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CÂMPUS DE JABOTICABAL

TRANSCRIPTOME STUDY OF MUSCLE TISSUE IN NELLORE CATTLE DIVERGENT FOR BEEF QUALITY

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TÍTULO DA TESE: TRANSCRIPTOME STUDY OF MUSCLE TISSUE IN NELLORE CATTLE DIVERGENT FOR BEEF QUALITY

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DADOS CURRICULARES DO AUTOR

Maria Malane Magalhães Muniz – Nasceu em Camocim, Ceará em 19 de Outubro de 1988, filha de Lucia Maria Magalhães e Manoel Gonçalves Muniz. Iniciou sua graduação em Zootecnia na Universidade Estadual Vale do Acarau (UVA), Câmpus Central de Sobral em agosto de 2009. Durante a graduação (2009-2012) participou de projetos científicos, foi bolsista EMBRAPA e CNPq, monitora na disciplina Melhoramento Genético Animal e recebeu certificado de Mérito Acadêmico por ter obtido o primeiro lugar na apresentação de trabalhos no curso de Bacharelado em Zootecnia-UVA. Em Março de 2013, iniciou seu mestrado no programa de pós-graduação em Ciências Animais da Universidade de Brasília- UNB, onde foi bolsista CNPq. Durante o mestrado dedicou-se a estudo de associação ampla do genoma para coloração de pelagem em ovinos da raça Morada Nova, sob orientação do pesquisador Dr. Samuel Rezende Paiva, recebendo o título de mestra em Março de 2015. Foi professora (2015-2016) nos programas de gradução em Agronomia e Medicina Veterinária no Centro Universitário ICESP - Águas Claras-DF. Em agosto de 2016 iniciou o Doutorado no programa de pós-graduação em Genética e Melhoramento Animal da Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, Câmpus de Jaboticabal (FCAV/Unesp) sob orientação do professora Dra. Lucia Galvão de Albuquerque, sendo bolsista CAPES e FAPESP. Realizou Estágio de Pesquisa no Exterior, no período de Setembro de 2018 a Março de 2020, na Universidade de Guelph, Canadá, onde desenvolveu parte do seu projeto de doutorado, sob orientação do professora Dra. Angela Cánovas. Durante o período de setembro/2018 à março/2019 obteve a Bolsa Estágio de Pesquisa no Exterior (BEPE)- FAPESP.

"O espírito que adquirir a virtude do perdão não achará dificuldade em mais nada. Haja o que houver aconteça o que acontecer, ele saberá administrar sua vida."

Chico Xavier

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ESTUDO DO TRANSCRIPTOMA DO TECIDO MUSCULAR DE BOVINOS DA RAÇA NELORE DIVERGENTES PARA CARACTERÍSTICAS DE QUALIDADE DE CARNE

RESUMO - As características de qualidade de carne, por serem de difícil mensuração, raramente são contempladas em programas de melhoramento genético. Porém, é necessário conhecer sobre os genes e os mecanismos moleculares que controlam essas características, isso poderá auxiliar na avaliação de animais de forma precoce. Objetivo desse estudo foi identificar e analisar genes e isoformas de mRNA diferencialmente expressos no tecido do músculo *Longissimus thoracis* de bovinos da raça Nelore, fenotipicamente divergentes para características cor da carne, marmoreio e maciez da carne (avaliada através das metodologias de WBSF e MFI) usando dados de RNA-Seq. Além disso, esse estudo visa a identificação dos efeitos de variantes estruturais em "splice sites" de transcriptos diferencialmente expressos em animais fenotipicamente divergentes para as características de color e marmoreio da carne. Uma média de 37 mil transcritos foram detectados sendo expressos no músculo de bovinos Nelore. Foram identificados 40 e 28 isoformas de mRNA (p-valor < 0,001) diferencialmente expressas para os fenótipos de WBSF e MFI, respectivamente. Além disso, foram identificados sendo diferencialmente expressos os mRNAs [WBSF (*SYNPO-201*) e MFI (*ANKRD23-202*)], e entre outras isoformas de mRNA relacionadas a genes que afetam a velocidade de degradação das fibras musculares durante o processo de envelhecimento da carne, como e.g. [para WBSF (*MYOT, CASQ1, TPM2, MB* e *SYNM*) e para MFI (*FGFRL1* e *NEB*)]. Na análise de enriquecimento, esses genes foram associados com funções biológicas relacionadas á processos oxidativos, produção de energia, contração muscular estriada, via de sinalização de HIF-1 e metabolismo de aminoácidos e derivados. Além disso, 14 e 15 isoformas de mRNA foram diferencialmente expressas (p-valor ≤ 0,001) entre os animais com alto marmorização da carne em relação ao grupo de animais com baixo marmorização, e entre o grupo de animais com coloração desejável em relação ao grupo de animais com coloração indesejável da carne, respectivamente. Entre elas foram encontrados mRNAs: *COL4A2-202, RPL30-203, MAPKAPK2-204, NEURL1-201, SCL16A6-201* e *JSP.1-204* que apresentaram variantes estruturais (e.g., variantes de nucleotídeo único, inserção ou exclusão) responsáveis pela a interrupção de *sites de splicing* nessas regiões. A análise de enriquecimento, realizada para a lista de isoformas de mRNA diferencialmente expressas para marmoreio e para coloração da carne identificou vias metabólicas comuns entre essas características, como por exemplo, a via de troca de O2/CO2 nos eritrócitos, biossíntese de tirosina e a via de degradação de fenilalanina. Possivelmente, o mecanismo de interação entre essas características dá-se através da oxidação lipídica mitocondrial que está intimamente relacionada à formação de mioglobina férrica, molécula importante para manter a cor fresca da carne vermelha. Os resultados obtidos nesse estudo mostram que as isoformas de mRNAs encontrados podem ser potenciais genes reguladores das características de maciez, coloração e marmoreio da carne bovina, as quais são características economicamente importantes para a indústria de carne.

Palavras-chave: Coloração da carne, isoforma de mRNA, Marmoreio, MFI, RNA-Seq, WBSF.

TRANSCRIPTOME STUDY OF MUSCLE TISSUE IN NELLORE CATTLE DIVERGENT FOR BEEF QUALITY

ABSTRACT - The measurement of meat quality traits are costly, because of that it is rarely included in breeding programs. However, it is necessary to know about the genes and the molecular mechanisms that control these traits, this may help in the evaluation of animals at an early stage.Thus, the identification of mRNA isoforms and the associated splice variants affecting meat quality traits in Nellore cattle could allow to better understanding the genetic architecture of these complex traits. The objective of this study was to identify and analyze genes and mRNA isoforms differentially expressed in the *Longissimus thoracis* muscle tissue of Nellore cattle, phenotypically divergent for meat color, marbling and meat tenderness (evaluated using WBSF and MFI methodologies) traits, using RNA-Seq data. In addition, to identify structural variants affecting splice sites of differencially expressed mRNA isoforms for marbling and meat color traits. An average of 37 thousand transcripts were detected in the Nellore muscle transcriptomic analysis. A total of 40 and 28 mRNA isoforms (p-value |<| 0.001) were differentially expressed between divergent groups for WBSF and MFI phenotypes, respectively. The differentially expressed mRNA isoforms [WBSF(*MYL1, MYL3, MYH1* and *MYBPC2*) and MFI (*MYL6*) traits], which are myosin encoders, was the family with most abundantly number of mRNA isoforms detected as diferencially expressed. In addition, we identified as DE [WBSF (*SYNPO-201* isoform*)* and for MFI (*ANKRD23-202* isoform) traits], and other mRNA isoforms related with genes affecting the speed fibers degradation during the meat aging process, such as [for WBSF (*MYOT, CASQ1, TPM2, MB* and *SYNM*) and for MFI *(FGFRL1* and NEB)]. The DE mRNA isoforms are transcripted by genes with biological functions related to oxidative process, energy production, striated muscle contraction, HIF-1 signaling pathway and metabolism of amino acids and derivatives. A total of 14 and 15 mRNA isoforms were differentially expressed (DE) (p-value ≤0.001) between the two groups of divergent bulls for marbling and color of meat traits, respectively. The DE mRNA for marbling trait (*COL4A2-202, RPL30-203, MAPKAPK2-204* and *NEURL1-201*) and color trait (*SCL16A6-201* and *JSP.1-204*) shown sites of splicing modified by structural variants as Single Nucleotide Variant (SNV), insertion or deletion (Indels). Enrichment analysis using the list of significant differentially expressed mRNA isoforms identified common metabolic pathways, such as O2/CO2 exchange in erythrocytes, tyrosine biosynthesis and phenylalanine degradation. The interaction mechanism among them, suggested that, mitochondrial lipid oxidation was closely related to ferric myoglobin formation, molecule important for maintaining fresh red meat color. The results obtained suggest potential key regulatory genes associated with economically important traits for the beef industry and for the consumer.

Keywords: meat color, mRNA isoforms, marbling, MFI, RNA-Seq, WBSF.

SUMMARY

CHAPTER 1 - GENERAL CONSIDERATIONS

1.1 INTRODUTION

Brazil is an important country in meat production and consumption of beef, being one of the highlights in the international agribusiness scenario (McManus et al. 2016). Most of the Brazilian herd is composite of Zebu breeds, notably among them, the Nellore breed that has been shown to be efficient in terms of rusticity and adaptability to the Brazilian pasture conditions, in addition to presenting excellent maternal ability (Bianchini et al., 2006). However, Zebu animals show a higher frequency of genes/alleles that are unfavorable to beef quality traits, such as tenderness and marbling, when compared to Taurine breeds (Crouse et al., 1993).

Tenderness is the sensorial trait most valued in meat quality to consumers (Miar et al., 2014). Color of meat and marbling are visual traits that determine the consumer purchase decision, since the decision is based on visual and olfactory appearance. However, these traits have low to moderate heritability, that hinders the genetic process by traditional animal breeding methods. Thus, functional genomics provided new opportunities for understanding the mechanisms and expression pattern of transcripts, in specific tissues, under different stages of animal development and production (Hamill et al., 2012; Jin et al., 2012).

The use of RNA sequencing technology (RNA-Seq), fundamental for the advances of gene expression studies, has being an important tool in the complex trait studies, providing a better understanding of their regulation mechanisms (Tizioto et al., 2015). Studies have been revealed an enormous amount of differentially expressed genes for various traits in different tissues of numerous species (Ramayo-Caldas et al., 2012; Ramayo-Caldas et al., 2014; Cesar et al., 2015; Piorkowska et al., 2015). Above all, this technology has greatly aided the identification of alternative splicing in genes involved in trait mechanisms of economic interest (Mardis, 2008), more accurately (Davey et al., 2011; Garber et al., 2011), standing out when compared to other techniques traditionally used for gene expression analysis (Tang et al., 2011).

The alternative splicing (AS) is an important process to control gene expression, increasing protein diversity, because a single transcribed gene can provide multiple proteins from this process (Wang et al., 2008). Thus, around 66.6% of genes are submitted to various

types or forms of AS, this fact provide the bovine transcriptome complexity (He & Liu, 2013). Zhou et al. (2014), studied the complexity of transcripts during adipose tissue development in cattle of different ages and sexes. They revealed over 50% of AS events were specifically expressed in adipose tissues, indicating a strong association of these events with adipose tissue regulation in specific physiological environments. Therefore, this is a complex and very important process for the expression of phenotypes related to productive traits of economic interest.

In addition, genomic studies have addressed the existence of a large structural variants number (Xu et al., 2002). These may affect protein structure, leading to different phenotypes for a given trait, or may not have visible effects at the trait level (Zhu et al., 2010). Currently, the largest genomic variation scientific approaches have been focusing in SNPs, small insertions or deletions, and copy number variation (CNV) (Boussaha et al., 2015). Thus, the structural variants identification that modify the splicing sites in Nellore muscle tissue will enhance differential expression gene studies, it could enable to understand functions and genome element mechanisms performance for meat quality traits.

1.2 GENERAL OBJECTIVE

Improve our understanding of the functional genetics underlying beef quality by studying the *Longissimus thoracis* muscle transcriptome and genomic structural variants.

1.2.1 SPECIFIC OBJECTIVE

- Use the RNA-Seq technique to find differentially expressed genes for the myofibrillar fragmentation index(MFI) and Warner-Bratzler Shear Force (WBSF) traits in divergent animal groups for these traits.

- Identify novel differentially expressed mRNA isoforms in *Longissimus thoracis* tissues, using different meat tenderness phenotypes, WBSF and MFI, to perform two differential expression analyses, and then integrate these results for identification of active pathways in the process of transforming muscle into meat.

- Annotate novel differentially expressed mRNA isoforms associated to meat tenderness.

- Identify candidate mRNA isoforms, as well as to identify potential structural variants (SNPs and Indels) associated with the splice sites effect in these differential expressed mRNA isoforms across groups of extreme animals for marbling and meat color traits.

1.3 LITERATURE REVIEW

1.3.1 Structure and muscular physiology

The muscular system is an organ consisting of skeletal, smooth and cardiac muscles (Listrat et al., 2016) . It is responsible for the body movement, permitting the posture stability and blood circulation throughout the body. Some of these muscles are controlled through the nervous system (e.g skeletal muscle) (Murphy et al., 2018), and other can be completely autonomous (e.g. cardic muscle).

The skeletal muscle consists of 90% muscle fibers, which are in turn composed of myofibrils, and 10% of connective and fat tissues (Listrat et al., 2016). Thus, this is a combination of muscle fibre sets are arragend in bundles that are surrounded by perimysium, and these several bundles form the fasciculus, which are surrounded by epimysium.

