without shade or under a sunshade with a UV filtration ratio of 50% (see Lobo-Jr. et. al, 2012 for additional information regarding the climate). Three groups were formed based on the weight to represent their frame size: light (376 ± 29 kg), intermediate (403 ± 16 kg), and heavy (457 ± 17 kg). The treatments were randomly assigned to the animals within each frame size (treated statistically as block). Forty-two animals were kept in individual pens to control feed intake and were conditioned for 20 days to receive the initial part of their concentrated diet in a small trough. Similarly, the animals were fed vitamin D_3 mixed with the concentrate by the end of the feedlot period. One animal was removed from the experiment due to lack of adaptation to the diet. At days 37 or 43 after the beginning of the experiment, vitamin D_3 supplementation commenced and lasted for 8 or 2 days, respectively. The three supplementation types and two sunlight exposure systems were combined into the six treatments: 1) no supplementation with vitamin D_3 and no shade (n = 7); 2) no vitamin and 50% shade (n = 7); 3) 2 days of supplementation and no shade (n = 7); 4) 2 days of supplementation and shade (n = 7); 5) 8 days of supplementation and no shade (n = 6), and 6) 8 days of supplementation and shade (n = 7). Vitamin D_3 supplementation was always 2×10^6 IU mixed with the concentrate.

Slaughter and sampling

The animals were transported to a commercial slaughterhouse at 70 km from the feedlot, fasted approximately for 12 h with free access to water. The slaughter followed the Brazilian Federal Inspection Service regulation and immediately after the exsanguination, small samples from *Longissimus thoracis et lumborum* were taken to determine gene expression, afterward, the carcasses were weighed and conditioned in a cold room (4 °C) for 24 h. After the cooling period, additional samples were taken from the same muscle between the 12^{th} thoracic and 5^{th} lumbar vertebrae and vacuum packed for the analyses of the shear force and myofibrillar fragmentation index (MFI). Samples for the gene expression analysis were promptly frozen in liquid nitrogen

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and stored at – 80 °C until total RNA extraction was conducted.

Gene expression

The total RNA was extracted from tissues using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The RNA concentration and quality were determined spectrophotometrically (HITACHI, U-200). To verify RNA integrity, 28s and 18s structural bands of rRNA were used as markers in 1% agarose gel submitted to electrophoresis. For cDNA synthesis, 1 µg out of total RNA was used as described in the protocol of the ImProm-II™ Reverse Transcription System kit (Promega, Madison, WI, USA), followed by dilution of each cDNA using purified water in a 1:4 concentration.

Primers were drawn using software Primer3 (Rozen & Skaletsky, 2000) (Table 1) in different exons to check possible contamination by genomic DNA. A difference in the 5′ extremity from the bovine CAST mRNA promotes the existence of four transcript types (type I, II, III, and IV), where the isoform types I, II, and III are specific for muscle, while type IV is specific for the testicles (Raynaud et al., 2005). The CAST gene and its variants underwent sequence alignment using the BioEdit program and three pairs of initiators in regions that differentiated the three variants were drawn based on this alignment. However, only two variants (CAST I and CAST II) were possible to be differentiated and a third called CAST T (total CAST) with the amplification of a region encompassing the three mRNA variants found in muscle tissue. We used the LightCycler® 480 Real-Time PCR model (Roche Diagnostics, Mannheim, Germany) for the expression ofCAPN1, CAST T, and CAST variants, as well as reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), eukaryotic elongation factor-1-alpha-2 (EEF1A2), and ribosomal protein L-19 (RPL-19). For each reaction, 2 µl of cDNA diluted 10 times, 2 mM of each initiator and 10 µl of SYBR Green Master I 2X were combined to a final sample volume of 20 µl. The amplification conditions for all oligonucleotides were 95 °C for 2 min for DNA denaturation in PCR first cycle (additional information regarding melting temperature are presented as supplementary table 1 and figure1). The other cycles were 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 3 min for the final extension in 40 cycles. Each experiment included a positive and a negative control (without cDNA). The gene expression results were generated and registered as cycle threshold (Ct) values. The lower the Ct value, the sooner mRNA detection in the gene occurs, indicating greater gene expression.

Phenotypic data: shear force and myofibrillar fragmentation index

The Warner-Bratzler Shear Force (WBSF) and MFI were applied as qualitative information on meat tenderness to understand its association with gene expression for each treatment as phenotypic data. The steaks were taken from the *Longissimus lumborum* muscle, vacuum packed, and stored at cold temperatures (1 to 4 °C) during the aging period. For WBSF, the analyses were performed at 1-, 7-, and 21-days

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Table 1. Gene and primers used in real time PCR

Gene[1]	NCBI Gene ID	Local[4]	Primer Sequence[5]	Product (bp)
CAPN1[2]	281661	Exon 4	F > 5'-CGCCTCCCTTACCCTCAA-3'	122
		Exon 5	R > 5'-CATCCACCCACTCACCAAAC-3'	
CAST I	281039	Exon 1xa*	F > 5'-TGCCCGCCACACCCAGGA-3'	109
		Exon 1z*	R > 5'-ACTGCTCCCAAGGCTTGTT-3'	
CAST II	281039	Exon 1xb*	F > 5'-TGCGTCTCTCGGAACACAT-3'	91
		Exon 1y*	R > 5'-AAGGCGATCCACTGGTTTTT-3'	
CAST T	281039	Exon 15*	F > 5'-ATGAGGAAACAGTCCCATCG-3'	95
		Exon 16-17*	R > 5'-CTGAAGAAAGACCCAAGCCC-3'	
GAPDH[3]	281181	Exon 1	F > 5'-GGCGTGAACCACGAGAAGTATAA-3'	194
		Exon 2	R > 5'-CCCTCCACGATGCCAAAGT-3'	
EEF1A2[2]	515233	Exon 6	F > 5'-GCAGCCATTGTGGAGATG-3'	196
		Exon 7	R > 5'-ACTTGCCCGCCTTCTGTG-3'	
RPL-19[3]	510615	Exon 1	F > 5'-GAAATCGCCAATGCCAAC-3'	410
		Exon 2	R > 5'-GAGCCTTGTCTGCCTTCA-3'	

[1]Genes identification: CAPN1 – calpain-1; CAST I – calpastatin variant I; CAST II – calpastatin variant II; CAST T – total calpastatin; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; EEF1A2 – eukaryotic elongation factor-1-alpha-2; RPL-19 – ribosomal protein G-19; [2]Zaros et al., 2007; [3]Tizioto et al., 2013; [4]Local with * represents exons identification for CAST that used the sequence in GenBank accession AH014526.2 GI 1036032130; [5]Primer sequence F = forward and R = reverse.

post-mortem immediately after each aging period was completed. The procedures followed recommendations from the American Meat Science Association (AMSA, 2015) and the WBSF for the respective steak was expressed in Newtons (N). For MFI, the analyses used beef samples frozen in liquid nitrogen after aging for 1 and 21 days. Approximately 4 g of muscle per steak was used, following procedures previously described by Culler et al. (1978). The readings of spectrophotometric absorbance at 540 nm for myofibrillar suspension with final protein concentration of 0.5 mg/ml were multiplied by 200 to represent the index.

Statistical analysis

The study comprised an experimental random block design with the three-frame size (blocks) and a factorial arrangement considering three vitamin D_3 supplementation schemes and two sunlight exposure systems. We used a mixed linear model to understand the combined analysis of vitamin D_3 supplementation and sunlight exposure effects as well as the interaction, that were considered as a fixed effect, while animals within each block interacting with sunlight exposure were considered as random effects. The WBSF and MFI were tested as repeated measures in time. The analysis was conducted using SAS software (University Edition, SAS Inst. Inc., Cary, NC, USA). For the gene expression analysis, after adjusting Ct, we applied a linear general model including the fixed effects, and the treatments were compared with the Tukey test at significance level of 5% ($P < 0.05$). To evaluate the results associated to RT-qPCR related to phenotypic data (WBSF and MFI) of aged beef, the measurements were used as dependent variables in the model. In the association analysis of gene expression of CAPN1 and CAST (CAST T, CAST I, and CAST II), two approaches were applied: 1) considering the expression of two genes (CAPN1 and CAST T) and 2) considering simultaneous expression of two genes in the model, however, with two variants (CAPN1, CAST I, and CAST II).