The myofibrils are composed of sarcomeres, which consist of thin actin filaments and thick myosin filaments (Roberts et al., 2016; Lange et al., 2020). These give striated appearance of skeletal muscle and forms the basic machinery necessary for muscle contraction. Thus, within the sarcomere, actin and myosin fibers overlap in a contractile motion towards each other. In addition, the endosarcomeric cytoskeleton acts as a third filament system that coexists with actin and myosin within the sarcomere (Waterman-Storer, 1991). The endosarcomeric cytoskeleton is composed of the elastic titin filaments, the inextensible nebulin system(Boudriau et al., 1993). In additon, theses mentioned structure, exist the exosarcomeric cytoskeleton, which is consisted of intermediate filaments (IF) composed of the proteins desmin, vimentin, and synemin, form (Waterman-Storer, 1991). Therefore, endosarcomeric and exosarcomeric cytoskeleton play important roles in the formation of muscle structure.

The sarcomeres are organized in A and I bands, M and Z line and H zone (Wilson & Hunt, 2008). The A-band contains the entire length of a single thick filaments, it contains both actin and myosin proteins. The I-bands are thin filaments (actin) that is not superimposed by thick filaments (myosin). The zone-H is is a paler region within of the band-A, that it is the zone of the thick filaments that has no actin. Within the H-zone is a thin M-line, it is formed of cross-connecting elements of the cytoskeleton. The M-band that anchor thick (myosin and associated proteins) filaments to the elastic filament system composed of titin. Thus, the M and Z lines holds together the thick myosin filaments and work for differentiates between each sarcomere, respectively (Lieber et al., 2017).

The muscle contraction is historically associated with the sliding and and the cross-bridge theories (Rassier, 2017). Based on these theories, the muscle contraction occours due to the thick filaments (myosin) of muscle fibers slide past by the thin (actin) filaments, generating tension in the muscle, while the two groups of filaments remain at relatively constant length. However, the contraction is not uniform across the sarcomere, the central position of the thick filaments becomes unstable and can shift during contraction. Thus, the actions of elastic proteins such as titin are possivebly maintain uniform tension across the sarcomere and pull the thick filament into a central position. Although titin has been associated with force regulation through phosphorylation and calcium binding (Leonard & Herzog, 2010). Thus, calcium ions are required for each cycle of the sarcomere. Calcium is released from the sarcoplasmic reticulum into the sarcomere when a muscle is stimulated to contract, the calcium uncovers the actin binding sites.

In addition, the cross-bridge is one of several molecular events that happened adjacents the sliding filament theory (Rassier, 2017). It is consisting larger structures along the myosin filament, called myosin heads, it has two binding sites, one for ATP and another for actin. Thus, it works as a myosin projection. This process consumes large amounts of adenosine triphosphate (ATP), the energy source of the cell. ATP binds to the cross bridges between myosin heads and actin filaments. The ATP is hydrolyzed by myosin, which uses the released energy to move itself, binding weakly to a part of the actin binding site (Manning, 2016).When ATP is used, it becomes adenosine diphosphate (ADP), it happens because muscles store little ATP, thus they must continuously replace the discharged ADP with ATP (Enoka & Pearson, 2013). Then, the remainder of the actin binding site is blocked by tropomyosin. However, with the ATP hydrolyzed, the cocked myosin head has ADP+ $_{Pi}$, and

two Ca^2 ions bind to troponin C on the actin filaments, which forms a protein complex allowing the tropomyosin to slide over and unblock the remainder of the actin binding site (Fenwick et al., 2017; Squire, 2019).

The muscle contraction could be described by length and tension that are two variable basics that determine the natural movements that underlie locomotor activity. Thefore, it is a multifaceted process that produces changes in length and tension in a time-varying manner (Fenwick et al., 2017). Based on that, there is contraction due to the muscle tension changes, but the muscle length remains the same,it is called isometric. In other hand, could occour contraction is isotonic, it happen when muscle tension remains the same throughout the contraction (Rio et al., 2017). Thus, when the muscle length shortens, the contraction is concentric; or the muscle length lengthens, the contraction is eccentric (Hessel et al., 2017).

Thus, the muscle fibers are the functional unit of the organ, leading to contraction and relaxation. There are 2 major classifications of skeletal muscle; these include Type I (slow oxidative) and Type II (fast twitch) (Pette & Staron, 1990). The type I fibers [slow fiber contraction] are called oxidative fibers, which are myoglobin and mitochondria rich and utilize mainly oxidative phosphorylation for energy production. It is typical of “red” muscles characterized. The type II fibers are glycolytic fibers [fast fibers], which have few mitochondria, low myoglobin content, and largely generate ATP through glycolytic metabolism (Khan et al., 2013). Thus, the classification of fibers types is essential to interpret the muscle development and the functional properties of different muscle. For this classification have been using type-specific molecular markers, such as myosin isoforms (Schiaffino, 2018).

1.3.2 Nellore beef quality

The beef industry faces many challenges, but it also has a great opportunity to satisfy the increasing global demand for high quality animal protein products. The meat quality is determining by combination of traits such as color, tenderness, fat content and marbling. These traits are composited by factors as succulence and flavour that have directly influence in the consumer`s choice, whom evaluates the meat quality cuts based on the physical aspects, according to the visual appearance (Jimenez & Fernandez, 2009). Researches conducted in

several countries, have been showing that consumers' willingness to pay more for additional quality (Polkinghorne and Thompson, 2010).

The tenderness is one of most valued beef quality traits by the meat market (Bolemam et al., 1997; Ishihara & Madruga et al., 2013; Miar et al., 2014). Among the methodologies using to evaluate meat tenderness, Warner-Bratzler shear force (WBSF), a mechanical tenderness measures, and myofibrillar fragmentation index (MFI), an enzymatic myofibrillar proteolysis quantification, are commonly used. Both methodologies present a high correlation with tenderness and have been frequently used due its precision in predicting meat tenderness (Hopkins et al., 2000). In addition, these measurements have high negative correlation each other; Olson & Parrish (1977) related a high correlation coefficient (-0.78) between MFI and WBSF measures. Other authors also found similar results (Culler et al., 1978; Malheiros et al., 2015).

The Warner-Bratzler shear force (WBSF) is the oldest one widespread to using to measure tenderness by shear force. It is widespread meat-specific protocol yet accepted to determine tenderness in various laboratories around the world (Bratzler, 1949). It measures the force applied to crush the cooked meat (shear force), thus when less force is applied to cut the cooked beef greater is the beef tenderness based on WBFS methodology. Some authors had used WBSF phenotypes to investigating beef tenderness in Nellore cattle by different prospection approaches, such as GWAS (Magalhaes et al., 2017; Castro et al., 2017) and gene expression studies (Tizioto et al., 2013; Malheiros et al., 2015; Tizioto et al., 2016; Fonseca et al., 2017; Gonçalves et al., 2018). However, this technique is only performed after slaughter of animal, thus WBFS becomes an expensive and time laborious methodology for the meat production system. The sample collect is usually performed 24 hours after animal`s slaughter because it needs to cool carcass for sampling; then, the cooked beef should be to cool at least 12 hours to measure shear force. In addition, preparation of 6-8 samples, with 1.27 cm in diameter each, taken parallel to the muscle fibers, are necessary to WBSF measurement (AMSA, 1995). Then therefore, it has been necessary the development of novel approaches to measure meat tenderness by a practical and efficient way, focusing to reduce research costs, and also present techniques able to be applied by the producers to identify and select animals enhancer for this trait.

The myofibrillar fragmentation index (MFI) indicates the intensity of myofibrillar proteolysis (Olson & Parrish, 1977), which is an indication of tenderness. Thus, as greater is

Z-line of the sarcomere degradation by endogenous enzymes should be greater the myofibrillar fragmentation index (Olson, 1976). Therefore, the facility fragmentation can be assessed from the intensity with which myofibrils are ruptured during the grinding process, which is simulating food chewing (Delgado et al., 2005). Thus, the MFI score ranges from 0 to 100, where over 60 have being considered extremely tender beef, scores between 50 and 60 is considered tender beef, and scores below 50 are considered non-tender. The MFI measures is a widely used method and can predict up to 50% of the tenderness variation (Hopkins et al., 2000). It could be applied when the muscle samples are not large enough to use the shear force method (Veiseth et al., 2001), or to measure tenderness in young animals, without slaughtering, the muscle sample could be collected by biopsy (Campos et al., 2016).

The correlation between MFI and tenderness is positive, the higher myofibrillar fragmentation index corresponds to greater tenderness of the meat. However, MFI has been used less frequently in studies of meat tenderness in Nellore cattle, these have been performed to evaluate the effect of SNPs in candidate genes such as *CAST* and *CAPN1* (Curi et al., 2009, 2010; Enriquez-Valencia et al., 2017). Guisti et al. (2013), used RT-PCR technique to investigate the expression profile of genes (*CAPN1, CAPN2, CAST, TG, DGAT1* and *LEP* gene) related to tenderness and another meat quality traits. Andrade et al. (2010), evaluated meat quality during maturation in Nellore and Red North cattle, observing a higher myofibrillar fragmentation index in Red North meat than in Nellore beef. Similarly, the shear force in Red North meat was lower than in Nellore meat.

The color and marbling are visual traits, usually these are main aspects evaluated by consumers, which prefer fresh and red color meat. The color beef trait is decisive in the moment of meat purchase by the consumer, who prefers fresh bright red meat (Guerrero et al. 2013). The color perception is given by the physical interaction between meat properties recorded by the human eye and light (Pepperell, 2011). Therefore, it is a subjective measure for the consumer market. The beef color mainly determined by the concentration and state of myoglobin (Mb) and hemoglobin (Hb) proteins along microstructure in the muscle. After bleed, myoglobin is main responsible for the pigmentation of meat. This protein can have three different chemical composition, known as deoxymyoglobin (Mb), oxygenated myoglobin (O^2Mb) and methamioglobin (MetMb) (Listrat et al., 2016). The reaction among these different myoglobin forms occurs in dynamic equilibrium, it is a reversible reaction, and constant conversion among these forms are depending presence or absence of oxygen. In

addition, there are many enzymes present in the muscle, which even after death of animal remain functional and using oxygen for energy metabolism and iron oxidation. (Smith & Carpenter, 1974). Beef color measure is evaluated by a colorimeter system, which emits primary colors (blue, green and red) and transforms the reflectance spectrum or transmittance from an object in three variables (a, b and l) by three-dimensional graph. Thus, any color can be measured by colorimeter or spectrophotometer measure with lamp settings adjusted to observer's pattern (MacDougall, 1982). Studies have been frequently performed to beef color investigation from non-genetic aspects, such as nutritional [e.g., effect of different diets (van-Cleef et al., 2017; Gesteira et al., 2018)] and post-mortem carcass treatment effects (Aroeira et al., 2017). However, this trait was rarely been investigated from genetic aspects, Chardulo et al., (2019) used real-time PCR technique to investigated gene and protein expression of myosin heavy chain (MyHC) for color and another meat quality in Nellore.

The marbling trait is an indicator of the proportion of intramuscular fat that is the fat interspersed in muscle fiber, this type of fat is desirable in some consumer's market (Williams, 2008). This trait is associated with organoleptic properties, such as taste and succulence. However, Nellore breed presents a low marbling percentage when compared to *Bos Taurus* (Cundiff, 2004). This fact evidences to needs genetic improvement of this trait in Nellore. The marbling measure is performed, directly on the beef samples, after slaughter of the animal. Another way, to measure marbling is using ultrasound technique, which has advantage to be performed without slaughter animals, and it has relatively low cost. However, this methodology does not replace traditional measurements that are performed directly on meat. Therefore, it has been used as an additional measure of marbling and also to evaluate breeding bulls, contributing to greater confidence in the predicting the animal's genetic potential for this trait (Waldner et al., 1992). The marbling is assessed to following a visual score scale with the aid of a meat cuts photographic standard. It is ranging from score 1, which means very little streaky fat in the meat cuts, to score 10, that is a large a streaky fat according to USDA Quality Grade (1997). This is one of most investigated meat quality trait; several studies have been performed for marbling and fat content of meat from a different prospections, such as genome-wide association studies (GWAS) (Magalhaes et al., 2017), gene expression (Cesar et al., 2015; Martins et al., 2015; Berton et al., 2016; Fonseca et al., 2020), and detection of structural variants (CNV) (de-Lemos et al., 2018).

These trait set have polygenic inheritance, which means the expression of these phenotypes are determinate for several factors genetic and non-genetic, such as gender, diet, age, and genetic group (Soares Filho et al., 2001). Currently, the understanding and control of these aspects have been great researchers' challenge. Thus, have been to increasing the search for methodologies and techniques able to potentialize the reliability and accuracy of selection methods of enhancer individuals to beef quality traits. In this sense, increase our knowledge about gene expression and regulatory elements involved in genetic mechanism of these traits have been a powerful tool for increase the accuracy of genomic selection (Magalhaes et al., 2017; de-Lemos et al., 2018; Fonseca et al., 2020)

1.3.2 Functional omic analysis

Functional genomics provided new opportunities for understanding the transcript`s mechanism and gene expression pattern in specific tissues at different stages of animal development and production (Hamill et al., 2012; Jin et al., 2012). Sequencing techniques have been important tools for complex trait studies, because it provides large-scale and reliable data for omics analysis (Tizioto et al., 2015), such as RNA-Seq technology that became a fundamental technique to transcriptomic investigation.

Transcriptomics studies have been showing an enormous amount of differentially expressed genes for different traits in several tissues of numerous animal species (Ramayo-Caldas et al., 2012; Cesar et al., 2015; Piorkowska et al., 2015). It has been aiding to identify alternative splicing patterns of genes involved in trait mechanisms of economic interest (Mardis, 2008; Davey et al., 2011; Garber et al., 2011; Nueda et al., 20180. In addition, it have been paving the way for new research such as coexpressed genes, regulatory elements and the identification of structural variants affecting splicing sites, which have been a way to integrate genomic and transcriptomics studies, enhancing the accuracy of gene prediction.