RESULTS AND DISCUSSION

The genes GAPDH, EEF1A2, and RPL-19 were tested as reference genes. All genes presented lower variation between treatments and had average Ct values of 20.05 (± 0.66), 24.20 (± 0.87), and 31.04 (± 0.86), respectively (Table 2).

The different sunlight exposure systems and interaction with vitamin D_3 supplementation did not alter CAPN1, CAST I, or CAST T gene expression (Table 3). However, sunlight exposure did alter ($P < 0.01$) CAST II expression. The CAST II gene expression was upregulated when animals received vitamin D_3 supplementation on 8 consecutive days before slaughter, regardless of exposure of the animals to sunlight (Figure 1). This gene expression was 3.5 times higher than the gene expression of animals that did not receive supplementation. When comparison was made between animals that received 2 or 8 days of vitamin D_3 supplementation, CAST II gene expression was 2.5 times higher for animals supplemented for 8 days.

Table 2. mRNA expression (Ct) of reference genes GAPDH, EEF1A2 and RPL-19 in *Longissimus lumborum* muscle from *Bos taurus indicus* cattle supplemented with vitamin D_3 and maintained in different sunlight exposure conditions[1]

Gene	GAPDH		EEF1A2		RPL-19	
Treatments[2]	No shade	Shade	No shade	Shade	No shade	Shade
Vit. 0	20.14	20.20	24.22	24.36	32.59	31.18
	(0.53)	(0.61)	(0.95)	(0.73)	(1.70)	(0.37)
Vit. 2	20.18	20.22	24.60	24.18	31.11	30.71
	(0.69)	(0.72)	(0.87)	(1.05)	(0.45)	(0.85)
Vit. 8	19.74	19.86	24.27	23.59	30.34	30.33
	(0.64)	(0.76)	(0.76)	(0.88)	(0.67)	(1.10)

[1]Least square means of Ct followed by standard error between parentheses; [2]Treatments: Vit. 0 = without vitamin D_3 feeding; Vit. 2 = vitamin D_3 supplementation at a dose of 2×10^6 IU for 2 consecutive days pre-slaughter; Vit. 8 = vitamin D_3 supplementation at a dose of 2×10^6 IU for 8 consecutive days pre-slaughter and animals submit to sunlight exposure (No shade) or no sunlight exposure (Shade).

Table 3. Effects of calpain system genes expression in *Longissimus lumborum* muscle from *Bos taurus indicus* cattle supplemented with vitamin D_3 and maintained in different sunlight exposure conditions

Gene[1]	CAPN1	CAST I	CAST II	CAST T
Source of Variation[2]	*P* > F			
Vitamin D (Vit)	0.191	0.346	**0.004**	0.142
Sunlight exposure (Sun)	0.383	0.250	0.115	0.186
Vit × Sun	0.343	0.824	0.344	0.683

[1]Gene specification: CAPN1 – μ-calpain; CAST I – calpastatin variant 1; CAST II – calpastatin variant II; CAST T – total calpastatin; [2]Treatments tested were with or without vitamin D_3 supplementation (Vit), with shade or without shade to protect animals from sunlight exposure (Sun) and the interaction between both (Vit × Sun).

CAPN1 expression did not change, possibly due to unaltered total plasma or muscular calcium concentration under vitamin D_3 supplementation in the experimental circumstances (Lobo-Jr. et al., 2012). Studies have reported increased μ-calpain activity 20 min *post-mortem* in the *Longissimus lumborum* muscle of *Bos taurus indicus* compared to *Bos taurus taurus* cattle even without changes in muscle calcium concentration (Montgomery et al., 2004).

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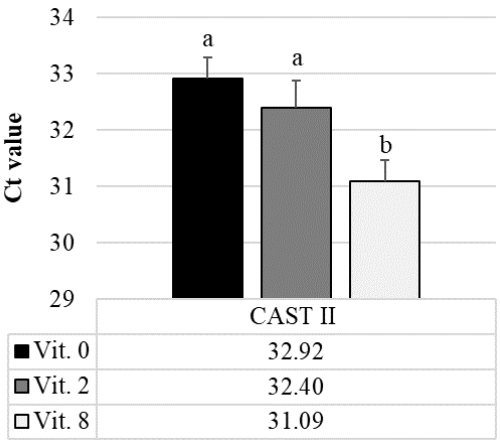