1.3.2.1 Gene expression analysis

Gene expression is a process where DNA nucleotide information synthesis becomes a functional product (Anjum et al., 2016). Thus, gene expression analysis is an important tool to characterize the molecular basis of phenotypic variation (Soneson & Delorenzi, 2013). The

gene regulation provides the control of amount and timing when gene elements may be transcript as cells needs. This process is vital to cells and organism (Meyer, 2016), because it gives flexibility to cell to adapt to variable external signals, such as environment and chemical factors, which can damage cell. Thus, knowledge of regulatory elements of gene expression are necessary to enhance tools, currently used to select animals for economic important traits in the livestock.

Gene expression profiles are highly tissue specific, which is essential to investigate gene expression levels in different tissues and how this expression levels affect the phenotypes. There have been performing many gene expression studies in bovine, those are using RNA-seq data, to investigate several reproductive traits (Chiwood et al., 2013; Reyes et al., 2015; Seo et al., 2016), as well as bovine diseases (McLoughlin et al., 2014; Fang et al., 2017). For carcass and meat quality, researches have been searching to fat content e marbling (Berton et al., 2016; Cesar et al., 2015; Zhang et al., 2018; Fonseca et al., 2020; Silva et al., 2020; Fonseca et al., 2020), meat tenderness (Giusti et al., 2013; Fonseca et al., 2017; Gonçalves et al., 2018; Malheiros et al., 2018). In addition, some studies have been investigating ribeye area (REA) and backfat thickness (BFT), which could be an indirect measure of meat yield (Silva-Vignato et al., 2017, 2019; Silva et al., 2020). However, meat color has been little investigated as its genetic aspects.

Alternative splicing (AS) is a common mechanism of eukaryotes. It is the main regulatory mechanism of expression, which could provide from a single gene different functional products (Joaquim & El- Hani, 2010). Thus, it is a great way to increase the transcriptome complexity and functional diversity (Xu, et al., 2002; Mallory et al., 2015; Nueda et al., 2018). The known about splicing role as a major source of protein diversity in genome (Wang et al., 2008) have been changing focus of researches, because this discovery allowed us to measure how complex it could be across different genomes.

The processing of pre-messenger RNAs, often produces multiple mRNA and protein isoforms that may have related, distinct or even opposing functions. It has acting to specific tissues and works into splicing networks to regulate tissue and organ development, because of that has paramount importance for physiological response or adaptation process of organism submitted to different environmental. The different mRNA isoforms, which are produce by AS events, could have distinct acting, such as stability, localization, or to be translated to stable protein isoforms with different structures and functions (Park et al., 2018). In the sense,

mRNA isoforms investigation has been recent, but it has been providing new insights to explore the genetic patterns of complex traits, such as meat quality traits.

1.3.2.2 Structural variants

Structural variants are chromosome structure variation on individuals level, which can affect a protein structure creating different phenotypes for a determinate trait (Escaramıs et al., 2015). However, major proportion of these variants do not produce visible modifications in the phenotype level, in other words it is silent mutations. Among these variations there are deletions, duplications, and replacement (Stephens et al., 2011; Elyanow et al., 2018). Thus, structural variant definition hasn`t implication on the frequency or phenotypical effects; it affects a larger portion of genome than single nucleotide polymorphism (Pang et al., 2010).

Structural variants are classified in microscopic and submicroscopic types. Microscopic variants consist in variation that could be detected with optical microscopes, e.g marker chromosome and variation in chromosome size (Nowell & Hungerford, 1960) and submicroscopic variants consist variations such as deletions, duplications and large-scale copy-number variants — collectively termed copy-number variants or copy-number polymorphisms — as well as insertions, inversions and translocations (Srebniak et al., 2018). These also have a mutation rate higher than microscopic structural variants, which become a challenge to detect accurately these structural variants type (Rimmer et al., 2014; Prodduturi et al., 2018). Feuk et al. (2006) showed that single nucleotide variant (SNV) mutation rate is higher than SNP and INDEL variant rates. The SNV could contribute significantly to generate spontaneous structural variants, which increases the likelihood of spontaneous small insertion/deletion polymorphisms (indels) within 100 kilobases of its event. Therefore, these variants have potential to affect gene dosage and could be related to expression of important beef quality phenotypes. The current knowledge about structural variants affecting complex traits, such as beef quality traits, is still unclear. Thus, the investigation of structural variant effects in splice sites could contribute to undertand of differential gene expression patterns.

SNV and insertion/ deletion (Indel) are primarily detected from DNA sequencing such as whole-genome, but RNA-seq is the most popular sequencing application as it contains much richer genomic information (Prodduturi et al., 2018). The sequencing of paired-end reads became more common, and they allow better alignment to the reference to allowing the

detection of small copy number variants (CNV) and the genome-wide characterization of breakpoints for all classes of structural variants (SV). Approaches focused to identify clusters of aberrantly mapped read pairs, suggesting the presence of SV breakpoints between the reads (Korbel et al., 2007), this provided several methods to identify different types of structural variants (Chen et al., 2009; Rausch et al., 2012; Tattini et al., 2015; Elyanow et al., 2018). In Cattle, studies have been focused on single nucleotide polymorphisms, small insertions, deletions and CNVs. However, studies investigate large insertions or deletions, tandem duplications, translocations, and inversions have been less common, despite their important impact to phenotypes (Boussaha et al., 2015).

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CHAPTER 2 - USE OF GENE EXPRESSION PROFILE TO IDENTIFY POTENTIALLY RELEVANT TRANSCRIPTS TO MYOFIBRILLAR FRAGMENTATION INDEX TRAIT[a]

ABSTRACT - The myofibrillar fragmentation index (MFI) is an indicative trait for meat tenderness. *Longissimus thoracis* muscle samples from the 20 most extreme bulls (out of 80 bulls set) for MFI (high (n=10) and low (n=10) groups) trait were used to perform transcriptomic analysis, using RNA Sequencing (*RNA-Seq*). An average of 24,616 genes were expressed in the Nellore muscle transcriptome analysis. A total of 96 genes were differentially expressed (p-value ≤0.001) between the two groups of divergent bulls for MFI. The *HEBP2* and *BDH1* genes were overexpressed in animals with high MFI. The *MYBPH* and *MYL6*, myosin encoders, were identified. The differentially expressed genes were related to increase of mitochondria efficiency, especially in cells under oxidative stress conditions. Those also were related to zinc and calcium binding, membrane transport, muscle constituent proteins, such as actin and myosin. Most of those genes were involved in metabolic pathways of oxidation-reduction, transport of lactate in the plasma membrane, muscle contraction. This is the first study applying MFI phenotypes in transcriptomic studies to identify and understand differentially expressed genes for beef tenderness. These results suggest that differences detected in gene expression between high and low MFI animals are related to reactive mechanisms and structural components of oxidative fibers under the condition of cellular stress. Some genes may be selected as positional candidate genes to beef tenderness, were *MYL6, MYBPH, TRIM63, TRIM55, TRIOBP* and *CHRNG* genes. The use of MFI phenotypes could enhancer results of meat tenderness studies.

Keywords: *Longissimus thoracis*, meat quality, MFI, Nellore.

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CAPÍTULO 2 - USO DO PERFIL DE EXPRESSÃO GÊNICA PARA IDENTIFICAR TRANSCRIÇÕES POTENCIALMENTE RELEVANTES PARA O TRAÇO DO ÍNDICE DE FRAGMENTAÇÃO MIOFIBRILAR

RESUMO - O índice de fragmentação miofibrilar (MFI) é uma característica indicativa da maciez da carne. Amostras do músculo *Longissimus thoracis* de 20 animais extremos (população= 80 touros) para MFI (alto (n = 10) e baixo (n = 10) groupos) foram utilizados para realizar a análise de genes diferencialmente expressos (DE), usando dados de sequenciamento de RNA (RNA-Seq). Foram identificados 24.616 genes sendo expressos do músculo de bovinos da raça Nelore. Um total de 96 genes foram diferencialmente expressos (p-valor < 0,001) entre os grupos divergentes para a característica MFI. Os genes *HEBP2* e *BDH1* foram superexpressos em animais com alto MFI. Foram DE,os genes *MYBPH* e *MYL6*, que são codificadores de miosina. Os genes diferencialmente expressos foram relacionados ao aumento da eficiência das mitocôndrias, principalmente em células sob estresse oxidativo. Esses também foram relacionados com à ligação de zinco e cálcio, transporte de membrana, proteínas constituintes do músculo (e.g. actina e miosina). Além disso, a maioria dos genes DE estiveram presentes em vias metabólicas de redução- oxidação, transporte de lactato na membrana plasmática e contração muscular. Esses resultados sugerem que as diferenças de expressão gênica detectadas entre os animais com alto e baixo MFI estão relacionadas a mecanismos reativos de componentes estruturais de fibras oxidativas sob a condição de estresse celular. Alguns genes podem ser selecionados como genes candidatos à maciez da carne bovina: *MYL6, MYBPH, TRIM63, TRIM55, TRIOBP* e *CHRNG* genes. Este é o primeiro estudo que utiliza fenótipos de MFI, como medida indicativa de maciez da carne para estudar o transcriptôma muscular de bovinos Nelore.

Palavras-chave: *Longissimus thoracis*, MFI, Nelore, qualidade de carne.

2.1 INTRODUCTION

A series of important aspects for consumers, such as visual, sensory, nutritional traits, food safety and animal welfare, have been considered for meat quality evaluation (Troy et al., 2010). Among these aspects, meat tenderness is one of the most valued trait in the meat market (Boleman et al., 1997; Miar et al., 2014).

The myofibrillar fragmentation index (MFI) is one of the methodologies used to evaluate the meat tenderness. This trait has been frequently used due to its precision in predicting meat tenderness (Hopkins et al., 2000) and, high correlation with tenderness, evaluated by the "Warner-Bratzler shear force" (WBSF). Phenotypically, MFI and WBSF are meat tenderness measurement proportionally inverse, i.e. when there is an increase in MFI, the shear force decreases (Olson et al., 1976). Olson & Parrish (1977) found a high coefficient of correlation (-0.78) between MFI and WBSF. Other authors also found similar results (Culler et al., 1978; Malheiros et al., 2015). The MFI has applicability, especially in cases where the muscle samples are not large enough for using shear force method (Veiseth et al., 2001), or to measure tenderness in young animals without slaughtering. For MFI analysis, a bit of muscle sample (3 grams) is necessary, which could be obtained by biopsy (Cervieri et al., 2005). Thus, we reduce the costs of tenderness phenotype measurements, which decreases generation interval and improves genetic gain.

Molecular studies have contributed greatly to the knowledge of complex traits, such as tenderness (Magalhaes et al., 2016; Braz et al., 2019; Silva et al., 2020), revealing genes and their mechanisms in the transcriptional and transductional levels. The RNA sequencing (RNA-Seq) technologies has contributed to the molecular genetic mechanism elucidation involved in the meat quality expression (Tizioto et al., 2013; Ramayo-Caldas et al., 2014; Tizioto et al., 2015; Cesar et al., 2015; Berton et al., 2016).

RNA sequencing generates millions of base pair information in a single step (Jin et al., 2012), which increase to the accuracy for identification of candidate genes, signaling pathways, differentially expressed genes and mRNA isoforms quantification. This technique have been improving transcriptomic studies (Tang et al., 2011; Cánovas et al., 2017; Cardoso et al., 2018), when compared to other techniques, especially for complex traits studies, as like meat tenderness (Lee et al., 2012; Tizioto et al., 2016; Fonseca et al., 2017). Zhang et al. (2011) investigated the gene expression profile of the *Longissimus dorsi* muscle in Qinchuan cattle (non-castrated males and females) at 36-months of age using *Bovine Genome Array*.

They found 142 differentially expressed genes related predominantly to the organization and collagen fibrils synthesis, regulating cell growth and development, and striated muscle contraction.

Differential gene expression analysis has been performed to several traits in cattle (McCabe et al., 2012; Yang et al., 2016; Fonseca et al., 2020) including beef tenderness (Fonseca et al., 2017). However, these studies have not used MFI phenotypes and RNA-Seq data for investigating beef tenderness in cattle. Thus, this study aim was to identify differentially expressed genes in *Longissimus thoracis* muscle tissue of Nellore bulls, phenotypically divergent for myofibrillar fragmentation index (MFI) trait using RNA-Seq data.

2.2 MATERIAL AND METHODS

2.2.1 Animal information and tissue sampling

Twenty animals extreme for MFI trait ((N=10 low) and (N=10 high) groups) were selected from an 80 Nellore bull set, belonging to the Nellore Qualitas breeding program. The animals belonged to the same contemporaries group, i.e. these animals were born in the same year, herd, and management system. The animals were slaughtered in a commercial slaughterhouse.

Longissimus thoracis muscle samples were collected between the 12th and 13th ribs of the left half carcass. Two samples were obtained at two time points: one sample at the time of slaughter, for RNA extraction, and another sample, 24 hours after slaughter, for MFI evaluation.

Student's t-test was used to verify the phenotype difference between the groups. The group of animals with low MFI had a mean value of 15.74 ± 2.14 and the animals with high MFI had an average of 56.16 ± 6.87. The groups were statistically different for the T test (p-valor = $2.69e^{-07}$).

2.2.2 Determination of myofibrillar fragmentation index (MFI)

The MFI determination was performed on samples without maturation, according to the methodology described by Culler et al. (1978), adapted by Protein Biochemistry Laboratory-

Chemistry and Biochemistry Department (IB) - UNESP, Botucatu, SP. We used 3 g of muscle sample from each animal evaluated. The samples were homogenized twice for 30 seconds in Ultra-turrax with shear stem (Marconi-MA102 / E), at 18,000 rpm, in 30 ml of myofibrillar fragmentation index buffer (TMFI) at 2 °C (100 mM KCl, 20 mM potassium phosphate, 1 mM EDTA, 1mM $MgCl_2$,1mM NaN_3, pH 7.0), with 30 seconds in ice between the first and second homogenization. After homogenization, samples were centrifuged at 1000Xg for 15 minutes at 2 °C, and the supernatant were discarded. The pellets were suspended in 30 ml of TMFI at 2 °C and homogenized with glass stick, centrifuged and supernatants were discarded. The pellets were suspended again in 7.5 ml of TMFI at 2 °C, subjected to the vortex until the samples became quite homogeneous and filtered on a polyethylene filter with a mesh of approximately 1 mm (Titan2 HPLC filter white – Thermo Fisher Scientific, Santa Clara, CA, USA, 2007). A total of 7.5 ml of TMFI at 2 °C was added in the filtered contents for washing the centrifuge tube and assisting in the filtration.

The Macro Biuret method (Gornall et al., 1949) was used to quantify myofibrillar proteins. The samples were homogenized by vigorous shaking and then the absorbance readings were taken at 540 nm wavelength in a spectrophotometer.

In the determination of MFI, samples were prepared with TMFI for a final volume of 8 ml and protein concentration of 0.5 mg / ml. Samples were homogenized by vigorous shaking and then read at 540 nm wavelength in a spectrophotometer. The spectrophotometer was calibrated having the TMFI solution as the blank. The MFI value was obtained by the following calculation: *MFI = Absorbance X 200.*

2.2.3 Total RNA extraction, concentration and integrity

RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used for the total RNA extraction, according to the manufacturer's recommendation. The RNA samples` purity were evaluated by determining at A260/230nm and A260/280nm ratios in a NanoDrop 1000 Spectrophotomether (Thermo Fisher Scientific, Santa Clara, CA, USA, 2007). The RNA samples integrity were assessed in the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA, 2009), and the RNA concentration and genomic DNA contamination were determined by Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA, 2010). All the parameters analyzed were considered satisfactory.

2.2.4 RNA sequencing, sequence processing, and alignment

Total RNA, from each sample, was used for cDNA library assembly, according to the protocol described in TruSeq RNA Sample Preparation kit® v2 guide (Illumina, San Diego, CA, USA). The libraries were quantified using a KAPA Library Quantification kit® (KAPA Biosystems, Foster City, CA, USA), according to Illumina's library quantification protocol. Sequencing (RNA-Seq) was performed on the HiSeq 2500 System platform (Illumina). Libraries were pooled to perform multiplexing sequencing process. Each molecule was sequenced at both ends (pair-end sequencing) to generate 100-base pairs end reads. We used the Cyverse platform (Goff et al., 2011) to perform the sequencing data analysis. The low-quality sequenced fragments were trimmed through the Sickle program (github.com/najoshi/sickle). The TopHat2 v2.0.9 program (Trapnell et al., 2009) was used to map the fragments, aligning with the bovine genome reference (UMD3.1) available in the NCBI database. For each library, a “. bam" file containing the alignment of the fragments was generated in relation to the genome reference.

2.2.5 Assembly and quantification of transcripts

The Cufflinks2 v2.1.1 program (Trapnell et al., 2012) was used to assemble the aligned fragments of each sample, as well as to estimate the number of transcripts in fragments per kilobase of transcript per million mapped fragments (FPKM). The results obtained, by sample, were concatenated in a single file through the program Cuffmerge2 v2. 1.1, which was used as reference to the differential gene expression test.

2.2.6 Differential gene expression test

Using the Cuffdiff2 v 2.1.1 program (Trapnell et al., 2012), the generated alignment files (“. bam") were separated into two contrasting groups for MFI. The FPKM values were obtained for each transcript to each sample. The corrections for false positive rates (FDR) were performed using the Benjamini-Hocheberg methodology (Benjamini and Hochberg, 1995), considering q-value for FDR less than 5%. The CummeRbund package (Trapnell et al.,

2012), implemented in R environment was used for exploration and visualization of the obtained data.

2.2.7 Enrichment analysis of differentially expressed genes

The Panther Classification System and Gene Ontology Consortium database was used (Ashburner et al., 2000) to annotate and interpret differentially expressed gene lists, additionally to determine the most relevant biological Pathways. The algorithm "Functional classification viewed in gene list" was used to annotate functional groups. Metabolic pathways involved with differentially expressed genes were mapped. The GeneMania (Warde-Farley et al., 2010) plugin of the Cytoscape program (Bindea et al., 2009) was used to generate networks of genes functionally grouped by non-redundant biological terms.

2.3 RESULTS

2.3.1 Processing, alignment, assembly of sequences and transcript quantification

An average of 530 million reads were obtained with 63X sequencing coverage (coverage for all transcripts of all the samples). The average reads per sample were 26.5 million and 90.2% were mapped in the genome reference. Detailed information can be found in Supplementary Table 1. A total of 24.616 genes were expressed in the *Longissimus thoracis* muscle of Nellore cattle.

The FPKM (normalizes and transformed values), box-plot graph and the plot of principal component analysis (PCA) (Figure 1 supplementary) were using to verify the quality of transcript distribution for each gene expressed. The quartiles distribution was uniform for both groups indicating good quality. In addition, the PCA showed the formation of different groups (high and low MFI), indicating differences in the expression of genes between the groups.

2.3.2 Differentially expressed genes analysis

In this analysis, 96 differentially expressed genes (q-value ≤ 0.05) were found. From those, 91 have known functions (Table 1). We verified 42 down regulated and 54 up regulated expressed genes in high MFI animals in relation to low MFI.

Table 1. Differentially expressed genes in the muscle of Nellore bulls, phenotypically distinct for myofibrillar fragmentation index (MFI)

Gene	Locus	FC(log2)*	p-value	q-value
SYT4	24:12950390-12960186	878.973	$5.00e^{-05}$	0.0092
BARX2	29:33240587-33255647	304.005	$5.00e^{-05}$	0.0092
PITX1	7:48063870-48069063	281.311	$5.00e^{-05}$	0.0092
RUNX1	1:148678709-148773773	239.155	$5.00e^{-05}$	0.0092
LBP	13:67874473-67910095	167.233	$5.00e^{-05}$	0.0092
PRR32	X:10981500-10983415	156.059	$5.00e^{-05}$	0.0092
GEM	14:72386750-72399660	137.505	$5.00e^{-05}$	0.0092
ITGB1BP2	X:84657970-84661552	0.733301	$5.00e^{-05}$	0.0092
TRIM55	14:32164441-32210852	0.707767	$5.00e^{-05}$	0.0092
MYL6	5:57486016-57489133	0.698886	$5.00e^{-05}$	0.0092
CHRND	2:120975091-120982350	132.432	$5.00e^{-05}$	0.0092
CHRNG	2:120988438-120993968	130.317	$5.00e^{-05}$	0.0092
SESN3	15:15502202-15523079	130.103	$5.00e^{-05}$	0.0092
TECRL	6:81511553-81653990	0.934109	$5.00e^{-05}$	0.0092
MYBPH	16:760055-768350	0.931705	$5.00e^{-05}$	0.0092
HOOK3	27:37278343-37358417	0.89023	$5.00e^{-05}$	0.0092
LIFR	20:35917478-35966671	0.874228	$5.00e^{-05}$	0.0092
MT2	18:24125365-24126333	0.860275	$5.00e^{-05}$	0.0092
DGAT2	15:55940756-55973229	0.825458	$5.00e^{-05}$	0.0092
MRPS6	1:669919-733729	0.823612	$5.00e^{-05}$	0.0092
MRC1	13:32544523-32644278	0.797647	$5.00e^{-05}$	0.0092
CAB39L	12:19016367-19079171	128.642	$5.00e^{-05}$	0.0092
FHL2	11:9253233-9300671	124.394	$5.00e^{-05}$	0.0092
LPL	8:67481088-67511227	109.709	$5.00e^{-05}$	0.0092
HEBP2	9:77215759-77222555	103.639	$5.00e^{-05}$	0.0092
CPE	17:546397-697915	102.864	$5.00e^{-05}$	0.0092
GHRH	13:66863224-66872531	90.901	$5.00e^{-05}$	0.0092
POSTN	12:24241936-24276486	13.187	$5.00e^{-05}$	0.0092
SLC16A7	5:53987908-54214799	12.839	$5.00e^{-05}$	0.0092
ZBED6	16:1400456-1403399	0.999538	$5.00e^{-05}$	0.0092

Continued Table 1

Gene	Locus	FC(log2)*	p-value	q-value
HR	8:69930955-69946715	0.979508	$5.00e^{-05}$	0.0092
LUM	5:21037442-21044658	0.955615	$5.00e^{-05}$	0.0092
RAD50	7:23062885-23169956	-0.634854	$5.00e^{-05}$	0.0092
ULK1	17:46171843-46193233	-0.6571	$5.00e^{-05}$	0.0092
KLHL21	16:47645899-47659012	-0.665355	$5.00e^{-05}$	0.0092
AFF4	7:46082729-46161887	0.660531	$5.00e^{-05}$	0.0092
ABHD4	10:22045426-22056366	-0.676873	$5.00e^{-05}$	0.0092
NAMPT	4:47597859-47635332	-0.683264	$5.00e^{-05}$	0.0092
RAB31	24:42256080-42305185	-0.706755	$5.00e^{-05}$	0.0092
PRKAG3	2:107509451-107516981	-0.714751	$5.00e^{-05}$	0.0092
PON3	4:12441125-12478659	-20.764	$5.00e^{-05}$	0.0092
CYFIP2	7:71003539-71115619	-103.533	$5.00e^{-05}$	0.0092
PM20D2	9:61848878-61861891	-109.655	$5.00e^{-05}$	0.0092
FAM184A	9:32425507-32497610	-117.073	$5.00e^{-05}$	0.0092
CRHR1	19:46456087-46483532	-140.319	$5.00e^{-05}$	0.0092
SLC7A8	10:21521554-21573888	-153.688	$5.00e^{-05}$	0.0092
KRT80	5:27886966-27910785	-163.479	$5.00e^{-05}$	0.0092
NF-M	8:73147852-73152844	-199.964	$5.00e^{-05}$	0.0092
HOXC9	5:26188848-26191440	-0.79009	$5.00e^{-05}$	0.0092
ITIH4	22:48615396-48630387	-0.798015	$5.00e^{-05}$	0.0092
HOXC8	5:26180270-26182515	-0.817086	$5.00e^{-05}$	0.0092
AGTPBP1	8:80235082-80392851	-0.818661	$5.00e^{-05}$	0.0092
ARRDC2	7:5101427-5105930	-0.726726	$5.00e^{-05}$	0.0092
FLNB	22:43674719-43815706	-0.743482	$5.00e^{-05}$	0.0092
CBR3	1:150144148-150153934	-0.884829	$5.00e^{-05}$	0.0092
DLGAP4	13:66204670-66292988	-0.885528	$5.00e^{-05}$	0.0092
ENSBTAG00000032057	3:28623390-28623702	159.936	$5.00e^{-05}$	0.0092
ENSBTAG00000046838	X:115892939-116081425	111.529	$5.00e^{-05}$	0.0092
ENSBTAG00000047029	11:47050710-47051142	325.212	$5.00e^{-05}$	0.0092
SLC16A6	19:62486091-62496100	-0.97691	$5.00e^{-05}$	0.0092
SCD5	6:99233278-99410753	0.764976	0.0001	0.0162
ANKRD2	26:18627372-18636269	-0.905512	0.0001	0.0162
SLC16A1	3:30533844-30563298	0.74882	0.0001	0.0162
DUSP7	22:49379907-49385352	-0.556285	0.0001	0.0162
SUOX	5:57639565-57643834	-0.669274	0.0001	0.0162
TRIOBP	5:110040662-110092020	-0.694815	0.0001	0.0162
SH3PXD2A	26:24413185-24469653	-0.588317	0.0001	0.0162
KLF15	22:61283171-61292866	-0.717645	0.0001	0.0162

Continued Table 1

Gene	Locus	FC(log2)*	p-value	q-value
SPOCK2	28:28304693-28329730	-0.923014	0.0001	0.0162
RNASE6	10:26402506-26404000	0.910141	0.0001	0.0216
MLLT3	8:23748585-24032951	-0.756579	0.0001	0.0216
CHMP4C	14:83201748-83231069	-0.88059	0.0001	0.0216
CD163	5:102245936-102278444	0.937006	0.0001	0.0216
PELI1	11:62471608-62528075	0.768861	0.0001	0.0216
TRIM63	2:127615049-127630608	-0.655643	0.0001	0.0216
ENSBTAG00000037937	15:46854182-46858574	-0.848581	0.0001	0.0216
ENSBTAG00000047008	12:69574458-69575727	0.767438	0.0001	0.0216
GBE1	1:28658789-28905833	0.664809	0.0002	0.0281
ACSM3	25:18605634-18656582	186.674	0.0002	0.0281
C7	20:33549494-33606517	0.642577	0.0002	0.0335
DMPK	18:53760688-53769611	0.639189	0.0002	0.0335
SCARF1	19:23309861-23319829	-0.598547	0.0002	0.0335
MAFA	14:2426856-2427912	-0.669037	0.0002	0.0335
PLCD4	2:107298619-107321320	-0.56151	0.0003	0.0380
ANKRD9	21:68908373-68911835	-0.571735	0.0003	0.0380
RTN4IP1	9:43832804-43884018	0.680154	0.0003	0.0380
FNTB	10:77421463-77502096	-0.584983	0.0003	0.0380
RGS16	16:65128480-65134057	0.792994	0.0003	0.0380
S100B	1:148009650-148016981	0.56887	0.0003	0.0434
RHOBTB3	7:97345763-97436844	-0.57421	0.0003	0.0434
CRACR2B	29:50710075-50739409	-0.667196	0.0004	0.0475
DENND5B	5:78850998-78966082	0.697926	0.0004	0.0475
TUBA1A	5:30821250-30825700	0.697759	0.0004	0.0475
LNPEP	7:98825232-98878164	0.680663	0.0004	0.0475

*FC (log2) = Fold change estimates are considering high MFI group in relation to the low.