Figure 1. mRNA expression (Ct) of calpastatin isoform II (± standard error) in *Longissimus lumborum* muscle from *Bos taurus indicus* cattle supplemented with vitamin D_3 and maintained in different sunlight exposure conditions. Treatments: Vit. 0 = without vitamin D_3 feeding; Vit. 2 = vitamin D_3 supplementation at a dose of 2×10^6 IU for 2 consecutive days pre-slaughter; Vit. 8 = vitamin D_3 supplementation at a dose of 2×10^6 IU for 8 consecutive days pre-slaughter; [a,b]Distinct lowercase letters significantly differ by Tukey test ($P < 0.05$).

Vitamin D_3 plays an important role in muscle calcium metabolism thus affecting meat tenderization, depending on the biological type of the animals. Crossbred steers with predominantly continental breed crosses treated with 5×10^6 IU vitamin D_3 for 9 days and harvested 2 days after the last vitamin dose showed lower WBSF-14 values than beef from animals without vitamin D_3 supplementation (Foote et al., 2001). Differences in the calpastatin activity between cattle biological types with increased activity are associated to higher genetic contribution of *Bos taurus indicus* compared to *Bos taurus taurus* (Pringle et al., 1997; Whipple et al., 1990). The lack of differences in CAPN1 expression and the increased CAST II expression in animals fed with vitamin D_3 supplements for 8 days challenge the hypothesis that vitamin D_3 promotes tenderization due to changes in the expression of calcium dependent muscular proteases. Studies show that modification of CAST gene expression plays a crucial role in regulating calcium dependent proteolysis in *Bos taurus indicus* (Souza et al., 2019). The changes in CAST II expression under vitamin D_3 supplementation may reflect the complex regulation process or an adaptation to changes in the CAPN1 activity due to a greater calcium influx in the muscle cell. Vitamin D positively altered the influx process in the sarcolemma muscle in chicken (de Boland, Gallego, & Boland, 1983).

Calcium metabolism *in vivo* plays an important role in muscle contraction and other processes; therefore, its homeostasis is regulated strictly. However, an increase in intracellular concentration activates responses compensation and inhibit protein degradation by calpain. In biological models with prolonged calcium homeostasis alteration using ionophores during the same period of the experiment, an increase in CAST mRNA was associated to a global mechanism involving greater calpastatin degradation, resulting in greater expression as compensation (Averna et al., 2003; Stifanese et al., 2010). In resting cells, calpastatin becomes available in the

cytosol only in response to calcium increase and free calpastatin in the cytosol is directly related to a reduction in its expression. Abundance of inhibitor molecules is a signal to reduce expression of these inhibitors; however, if calpastatin is degraded due to prolonged calpain activation, inhibition of the calpastatin synthesis is removed (Averna et al., 2003). A similar situation was observed in kidneys of hypertensive rats in which calpastatin degradation was associated to high expression levels (Averna et al., 2001). Additionally, persistent alterations in calcium homeostasis in muscles of transgenic mice caused by continuous proteolytic degradation involved a conservative calpastatin degradation due to the formation of active 15 kDa fragments in addition to a four-fold increase in inhibitor mRNA abundance (Stifanese et al., 2010). The ~~a~~—longer vitamin D supplementation time changes this scenario, as calpastatin inhibits the μ-calpain activity and triggers the signal for increased protease expression, corroborating the hypothesis of reciprocal CAPN1 and CAST expression control. This was observed in cows supplemented with vitamin D_3 for 14 days before slaughter that presented muscles with 4 to 5 times greater CAPN1 and CAPN2 mRNA abundance and a reduction between 14 and 29% in CAST expression (Cho et al., 2006).

The association analysis uses gene expression to obtain the estimated values of phenotypic characteristics, represented in our study by WBSF of beef aged 1-, 7-, or 21-days *post-mortem* (WBSF-1, WBSF-7, or WBSF-21) or by MFI at 1- or 21-days *post-mortem* (MFI-1 or MFI-21). These analyses are relevant as they included both genes that are central to the proteolysis process: CAPN1 and CAST. The first approach revealed that CAST T did not show ($P > 0.05$) any effects associated with tenderness phenotypes. Nevertheless, CAPN1 showed a negative association to tenderness for beef aged for 7 days *post-mortem*, which was an unexpected result. The result showed that as 1 Ct cycle increased, or as CAPN1 gene expression decreased, there was a 3.25 N reduction in WBSF (Table 4).