The myosin encoder` genes, *MYL6* (myosin light chain 6) and *MYBPH* (myosin binding protein H), as well as two E3 ubiquitin ligase encoders, *TRIM63* (Tripartite Motif Containing 63) and *TRIM55* (Tripartite Motif Containing 55*)* genes, and *CHRNG* (Cholinergic Receptor Nicotinic Gamma Subunit) gene (Table 1) were up regulated across high MFI in relation to low groups, except for the *TRIM63* gene that was down regulated. These genes are related directly with skeletal muscle functions and muscle constituents.

When we analyzed these five genes (*TRIM63, MYL6, MYBPH, TRIM55* and *CHRNG)* were observed different interactions among them, such as physical interactions (67%), co-

expression (13.50%), prediction (6.35%), co-localization (6.17%), pathways (4.35%), and genetic interactions (1.40%) and shared protein domains (0.59%). In the network (Figure 1), we considered just co-expression interaction links among these genes and their co-expressed genes. Thus, we have shown the contribution of each one of them for expression of the seven most significant muscle functions (FDR |<| 0.05) detected in this study.

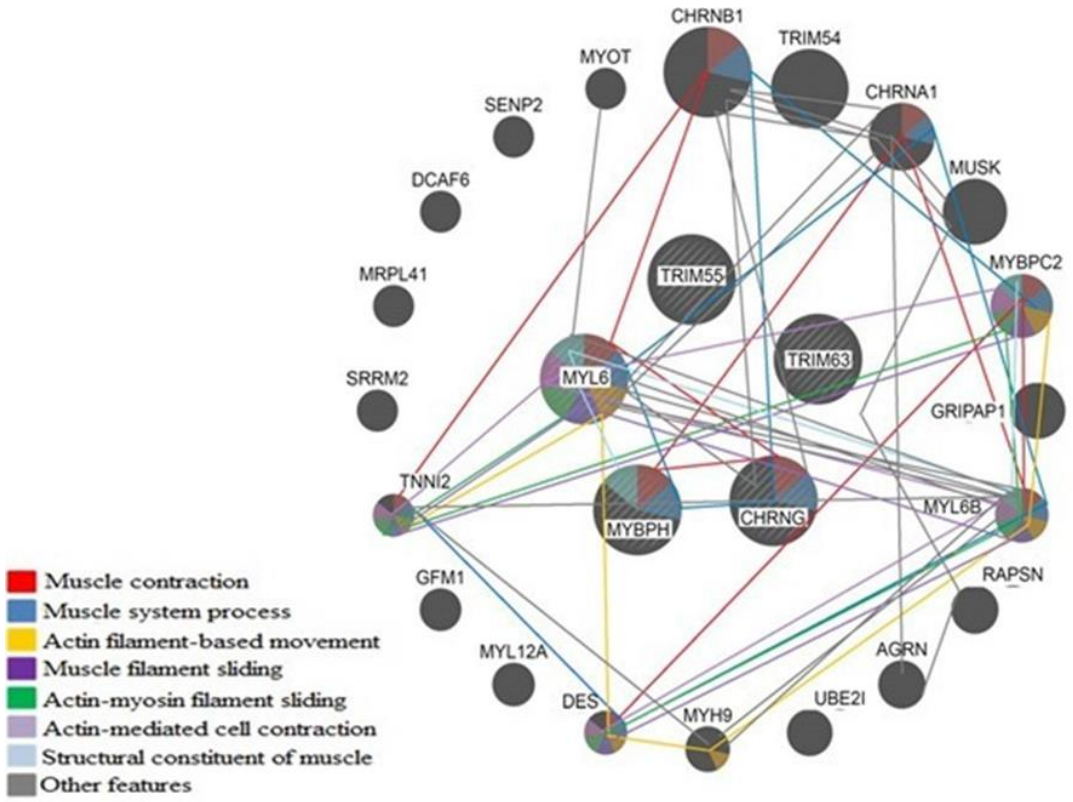

Figure 1. Integrative network of differentially expressed genes (*TRIM63, MYL6, MYBPH, TRIM55* and *CHRNG)* in muscle tissue of tender beef animals in relation to tough beef animals. DE genes inside circle and co-expressed genes form the outer circle.

The *TRIM63, MYL6, MYBPH, TRIM55* and *CHRNG* genes have been co-expressed with 213 genes across the bovine reference genome. All these genes work together in muscular system processes; also, they genes were linked with 219 genes associated specifically with muscle contraction mechanisms. Additionally, myosin encoders (not DE genes in this study), such as *MYL6B* (myosin light chain 6B) and *MYH9* (myosin heavy chain 9) are linked in this gene network. These genes are related to muscle function, structure and mechanisms (Zhang et al., 2006; White et al., 2016; Fisher et al., 2017), which could be key genes for muscle fiber degradation studies.

Functional annotation and enrichment analysis were performed on a list with all differentially expressed annotated genes (up and down regulated). These genes were playing in 15 pathways, which we considered for their functional classification (Table 2).

Table 2. Principal signaling pathways (p-value | <| 0.05) for differentially expressed genes in animals divergent for myofibrillar fragmentation index trait.

Accession	Pathway Name	Genes*
P04385	Histamine H1 receptor mediated signaling pathway	PLCD4
P04391	Oxytocin receptor mediated signaling pathway	PLCD4
P04394	Thyrotropin-releasing hormone receptor signaling pathway	PLCD4
P04374	5HT2 type receptor mediated signaling pathway	PLCD4
P04395	Vasopressin synthesis	CPE
P05726	2-arachidonoylglycerol biosynthesis	LPL
P00044	Nicotinic acetylcholine receptor	CHRNG CHRND
P00027	Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	RGS16
P06664	Gonadotropin-releasing hormone receptor pathway	PITX1 SYT4 TUBA1
P04380	Corticotropin releasing factor receptor	CRHR1
P00026	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	RGS16
P00046	Oxidative stress response	DUSP7
P00031	Inflammation mediated by chemokine and cytokine	PLCD4
P06959	CCKR signaling map	CPE
P00034	Integrin signaling pathway	FLNB

*Genes= genes from the input

2.4 DISCUSSION

Among the highest differential expression genes (p-value ≤ 0.0001), important genes related with processes of muscle transformation to meat, as the up-regulated genes, *FHL2* (Four and A Half LIM Domains 2), *HEBP2* (Heme Binding Protein 2), *BDH1* (3-Hydroxybutyrate Dehydrogenase 1) and *DGAT2* (Diacylglycerol O-Acyltransferase 2) were observed. They encode proteins from different families; however, all those are predominantly

present in the cytoplasm, where they contribute to mitochondrial efficiency and cytoplasm mechanism.

The *FHL2* gene encodes a member of the four-and-a-half-LIM-only protein family, and it is thought to have a role in the assembly of extracellular membranes. Hayashi et al. (2009) used *FHL2*, as candidate gene to identify a novel antiangiogenic factor from the human heart cDNA library. They related the expression of this gene in the vascular endothelial cells, where supposedly it may interact with *SK1* (Sphingosine Kinase-1), producing sphingosine1-phosphate (S1P), a bioactive sphingolipid that acts as a potent angiogenic mediator. Heme-binding protein 2 gene (*HEBP2*) encodes a protein that increases the permeability of the outer and inner mitochondrial membrane, especially under oxidative stress conditions, allowing an efficient transformation process of living tissues (Szigeti et al., 2010). One of signaling pathways found in this study was the oxidative stress response pathway. This occurs because of the imbalance between an oxidant molecule and an antioxidant, which induces bimolecular damage due to free radicals (Rahman, 2007). The *BDH1* gene encodes a short chain dehydrogenase/reductase (NCBI, 2020). Uchihashi et al. (2017) studying the overexpression of *BDH1* in the mice cardiac muscle to improve oxidative stress, reported that *BDH1* transgenic mice were resistant to fibrosis, contractile dysfunction, and oxidative damage. Therefore, this gene may be contributing to complementary functions of cellular control mechanisms involved in the transformation of the muscle to flesh/meat. The *DGAT2* gene encodes a protein involved in the triacylglycerol biosynthesis pathway. In bovine, this protein has been related to dietary fat uptake, lipid synthesis and storage (Winter et al., 2003; Stone et al., 2008; Li et al., 2009).

The down-regulated expressed genes, *AGTPBP1* (ATP/GTP Binding Protein 1), *DLGAP4* (LG Associated Protein 4) and *TRIOBP* (TRIO and F-Actin Binding Protein), as well as the up-regulated expressed genes *BARX2* (BARX homeobox 2) and *SUOX* (Sulfite Oxidase) have an important role in the functional and structural cell organization. It seems that these genes play together in muscle processes. The AGTPBP1 protein contains nuclear localization signals and an ATP/GTP-binding motif, and has metallocarboxypeptidase activity (Zhao et al., 2012). This activity is important to remove gene-encoded polyglutamates from the carboxy-terminus of target proteins, such as *MYLK* gene, which encodes a myosin protein (main muscle component). The DLGAP4 protein can be characterized as a signaling molecule and interacts with potassium channels and receptors as well as with other signaling molecules

(Wu et al., 2012). The TRIOBP protein is related to the actin cytoskeleton organization through the direct binding and stabilization of filamentous F-actin (Lee et al., 2018). The BARX2 protein is a homeobox transcription factor family member, which is related to control of cell adhesion and actin cytoskeleton remodeling in myoblast fusion and chondrogenesis (Stevens et al., 2004). The *SUOX* provides instructions for making an enzyme called sulfite oxidase (Jonhson, 2003). It seems that this set of genes are acting to facilitate the muscle structure development.

The genes *HEBP2, BDH1* and *SUOX* among other genes differentially expressed in this study were present in metabolic pathways that maximize the efficiency of mitochondrial activities (Szigeti et al., 2010). In addition, they integrate pathways of oxidation-reduction, transport of lactate, zinc and calcium bindings and metabolism of oxidative fibers. These processes are directly linked to the transformation of muscle to meat, and are critical to the ultimate tenderness of the meat.

The up-regulated genes, *TRIM55* (Tripartite Motif Containing 55), *SESN3* (Sestrin 3), *LUM* (Lumican), *CHRNG* (Cholinergic Receptor Nicotinic Gamma Subunit), *MYBPH* (Myosin Binding Protein H) and *MYL6* (Myosin Light Chain 6), as well as the down-regulated, *TRIM63* (Tripartite Motif Containing 63, *DUSP7* (Dual Specificity Phosphatase 7) and *FLNB* (Filamin-B), have an important role on the muscular mechanisms, which could be related to tenderness, among other meat quality traits (Krakow et al., 2004; Li et al., 2010; Zhang et al., 2012; White et al., 2016; Fisher et al., 2017).

The *TRIM55* gene product is temporarily associated with microtubules, myosin, and titin. This association occurs just during the assembly of the muscular sarcomere (Dixon et al., 2015). The CHRNG protein is an acetylcholine receptor of muscle type (Azim et al., 2012). The *MYL6* and *MYBPH* genes had induced expression in animals with high MFI. These genes encode a myosin alkali light chain and myosin binding protein H that are playing in the cellular motor mechanisms and muscle structural constitution, respectively (Li et al., 2010; Park et al., 2011). The myosin are the most abundant proteins in the muscle (Deshmukh et al., 2015), and play a key role in muscle growth and contraction. The myosin heavy chain isoforms are responsible for the muscle contractile properties (Asmussen et al., 2003).

The *TRIOBP*, *BARX2* and *FLNB* genes encode implicated proteins in the actin cytoskeleton formation. The cytoskeleton contributes to the muscle contraction, where it is sliding the filaments of myosin over those of actin. In addition, important up-regulated genes

related to collagen fibrils (*LUM* gene) and hormone receptors (*FHL2* gene), as well as the down-regulated, zinc binding gene (*AGTPBP1*) were differentially expressed. These proteins are important for construction of different muscle types; differences in animals' growth rates, as well as in humans, depend of different types of muscle fibers (Baillie and Garlick, 1991). Skeletal muscle is formed by myofibrils, which are composed for actin filaments (thin filaments) and myosin filaments (thick filaments) arranged longitudinally, organized by several cytoskeleton proteins in a symmetrical and parallel form (Luther, 2009). It is suggesting an interaction among these genes and constituent proteins of muscle, the genetic segregation among those could regulate to different muscle types, which could be associated to meat tenderness.

The *TRIM63* gene encodes an E3 ubiquitin ligase, which is a necessary component for ubiquitin proteasome formation (Myung et al., 2001). It is located in the network formed by line Z and M of myofibrils, and works as marks for cells to be degraded. This protein is required to the degradation of heavy and light myosin chain proteins, binding myosin protein, and creatine kinase in the muscle (Steinberg, 2013). Moreover, this gene also plays an important role in skeletal and cardiac muscle atrophy (Bodine and Baehr, 2014).

The functional analysis was performed on a list of DE genes using Gene Ontology. Here, we were identified molecular process and pathways involved in oxidation process, such as plasma membrane lactate transport, calcium and zinc ion bindings, gonadotrophin and nicotinic acetylcholine receptors, corticotropin releasing factor receptor, vasopressin synthesis, G protein heterotrimer (alpha Gi, Gs, Gq and Go pathways). These pathways and molecular processes are necessary for muscle transformation to meat (Listrat et al., 2016).

The enrichment analysis revealed that these genes were related with external and internal mitochondrial membrane permeability increase, especially in cells under oxidative stress conditions. In additional, were related to zinc binding and synthesis of triglycerides in its final reaction, when triacylglycerol is covalently linked to long chain fatty acyl-CoAs (Szigeti et al., 2010; Renaville et al., 2018; Lorda-Diez et al., 2018).

The structure and muscle composition, as well as the change kinetics that occur in muscular system of animals throughout of them life, vary according to species and breeds. In cattle, the relation between muscle fiber type and meat tenderness is rather complex, changing widely according to breeds; sex, age and muscle type (Ellis-Oury et al., 2013). Moreover, conditions associated to animal's management from birth to slaughter, and carcass processing

are essential for the good meat quality. According to Hocquette et al. (2014) muscle fiber types could change among of the different muscles in the body, it difficult to determinate the musculature pattern related specifically to beef tenderness. These authors reported a significant variability between *Longissimus thoracis* and *Semitendinosus* muscles of bulls, and oxidative fibers predominance into *Longissimus thoracis* muscle of cattle. Several meat tenderness studies (Gagaoua et al., 2015; Tizioto et al., 2016) identified many genes related to mitochondrial properties and oxidative stress. Fonseca et al. (2017) used WBFS measurement, as meat tenderness phenotypes, to identify differentially expressed genes in the same Nellore population used in this study. We did not find differentially expressed genes in common with this study; however, both studies identified genes were playing in common pathways, and it had similar functions, which were related to catalytic activity, enzymatic proteolysis activity, degradation of myofibrillar proteins, metabolic pathway as actin cytoskeletal organization and myosin. It is suggesting that WBSF and MFI phonotypes could be used as complementary measures to gene expression investigation for beef tenderness.

In a genome-wide association study (GWAS) performed to meat tenderness using the same database used in this study, Magalhães et al. (2016) reported that regions located in the chromosomes 5, 7, 10, 14 and 21 explained 3.80% of the additive genetic variance for tenderness in Nellore cattle. In our study, we observed that 28.12% of differentially expressed genes detected (Table 1) were located into these chromosomes. Same of those genes were directly related to muscle constituents, such as *MYL6* and *TRIM55* genes. In another meat tenderness GWAS study for Polled Nellore cattle were identified significant SNPs ($P< 0.0001$) in the chromosomes 15, 16, and 25 (Castro et al., 2017). Those authors related the identified genes with protein phosphorylation, protein serine/threonine kinase activity, calcium ion binding, and growth factors. These biological processes also are important to meat tenderness (Castro et al., 2014).

Most of the meat quality traits, especially meat tenderness (measured by WBSF or MFI methodologies) have been presenting significant genetic differences among bovine breeds (Xie et al., 2012). However, within breed the genetic variance are smaller, which have been challenge identifying best animals for complex trait, such as meat tenderness. Traditional selection is a very efficient approach in the animal improvement process; it also requires a large phenotypic data amount (van-Marle-Koster & Visser, 2018). Collecting data from many animals is time consuming and costly, mainly for traits such meat tenderness.

Thus, the knowledge of gene expression could be a tool that may aid metabolic pathways understanding and gene mechanisms act that regulates this trait (Fonseca et al., 2017). It may, possibly contribute to reduce the need for large datasets, mainly for traits that are measured after the animal has been slaughtered, such as meat tenderness, when evaluated by WBSF measurement.

2.5 CONCLUSION

The DE gene identified in this study were closely related to biochemical processes involved in muscle transformation to meat. Among the genes may be selected as positional candidate genes to beef tenderness, are myosin encoders (*MYL6* and *MYBPH*), and tripartite motif encoders (*TRIM63* and *TRIM55), TRIOBP* and *CHRNG* genes. These genes encode muscle specific proteins, or they are playing important roles to the muscle contraction, which is a paramount factor during the meat maturation process. This result indicate that meat tenderness may be controlled by specific genomic regions in this population of Nellore cattle.

This study suggests the use of MFI phenotypes as a potential indicative of beef tenderness. It could be used to perform transcriptomic investigates for meat tenderness trait, which could provide the discovery of new candidate genes for tenderness trait.

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2.7 SUPPLEMENTARY MATERIAL

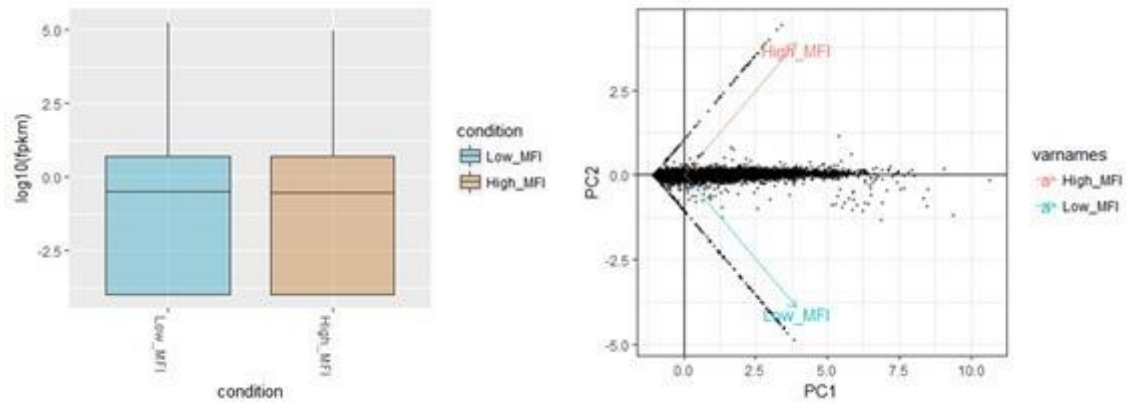

Figure S1. (a) Boxplot of the FPKM log10 of the expression values for the evaluated groups. (b) Principal components analysis (PCA) of the transcripts found in the groups with high MFI (Red) and low MFI (Blue).

Table S1. Sample identification (ID), phenotype of the sample (Phenotype). MFI value number of aligned transcripts in pairs (N Reads) and percentage of transcripts aligned in pairs (% of reads).

ID	Phenotype	MFI	N Reads	(%) of reads
5042	High	42.8	14,097,711	82.3
5078	High	43.4	31,369,007	92.0
5019	High	46.1	11,498,431	82.3
5039	High	47.9	28,302,040	91.0
5045	High	50.0	23,103,200	90.5
5020	High	53.9	24,469,269	90.7
5052	High	55.5	27,921,731	91.8
5103	High	57.9	29,142,454	92.9
5046	High	65.8	31,536,799	90.7
5054	High	68.1	27,916,395	91.6
Average group	High	53.14	24,935,704	89.58
5064	Low	13.8	32,740,303	91.9
5119	Low	15.9	31,737,792	92.0
5034	Low	19.2	23,704,428	91.0
5068	Low	19.3	29,078,147	91.8
5062	Low	19.9	31,400,341	92.0
5005	Low	20.3	32,131,703	91.1
5097	Low	20.6	26,676,373	90.9
5117	Low	22.2	27,973,797	92.1
5050	Low	23.8	14,265,931	82.5
5026	Low	24.3	31,126,706	92.1
Average group	low	19.93	28,083,552	90.74

CHAPTER 3 - IDENTIFICATION OF NOVEL mRNA ISOFORMS ASSOCIATED WITH MEAT TENDERNESS USING RNA-SEQUENCING DATA IN NELLORE CATTLE

ABSTRACT - The Warner-Bratzler shear force (WBSF) and myofibrillar fragmentation index (MFI) are complementary methodologies commonly used to measure beef tenderness. To identify mRNA isoforms differentially expressed became an important tool to provide new insights to better understand the transcripts involved in the regulation of the meat tenderness. Longissimus thoracis muscle samples from the 20 most extreme bulls (out of 80 bulls set) for WBSF (tender (n=10) and tough (n=10) groups) and MFI (high (n=10) and low (n=10) groups) traits were collected to perform transcriptomic analysis using RNA-Sequencing. The CLC Genomics Workbench was used to align the fragments against the bovine reference genome ARS.UCD1.2 using the large gap mapping tool approach of each sample and differential expression analysis (DE). An average of 113,471 transcripts were expressed in the Nellore muscle transcriptome. A total of 40 and 28 mRNA isoforms (p-value |<| 0.001) were differentially expressed between divergent groups for WBSF and MFI phenotypes, respectively. The differentially expressed mRNA isoforms [WBSF (MYL1, MYL3, MYH1 and MYBPC2) and MFI (MYL6) traits], which are myosin encoders, was the family with most abundantly number of mRNA isoforms detected as differentially expressed. In addition, we identified as DE [WBSF (SYNPO-201), [MFI (ANKRD23-202) isoforms], and other mRNA isoforms related to genes affecting the speed fibers degradation during the meat aging process, such as [WBSF (MYOT, CASQ1, TPM2, MB and SYNM), and MFI (FGFRL1 and NEB)]. The DE mRNA isoforms are transcripted by genes with biological functions related to oxidative process, energy production, striated muscle contraction, HIF-1 signaling pathway and metabolism of amino acids and derivatives. The results suggest that the identified mRNA isoforms could be used as potential candidate regions to select animals in order to improve tenderness in beef cattle.

Keywords: maturation, MFI, novel transcripts, RNA-Seq, WBSF

CAPÍTULO 3 - IDENTIFICAÇÃO DE NOVAS ISOFORMAS de mRNA ASSOCIADA A MACIZ DA CARNE USANDO DADOS SEQUENCIAMENTO DE RNA EM BOVINOS NELORE

RESUMO - A força de cisalhamento Warner-Bratzler (WBSF) e o índice de fragmentação miofibrilar (MFI) são metodologias complementares comumente usadas para medir a maciez da carne bovina. Nesse estudo foram utilizadas amostras do músculo *Longissimus thoracis* de 20 animais extremos para os fenótipos de WBSF (grupos macia (n = 10) e dura (n = 10)) e MFI (alta (N = 10) e baixa (N = 10)) grupos), as amostras foram selecionados a partir de um conjunto amostral composta por 80 animais. Além disso, foram utilizados dados de RNA-sequencing (RNA-Seq), os quais foram processados e analizados através de ferramentas de transcriptomica implementado na plataforma CLC Genomics. Assim, os fragmentos de cada amostra foram alinhados ao genoma bovino de referência (ARS.UCD1.2). Nesse estudo, foram encontrados uma média de 113.471 transcritos sendo expressos no músculo *Longissimus thoracis* de animais Nelore. Foram identificados 40 e 28 isoformas de mRNA (P-valor < 0,001) diferencialmente expressas para os fenótipos de WBSF e MFI, respectivamente. Além disso, identificamos como isoformas DE [WBSF (*SYNPO-201*) e MFI (*ANKRD23-202*)], entre outras isoformas de mRNA relacionadas a genes que afetam a degradação das fibras de velocidade durante o processo de envelhecimento da carne, como [WBSF (*MYOT, CASQ1, TPM2, MB* e *SYNM*) e MFI (*FGFRL1* e *NEB*)]. Na análise de enriquecimento, esses genes foram associados com funções biológicas relacionadas ao processo oxidativo, produção de energia, contração muscular estriada, via de sinalização de HIF-1 e metabolismo de aminoácidos e derivados. Portanto, esses resultados sugerem que as isoformas de mRNA identificadas podem ser possíveis regiões candidatas para a seleção de animais com carne macia.

Palavras- chave: mRNA isoformas, MFI, novos transcritos, RNA-Seq, WBSF

3.1 INTRODUCTION

RNA sequencing (RNA-Seq) technology has been of paramount importance to improve the accuracy of results in transcriptional studies, contributing to elucidate molecular mechanisms involved in the expression of complex traits (Cánovas et al., 2014a; Wickramasinghe et al., 2014; Silva et al., 2020; Fonseca et al., 2020). Although various gene expression studies have been carried out for complex traits in livestock animals (Tizioto et al., 2016; Berton et al., 2016; Fonseca et al., 2019), studies focusing on the investigation of mRNA isoforms in beef cattle are still scarce in the literature. In this context, using gene expression studies based on RNA-Seq to investigate mRNA isoforms in beef cattle can help to identify the biological mechanisms and key genes associated with meat tenderness. In pigs, Cardoso et al. (2018) reported that 10.9% of transcripts expressed in skeletal muscle of Duroc animals were produced through splicing alternative process, creating different mRNA isoforms from a specific region in the genome.

Meat tenderness measurements have usually been taken using the Warner-Bratzler shear force (WBSF) method (e.g., Tizioto et al., 2013; Tizioto et al., 2016; Fonseca et al., 2019). Similarly to WBSF, the myofibrillar fragmentation index (MFI) can also be used to evaluate meat tenderness. While WBSF is a mechanical measurement, MFI is based on enzymatic proteolysis quantification. This last measure is easier to obtain than WSBF since it requires a smaller meat sample (only 3 grams of meat), which can be collected by biopsy, without slaughtering the animals. Nevertheless, the correlation between phenotypes measured based on both approaches is usually high (Hopkins et al., 2000; Olson et al., 1976). For instance, Olson & Parrish (1977) estimated high negative correlation coefficient (-0.78) between measurements made using MFI and WBSF, which is similar to results reported by other authors (Culler et al., 1978; Malheiros et al., 2015).

In this context, the main hypothesis of the current study is that these different tenderness phenotypes show different expression patterns. Henceforth, the goal of this study was to identify novel mRNA isoforms differentially expressed in Longissimus thoracis tissues of Nellore cattle, using tenderness phenotypes measured using both WBSF and MFI methods. In addition, mRNA isoforms found in this study were integrated to different functional analysis in order to identify active metabolic pathways and transcripts involved in the meat maturation process.

3.2 MATERIAL AND METHODS

3.2.1 Samples collection and phenotype measurements

Animals were selected from a population of 80 Nellore bulls belonging to the Capivara farm located in the São Paulo state, Brazil, which participated in the Nellore Qualitas breeding program. All 80 Nellore bulls belonged to the same contemporary groups, i.e. they were born in the same year and herd, and they were kept in the same management group from birth to yearling. Animals were slaughtered with an average age of 24 months, in a commercial slaughterhouse (Fonseca et al., 2017).

The evaluation of tenderness using the WBSF method was performed using the mechanical salter Warner- Bratzler Shear Force device, as proposed by Bratzler (1949). The evaluation of tenderness using the MFI method was performed as described by Culler et al. (1978). In this context, the Macro Biuret method (Gornall et al., 1949) was used to quantify myofibrillar proteins. The samples were homogenized by vigorous shaking and then read in the spectrophotometer at 540 nm wavelength. The spectrophotometer was calibrated using the Tampan myofibrillar fragmentation index (TMFI) solution as blank. The final MFI value was calculated as MFI = Absorbance X 200.