From the second approach, considering the expression of both CAST variants and CAPN1 simultaneously, CAPN1 or CAST I expression did not have any effects ($P \geq 0.08$ and $P \geq 0.17$, respectively) that was related to shear force. However, CAST II expression showed an effect associated to WBSF-1, WBSF-7, and WBSF-21 days (Table 5). As 1 Ct cycle increased, or as CAST II gene expression was reduced, WBSF-1 increased to 8.91 N in beef. Similar results were obtained for beef aged 7- and 21-days *post-mortem*, where 1 Ct cycle increased WBSF to 4.61 and 8.17 N, respectively. As calpastatin has an inhibitory effect on the proteolytic system, this result was not expected because it contradicts the action mode of the calpain system in the tenderization process during beef aging.

Table 4. µ-Calpain (CAPN1) and total calpastatin (CAST T) genes expression contribution associated to tenderness phenotype identified by Warner-Bratzler shear force (WBSF) in beef *Longissimus lumborum* aged 1-, 7- and 21-days *post-mortem* from *Bos taurus indicus* cattle

	WBSF-1[1]			WBSF-7[2]			WBSF-21[3]		
Gene[4]	Estimated	SE	*P* value	Estimated	SE	*P* value	Estimated	SE	*P* value
CAPN1	0.028	0.163	0.86	-0.331	0.135	**0.02**	-0.077	0.154	0.62
CAST T	-0.056	0.245	0.82	-0.062	0.175	0.73	-0.111	0.235	0.64

[1]WBSF-1 in beef aged 1-day *post-mortem*; [2]WBSF-7 in beef aged 7 days *post-mortem*; [3]WBSF-21 in beef aged 21 days *post-mortem*; [4]CAPN1 = µ-calpain mRNA and CAST T = all calpastatin mRNA in muscle extract.

Table 5. µ-Calpain (CAPN1) and calpastatin variants (CAST I and CAST II) genes expression contribution associated to tenderness phenotype identified by Warner-Bratzler shear force (WBSF) in beef *Longissimus lumborum* aged 1-, 7- and 21-days *post-mortem* from *Bos taurus indicus* cattle

	WBSF-1[1]			WBSF-7[2]			WBSF-21[3]		
Gene[4]	Estimated	SE	*P* value	Estimated	SE	*P* value	Estimated	SE	*P* value
CAPN1	0.016	0.114	0.90	-0.237	0.135	0.09	-0.098	0.116	0.41
CAST I	0.193	0.145	0.20	0.133	0.158	0.42	0.211	0.153	0.18
CAST II	0.909	0.245	≻≺ **0.001**	0.470	0.193	**0.03**	0.833	0.189	≻≺ **0.001**

[1]WBSF in beef aged 1-day *post-mortem*; [2]WBSF in beef aged 7-days *post-mortem*; [3]WBSF in beef aged 21-days *post-mortem*; [4]CAPN1 = calpain-1 mRNA, CAST I and II = calpastatin mRNA specific for variant I and II in muscle extract.

In the association analysis, CAST T association with MFI showed no effects ($P \geq 0.31$) on beef aged for 1- and 21-days *post-mortem* (Table 6) or when using CAST I and CAST II (Table 7). The CAPN1 showed an unexpected association between gene expression and MFI ($P < 0.05$). Regardless of the approach, an inverse relationship was found between MFI-21 and gene expression. As 1 Ct cycle increased, which reduced CAPN1 gene expression, the fragmentation index increased by approximately 0.56. The same was observed in the second approach using MFI-21 with an increase of 0.59 in the index.