Student's t-test was used to verify the statistical difference between groups. Average (and SD) values for MFI were 15.74 (2.14 – tough group) and 56.16 (6.87 – tender group). Based on the Student's t-test, both MFI groups were statistically different (p-valor = $2.69e^{-07}$). Average (and SD) values for WBSF in the tender and tough groups were 4.11kgf (0.30) and 8.93kgf (1.23), respectively. The WBSF groups were also statistically different based on the t-test (p-valor = $2.58\ e^{-07}$).

Samples of the *Longissimus thoracis* muscle were collected between the 12th and 13th ribs of the left half carcass from the 20 most divergent Nellore bulls for both WBSF and MFI phenotypes [i.e., one group of tough (n=10) and one group of tender (n=10) for each trait; 40 animals potentially selected in total]. Two samples were collected at two different times: one sample at the time of slaughter (used for RNA extraction), and another sample 24 hours after slaughter (used for evaluation of tenderness using the WBSF and MFI methods).

3.2.2 RNA isolation and library construction

The RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia-CA, USA) was used to extract the total RNA from 80 muscle samples of Nellore bulls set used in this study, according to the manufacturer's recommendation. The purity of the RNA samples was determined at A260/230nm and A260/280nm ratios using the NanoDrop 1000 Spectrophotomether (Thermo Fisher Scientific, Santa Clara, CA, USA, 2007). The integrity of the RNA samples was assessed in the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA, 2009), and the RNA concentration and genomic DNA contamination were determined using the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA, 2010) (Fonseca et al., 2017). RNA integrity number (RIN) values were higher than 7.0 for all muscle samples, indicating good RNA quality (Cánovas et al., 2013).

Total RNA content from each sample was used to prepare the cDNA libraries using the TruSeq RNA Sample Preparation kit® v2 guide (Illumina, San Diego, CA, USA), following the manufacturer's recommendation. Sequencing libraries were quantified using the KAPA Library Quantification kit® (KAPA Biosystems, Foster City, CA, USA), according to Illumina's library quantification protocol. Paired-end (2 x 100 pb) reads were sequenced using the HiSeq 2500 sequencer (Illumina, San Diego, CA, USA).

3.2.3 Sequence assembly and quantification

Quality control analysis were performed using the CLC Genomics Workbeach software 12.0 (CLC Bio, Aarhus, Denmark), following the parameters described in Cánovas et al. (2014b). The “Large Gap Read Mapping” tool was used to map the paired-end sequence reads according to the annotated reference genome ARS.UCD1.2 (ftp://ftp.ensembl.org/pub/release-95/genbank/bos_taurus/). In summary, this tool allows to map the RNA-Seq reads that span introns without requiring prior transcript annotations, which gave us support to perform the transcript discovery analysis.

The transcript discovery analysis were performed using the “Transcript Discovery” tool, which uses an algorithm that takes large gap reads mapping tracks. This algorithm uses gene and transcript annotations as input, and produces optimized gene and transcript track annotations as outputs (Cardoso et al, 2018). Thus, for each gene region, there is a set of

transcript annotations that can explain the observed exons and splice sites in this region (Cardoso et al, 2018).

Then, the RNA- Sequencing analysis tool was used to align the sequences of each sample against the created tracks (genes and transcripts tracks) using the bovine reference genome as map, which increase the accuracy of alignment, following these parameters: end-to-end with 0.8 length fraction [the minimum percentage of the total alignment length that must match the reference sequence at the selected similarity fraction] and 0.8 similarity fraction [the minimum percentage identity between the aligned region of the read and the reference sequence]; two mismatches; and three insertions and three deletions per read were allowed. In order to facilitate the comparisons groups, considering independently the MFI and WBSF criteria. The transcript levels were quantified in reads per kilobase per million mapped reads (RPKM), and transformed to log10 (Allison et al., 2006; Mortazavi et al., 2008). All the "Large Gap Read Mapping", "Transcript Discovery" and "RNA- Sequencing analysis" tools are also implemented in CLC Genomics Workbench environment (CLC Bio, Aarhus, Denmark).

3.2.4 Differential mRNA isoform expression

Differential mRNA isoform expression analyses were performed using the CLC Genomics Workbench software 12.0 (CLC Bio, Aarhus, Denmark). Thus, these empirical statistical analysis were performed using the follow parameters: read coverage > 5.0; estimate tag-wise dispersions; comparisons against the genome reference and large gap mapping tracks (considering extreme groups for each trait); and FDR correction for the p-value (Cardoso et al., 2018).

3.2.5 Functional enrichment analysis and gene annotation

Functional annotation was performed through the TopFun tool implemented in the ToppGene software website (https://toppgene.cchmc.org/), using GO: Biological Process and pathway analysis (Chen et al., 2009). The analysis was performed based on mRNA isoforms and annotated genes list. Biological processes and pathways significantly associated with the gene lists were determined based on their p-values and FDR (Ashburner et al., 2000). The GO

analysis was performed taking into account three GO categories: biological process, molecular function, and cellular component (Cánovas et al., 2012; Cardoso et al., 2018). Thresholds of p-value ≤0.01, FDR ≤0.05 and minimum of 5 genes involved in the process or pathway were applied to filter significant genes by function, pathway, and network effects (Cardoso et al., 2017).

To annotate novel mRNA isoforms of unknown genes, their coding sequence was obtained from the Genome Data Viewer tool, which is implemented in the National Center of Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/genome/gdv/browser/genome/?id=GCF_002263795.1). Thereafter, the 'Nucleotide BLAST' method available on the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to detect similarities between the nucleotide sequences from the novel mRNA isoforms and the nucleotide collection (nt) available in the database. The nt consists of GenBank, EMBL, DDBJ, PDB, and RefSeq sequences data, but it excludes EST, STS, GSS, WGS, TSA, patent sequences, phase 0, 1, and 2 HTGS sequences, and sequences longer than 100Mb. The database is non-redundant, i.e., identical sequences are merged into one entry (but it keeps the accession, GI, title and taxonomy information from each entry).

3.3 RESULTS

3.3.1 Sequence assembly and quantification

A total of 47.5% of the animals selected for WBSF were also selected for MFI (Supplementary Table S1 and S2). This could be expected since the genetic correlation estimated between these two traits, measured in a larger data set including our animals, was -0.76 (Supplementary Table S3).

The RNA-Seq generated an average of 48 million paired-end reads per sample, from which 88.65 % were mapped in pairs. In addition, 91.87% and 8.13% of the reads were mapped in genic and intergenic regions, respectively (Supplementary Table S1 and S2). From both differential expressed mRNA isoforms analysis performed, an average of 33,455 genes (read coverage > 5) were being expressed in the muscle tissues of Nellore cattle. These genes were producing an average of 113,471 transcripts, which correspond to an average of 3.4 transcripts per gene. From the total number of mRNAs found, 40 and 28 mRNA isoforms

were differentially expressed between groups (p-value < 0.001), for phenotypes measured using the WBSF and MFI methods, respectively.

3.3.2 Differential mRNA isoform expression

Using the *large gap read mapping* approach to search for differentially expressed mRNA isoforms (Table 1), a number of transcripts (113,471) was found in this study. The number of annotated mRNA isoforms found (WBSF= 2 and MFI= 7) was smaller when the number of novel transcripts detected (WBSF= 38 and MFI= 21).

A total of 72.5% and 46.42% of identified transcripts were novel mRNA isoforms related to known genes, for WBSF and MFI phenotypes, respectively. On the other hand, 22.5% and 28.57% were novel transcripts of likely non-annotated genes, for WBSF and MFI, respectively. Three types of mRNA isoforms were identified when comparing the tender and tough (or tough *vs* tender) groups used in this study: 1) annotated mRNA isoforms (2 found using WBSF and 7 using MFI); 2) novel transcript lengths with annotated genes (29 using WBSF and 13 using MFI); and 3) novel transcript lengths with no annotated genes (9 using WBSF and 9 using MFI).

Differentially expressed annotated mRNA isoforms

For WBSF, two known mRNA isoforms were differentially expressed between groups: *SYNPO-201* (synaptopodin) and *RPS7-201* (ribosomal protein S7). Both mRNA isoforms were significantly up-regulated (p-value <0.001; FC > 2) in the tough beef group compared to the tender. For MFI, seven annotated mRNA isoforms were differentially expressed. From these, four [i.e., *RGMB-201* (repulsive guidance molecule BMP co-receptor b), *ZBTB18* (zinc finger and BTB domain containing 18), *DCTN2* (dynactin subunit 2), and *NKD2* (NKD inhibitor of WNT signaling pathway 2)] were up-regulated in the tender beef group compared to the tough. However, three annotated mRNA isoforms [i.e., *RAB1A-201* (RAB1A, member RAS Oncogene Family), *SRGN-201* (Serglycin), and *ANKRD23-202* (Ankyrin Repeat Domain 23)] were down-regulated in the tender beef group compared to the tough (Table 1).

Table 1. Annotated mRNA isoforms identified as differentially expressed (p-valor < 0.01) between divergent groups using phenotypes measured based on the Warner-Bratzler shear force (WBSF) and myofibrillar fragmentation index (MFI) methods.

WBSF						
Gene ID	Transcript ID	Transcript name	Length (bp)	Position	p-value	Log2(FC) [1]
ENSBTAG00000013744.5	ENSBTAT00000018267.4	SYNPO-201	3734	7: 61971867-62026834	$6.11e^{-05}$	270.83
ENSBTAG00000016224.4	ENSBTAT00000084857.1	RPS7-201	849	8: 110987195-110992673	$9.94\ e^{-05}$	21.23
MFI						
Gene ID	Transcript ID	Transcript name	Length (bp)	Position	p-value	Log2(FC)[1]
ENSBTAG00000000385.6	ENSBTAT00000076393.1	ZBTB18-202	782	16:33152350-33155595	$6.32\ e^{-04}$	12.56
ENSBTAG00000034435.4	ENSBTAT00000048774.4	NKD2-201	1218	20:71307540-71311025	$9.78\ e^{-04}$	8.12
ENSBTAG00000011864.6	ENSBTAT00000015746.6	RGMB-201	1194	7:98014273-98032844	$3.08\ e^{-04}$	3.77
ENSBTAG00000010624.5	ENSBTAT00000014053.5	DCTN2-203	2054	5:55932943-55946889	$6.56\ e^{-04}$	2.95
ENSBTAG00000017863.5	ENSBTAT00000023743.5	SRGN-201	1166	28:25474774-25490574	$1.73\ e^{-04}$	-5.86
ENSBTAG00000016720. 6	ENSBTAT00000022227.6	RAB1A-201	1415	11:63471792-63500625	$3.05e^{-05}$	-19.54
ENSBTAG00000010849.6	ENSBTAT00000074804.1	ANKRD23-202	1146	2801346-2805847	$9.61\ e^{-04}$	-30.52

[1]Log2(FC)= Fold change (log2)

Differentially expressed mRNA isoforms (novel transcript length) with annotated genes

Novel differentially expressed mRNA isoforms with annotated genes for beef tenderness, evaluated using the WBSF and MFI methods, and were showed in Tables 2 and 3 [e.g. the alignment view of mRNA could be seen in the supplementary Figure S1 and S2], respectively. A total of 38 novel differentially expressed mRNA isoforms were found using WBSF, and 21 were found using MFI. Using the phenotypes measured based on the WBSF method, a significant number mRNA isoforms was observed to be related to myosin's encoders, e.g., *MYH1* (myosin heavy chain 1), *MYL1* (myosin light chain 1), *MYBPC2* (myosin binding protein C, fast type), and *MYL3* (myosin light chain 3). These mRNA isoforms were up-regulated in the tough beef groups compared to the tender beef groups (Table 2).

A total of 37 up-regulated mRNA isoforms were found in the tough beef groups compared to the tender beef animals when using the WBSF method to classify the samples. These mRNA isoforms were related to genes associated to cystoskeletal protein, skeletal muscle-specific member of the calsequestrin protein family, beta-tropomyosin, and hemoprotein functions (NCBI, 2019), e.g. *MYOT* (myotilin), *CASQ1* (Calsequestrin 1), *TPM2* (Tropomyosin 2) and *MB* (Myoglobin). In addition, some energy promoters for development of cell functions, such as *UQCRC2* (Ubiquinol-Cytochrome C Reductase Core Protein 2), *LDHA* (Lactate Dehydrogenase A), *CKMT2* (Creatine Kinase, Mitochondrial 2), *ATP5F1E* (ATP Synthase Peripheral Stalk-Membrane Subunit B), ATP5ME (ATP Synthase Membrane Subunit E), *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase), PGK1 (Phosphoglycerate Kinase 1), *ALDOA* (Aldolase, Fructose-Bisphosphate A), and *ATOX1* (Antioxidant protein 1 copper chaperone), were also found to be up-regulated in this analysis.

The mRNA isoform associated with the *SYNM* (Synemin) gene was the only down-regulated in muscle tissue of tough beef in relation to tender beef animals, using WBSF phenotype.

Table 2. Differentially expressed novel mRNA isoforms with annotated genes (p-value ≤ 0.01) in Longissimus thoracis muscle of tough beef animals in relation to tender beef animals, using phenotypes measured based on the Warner-Bratzler shear force (WBSF) method.