Table 6. µ-Calpain (CAPN1) and total calpastatin (CAST) genes expression contribution associated to tenderness phenotype identified by myofibrillar fragmentation index (MFI) in beef *Longissimus lumborum* aged 1- and 21-days *post-mortem* from *Bos taurus indicus* cattle

	MFI 1[1]			MFI 21[2]		
Gene[3]	Estimated	SE	*P* value	Estimated	SE	*P* value
CAPN1	-0.290	0.211	0.18	0.557	0.211	**0.02**
CAST T	-0.235	0.311	0.46	-0.142	0.329	0.67

[1]MFI in beef aged 1-day *post-mortem*; [2]MFI in beef aged 21-days *post-mortem*; [3]CAPN1 = calpain-1 mRNA and CAST T = all calpastatin mRNA in muscle extract.

Table 7. µ-Calpain (CAPN1) and calpastatin (CAST) variants genes expression contribution associated to tenderness phenotype identified by myofibrillar fragmentation index (MFI) in beef *Longissimus lumborum* aged 1- and 21-days *post-mortem* from *Bos taurus indicus* cattle

	MFI 1[1]			MFI 21[2]		
Gene[3]	Estimated	SE	*P* value	Estimated	SE	*P* value
CAPN1	-0.299	0.205	0.16	0.593	0.206	**0.01**
CAST I	-0.053	0.251	0.84	0.084	0.245	0.74
CAST II	-0.186	0.319	0.57	-0.079	0.330	0.82

[1]MFI in beef aged 1-day *post-mortem*; [2]MFI in beef aged 21-days *post-mortem*; [3]CAPN1 = calpain-1 mRNA and CAST T = all calpastatin mRNA in muscle extract.

The negative relationship found between CAPN1 Ct values and WBSF-7, regardless of the approach, as well as the positive relationship between CAST II Ct values and WBSF at all aging times revealed a possible mismatch between mRNA abundance and the activity of calpain components in specific physiological conditions. These observations point to a model related to

greater μ-calpain activity associated to lower momentary gene expression, that is, lower mRNA abundance. An increased μ-calpain activity could cause greater calpastatin degradation and increase in CAST mRNA. This higher abundance in CAST mRNA, observed immediately after a short period of vitamin D_3 supplementation, does not imply a calpastatin level alteration that could be effective in the proteolysis inhibition. In our study, more than 2 days of vitamin D_3 supplementation was required to change the CAST II mRNA abundance, which might then take several days to elicit an increase in protein level. The delay in days between changes of mRNA abundances and protein levels are especially longer in proteins with half-lives of several days, since protein degradation is an important factor to determine changes of the protein level after transcription (Hargrove, Hulsey & Beale, 1991). Proteins in the calpain system, including calpastatin, have long half-lives (Zhang, Lane, & Mellgren, 1996); therefore, this condition is compatible with greater protein degradation, lower WBSF, and greater MFI associated to lower CAPN1 and greater CAST II mRNA abundances. The difference in calpastatin between biological types that reduces tenderness is associated to CAST gene allele modification (Curi et al., 2010) and, although genetic associations have not been addressed in our study, polymorphism in the CAST gene seems to be related to alterations of mRNA levels, mainly CAST II, as reported by Nattrass et al. (2014). These authors verified that animals with one or two favorable alleles to tenderness showed low mRNA levels to the CAST II variant in comparison to animals without favorable alleles, which in turn presented a 14% increase in mRNA levels and reduced tenderness. Moreover, the same authors reported greater prevalence of unfavorable alleles in *Bos taurus indicus* cattle associated to greater expression of calpastatin. This condition seems to be enough to reduce tenderization and may preclude the effectiveness of short-term vitamin D supplementation to improve tenderness in *Bos taurus indicus* cattle (Lobo-Jr. et al., 2012; Pedreira et al., 2003; Tipton et al., 2007). Nonetheless, it seems that vitamin D improves muscle proteolysis causing a negative and positive feedback control in CAPN1 and CAST II transcripts expression, respectively.

The apparently contradictory association should be considered with caution because there is no data on enzyme activity to determine the cause/effect relationship between gene expression and the tenderization process. The gene expression of CAPN1 and CAST variants associated to WBSF values and MFI in bovine muscle should be evaluated in a greater number of samples to confirm the results of our study.

Our study showed vitamin D_3 supplementation for 8 consecutive days before slaughter in *Bos taurus indicus* cattle affected the calpain system. Although CAPN1 expression was not affected, CAST II expression increased. The association analysis between the calpain system mRNA and tenderness traits revealed a possible complex negative feedback system control involving calcium homeostasis in muscle cells.

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