Feature ID	Gene name	mRNA Length(bp)	Position	p-value	Log2 (FC)[1]
ENSBTAG00000014731.6	GAPDH	2041	5:103869738-103874335	1.33 e^{-05}	7,513.17
ENSBTAG00000011424.6	TPM2	3809	8:59855588-59864645	1.97 e^{-05}	6,372.02
ENSBTAG00000005333.3	MB	2579	5:73812939-73825226	8.31 e^{-06}	3,726.20
ENSBTAG00000009707.6	MYL1	173	2:98112898-98113802	6.13 e^{-05}	2,069.69
ENSBTAG00000012927.5	ALDOA	3212	25:26211040-26218476	4.96 e^{-05}	961.86
ENSBTAG00000018204.5	MYH1	7722	19:29481385-29507064	4.11 e^{-06}	152.55
ENSBTAG00000008394.5	MYL3	1617	22:52637863-52644137	2.24 e^{-05}	120.52
ENSBTAG00000007782.4	MYOT	1624	7:49353654-49357528	7.94 e^{-06}	120.50
ENSBTAG00000052508.1	SEM1	11917	4:14044426-14080582	2.87 e^{-05}	115.85
ENSBTAG00000001003.3	CKMT2	1849	7:81260432-81275405	3.55 e^{-05}	70.90
ENSBTAG00000021651.5	UQCRC2	408	25:19657186-19661301	2.4 e^{-05}	63.65
ENSBTAG00000039208.2	ATP5F1E	5268	13:57359506-57364774	5.88 e^{-05}	54.07
ENSBTAG00000000894.6	PGK1	4503	X:74242014-74261087	2.22 e^{-05}	54.02
ENSBTAG00000008683.6	LDHA	1486	29:26290181-26291667	1.05 e^{-05}	45.83
ENSBTAG00000020223.5	CASQ1	3455	3:9474290-9485522	1.87 e^{-05}	38.29
ENSBTAG00000017496.3	ATP5ME	882	6:117650605-117652332	3.64 e^{-05}	36.57
ENSBTAG00000049306.1	MSRB1	6697	25:1497741-1507740	8.63 e^{-05}	34.27
ENSBTAG00000020080.6	MYBPC2	1750	18:56581088-56588653	2.11 e^{-05}	32.50
ENSBTAG00000009707.6	MYL1	17729	2:98090933-98126281	4.32 e^{-05}	31.73
ENSBTAG00000020080.6	MYBPC2	2378	18:56590019-56603457	1.51 e^{-05}	30.25
ENSBTAG00000008340.5	ATOX1	530	7:62942151-62957587	2.81e^{-07}	27.44

Continued Table 2

Feature ID	Gene name	mRNA Length(bp)	Position	p-value	Log2(FC)[1]
ENSBTAG00000011843.5	EEF1G	1990	29:40915677-40917889	6.13 e^{-05}	25.84
ENSBTAG00000009663.5	YBX3	3269	5:98873211-98879194	1.44 e^{-05}	22.99
ENSBTAG00000020733.3	RPS15A	7860	25:16416457-16424317	5.67e^{-05}	20.99
ENSBTAG00000034529.3	HMGA1	1887	23:8313809-8322718	2.84 e^{-06}	19.66
ENSBTAG00000052337.1	RPS29	2721	10:42625568-42629847	3.63 e^{-05}	18.08
ENSBTAG00000019543.6	ELOB	1267	25:2281094-2285801	6.21 e^{-07}	17.15
ENSBTAG00000018071.5	TMOD4	3293	3:19636142-19642311	6.06 e^{-05}	12.08
ENSBTAG00000011752.6	SYNM	10390	21:7576436-7606637	9.25 e^{-05}	-55.05
ENSBTAG00000008340.5	ATOX1	530	7:62942151-62957587	2.81e^{-07}	27.44
ENSBTAG00000011843.5	EEF1G	1990	29:40915677-40917889	6.13 e^{-05}	25.84
ENSBTAG00000009663.5	YBX3	3269	5:98873211-98879194	1.44 e^{-05}	22.99
ENSBTAG00000020733.3	RPS15A	7860	25:16416457-16424317	5.67e^{-05}	20.99
ENSBTAG00000034529.3	HMGA1	1887	23:8313809-8322718	2.84 e^{-06}	19.66
ENSBTAG00000052337.1	RPS29	2721	10:42625568-42629847	3.63 e^{-05}	18.08
ENSBTAG00000019543.6	ELOB	1267	25:2281094-2285801	6.21 e^{-07}	17.15
ENSBTAG00000018071.5	TMOD4	3293	3:19636142-19642311	6.06 e^{-05}	12.08
ENSBTAG00000011752.6	SYNM	10390	21:7576436-7606637	9.25 e^{-05}	-55.05

[1]Log2(FC)= Fold change

For the phenotypes measured based on MFI (Table 3), mRNA isoforms associated with genes related to muscle constituents (NCBI, 2020), such as *NEB* (Nebulin), *FGFRL1* (Fibroblast Growth Factor Receptor like 1), *NUMA1* (Nuclear Mitotic Apparatus Protein 1), and *DMPK* (DM1 Protein Kinase) were up-regulated in the tender beef groups compared to the tough beef groups. Furthermore, mRNA isoforms associated with genes related to regulatory cell mechanism and DNA-binding transcription factor activity (NCBI, 2020), such

as *PITX1* (Paired like Homeodomain 1) and *FAM204A* (Family with Sequence Similarity 204 Member A), were also up-regulated in tender beef groups. Only one novel mRNA isoform associated with myosin encoder [*MYL6* (myosin light chain 6)], was found when using the MFI, which was also up-regulated in the tender beef group (Table 3).

Using MFI, six down-regulated novel mRNAs isoforms (Table 3) were found to be associated with genes related to catalysis, activation and regulation of cells, such as *MARCH6* (Membrane Associated Ring-CH-Type Finger 6), *TEAD1* (TEA Domain Transcription Factor 1), and cell transporter`s factor encoders, such as *SLC7A8* (Solute Carrier Family 7 Member 8, and *TMEM160* (transmembrane Protein 160).

Table 3. Differentially expressed novel mRNA isoforms with annotated genes (p-value ≤ 0.01) in *Longissimus thoracis* muscle of tender beef animals in relation to tough beef animals, using phenotypes measured based on the myofibrillar fragmentation index (MFI) method.

Feature ID	Gene name	mRNA Length(bp)	Position	p-value	Log2(FC)[1]
ENSBTAG00000005057.6	FAM204A	407	26:38604971-38611934	3.79 e^{-05}	26.60
ENSBTAG00000004602.6	PITX1	2352	7:46474413-46481011	6.52 e^{-05}	28.26
ENSBTAG00000006907.7	NEB	4407	2:44569189-44605165	2.01 e^{-04}	17.38
ENSBTAG00000018449.6	NUMA1	1268	15:51642814-51645001	4.48 e^{-04}	11.30
ENSBTAG00000027464.4	FGFRL1	2784	6:117346082-117350247	6.44 e^{-04}	3.45
ENSBTAG00000010799.6	MYL6	811	5:57161769-57164986	3.51e^{-07}	2.49
ENSBTAG00000013347.6	DMPK	3594	18:53309572-53320117	5.00 e^{-04}	2.21
ENSBTAG00000032657.4	TEAD1	8130	15:39740847-39748977	3.67 e^{-05}	-2.59
ENSBTAG00000003536.5	MARCH6	5075	20:62819170-62843802	3.81 e^{-07}	-3.44
ENSBTAG00000007415.6	SLC7A8	6491	10:21683778-21737239	2.18 e^{-04}	-4.61
ENSBTAG00000007415.6	SLC7A8	5512	10:21700393-21737239	6.50 e^{-04}	-4.66
ENSBTAG00000007415.6	SLC7A8	5074	10:21725297-21737239	2.54 e^{-04}	-4.83
ENSBTAG00000015579.4	TMEM160	1043	18:54094486-54097330	2.59 e^{-04}	-16.63

[1]Log2(FC)= Fold change

Annotation of novel differentially expressed mRNA isoforms associated with non-annotated genes

Novel mRNA isoforms associated with non-annotated genes are shown in Tables 4 and 5, for phenotypes measured based on WBSF and MFI, respectively. A total of nine novel mRNA isoforms associated with non-annotated genes were identified using the WBSF (Table 4), and nine were identified using the MFI (Table 5) method. In addition, it was observed that the majority of novel mRNA isoforms were long-length transcripts (>1300 bp). Some of those mRNA seems to be gene clusters [e.g. the alignment view of novel mRNA that be seen in the supplementary Figure S3 and S4], which could suggest a polycistronic mRNAs, situation wheere two genes or more are expressed from a single promoter. The sequences of these novel mRNA isoforms were compared by similarity to the nucleotide collection, using the BLAST tool (NCBI, 2020). Tables 4 and 5 show the percentage of similarity found between them, for the analysis using phenotypes measured based on the WBSF and MFI methods, respectively.

The *Gene_3524_4* mRNA isoform (Table 5) was up-regulated in the tender beef group compared to the tough beef group, and it was in overlapping with the *COL4A2* (Collagen, type IV, alpha 2) gene in the ARS-UCD1.2 bovine genome (NCBI, 2020). This gene had 100% of similarity with the "PREDICTED: *Bos taurus* collagen type IV alpha 2 chain (*COL4A2*)" mRNA sequence.

The novel mRNA differentially expressed, *Gene_3967_2* and *Gene_1479_3*, were up-regulated in tough beef animals in relation to tender beef animals, those mRNAs presented 79.89% and 86.76 % of similatity with *Homo sapiens* partial TTN gene for titin and *Homo sapiens* titin (TTN), Ref Seq Gene (LRG_391) on chromosome 2, respectivally. The TTN (titin) is gene encodes a large abundant protein of striated muscle. The product of this gene is allow the elasticity of band-I, which contains two regions of tandem immunoglobulin domains on either side of a PEVK region that is rich in proline, glutamate, valine and lysine (GeneCards, 2020). Thus, Collagen and titin are very important proteins in the muscular constitution, as well as to beef tenderness.

Table 4. Differentially expressed novel mRNA isoforms (p-valor < 0.01) with non-annotated genes identified as being between divergent groups, using phenotypes measured based on the Warner-Bratzler shear force (WBSF) method.

Feature ID	mRNA Length(bp)	Position	p-value	Log2 (FC)[1]	Predicted gene	Cover (%)	E-Value	Identity (%)	Pred. gene* Acession
Gene_3916_3	3000	19:48082109-48091908	8.23 e^{-05}	320.39	Bos mutus isolate yakQH1 chromosome 19	100	0	99.10	CP027087.1
Gene_1371_1	697	19:55052578-55053803	6.08 e^{-05}	284.87	Bos mutus isolate yakQH1 chromosome 19	100	0	99.10	CP027087.1
Gene_2735_1	1447	6:58750827-58774420	6.64 e^{-05}	270.03	Bos taurus isolate Dominette_000065F genomic sequence	39	0	95.57	KX592814.1
Gene_992_3	2042	18:35881322-35883364	6.77 e^{-05}	199.16	Bos mutus isolate yakQH1 chromosome 18	100	0	98.73	CP027086.1
Gene_797_4	374	15:29943886-29951505	4.6 e^{-05}	108.34	Bos mutus isolate yakQH1 chromosome 15	100	0	98.62	CP027083.1
Gene_4316_1	737	27:36675312-36681258	2.26 e^{-05}	77.82	Bos mutus isolate yakQH1 chromosome 27	100	0	98.78	CP027095.1
Gene_3916_1	3091	19:48082109-48091908	8.39 e^{-05}	66.25	Bos mutus isolate yakQH1 chromosome 19	96	0	99.06	CP027087.1
Gene_4330_1	4784	28:29286693-29291477	4.21 e^{-05}	30.66	Bos mutus isolate yakQH1 chromosome 28	100	0	98.96	CP027096.1
Gene_2734_1	4708	6:58144953-58159271	4.67 e^{-05}	20.50	PREDICTED: Bos taurus family with sequence similarity 114 member A1 (FAM114A1), transcript variant X5, mRNA	21	0	100	XM002688202.6

[1]Log2(FC)= Fold change (log2)
*Acession number of predicted gene

Table 5. Differentially expressed novel mRNA isoforms with non-annotated genes identified between divergent groups, using phenotypes measured based on the myofibrillar fragmentation index (MFI) method.

Feature ID	mRNA Length(bp)	Position	p-value	Log2 (FC)[1]	Predicted gene	Cover (%)	E-Value	Identity (%)	Pred. Gene* Acession
Gene_3967_2	1386	2:18200835-18207860	3.16 e^{-05}	39.76	Homo sapiens partial TTN gene for titin	73	0	79.89	AJ277892.2
Gene_2167_2	151	26:23984354-23987754	9.42 e^{-04}	11.96	Ovis canadensis canadensis isolate 43U chromosome 22 sequence	99	0	91.22	CP011907.1
Gene_1908_1	3967	23:27645743-27657003	4.61 e^{-05}	7.79	B.taurus DNA sequence from clone CH240-264I3, complete sequence	100	0	100	FQ482107.2
Gene_2167_2	151	26:23984354-23987754	9.42 e^{-04}	11.96	Ovis canadensis canadensis isolate 43U chromosome 22 sequence	99	0	91.22	CP011907.1
Gene_3524_4	1874	12:85148989-85154030	7.23 e^{-04}	4.41	PREDICTED: Bos taurus collagen type IV alpha 2 chain (COL4A2), mRNA	35	0	100	XM025000171.1
Gene_1479_3	587	2:18145596-18146334	2.01 e^{-04}	3.56	Homo sapiens titin (TTN), Ref Seq Gene (LRG_391) on chromosome 2	100	0	86.76	NG011618.3
Gene_1068_1	1547	18:46466867-46469534	7.93 e^{-04}	2.38	Bos mutus isolate yakQH1 chromosome 18	100	0	99.10	CP027086.1
Gene_4708_4	3661	7:95068807-95094694	1.13 e^{-05}	-4.11	PREDICTED: Bos taurus Rho related BTB domain containing 3 (RHOBTB3), transcript variant X1, mRNA	14	0	99.97	XM002689396.6
Gene_1409_1	1780	19:50821437-50825531	2.74 e^{-04}	-6.29	Bos mutus isolate yakQH1 chromosome 19	93	0	98.75	CP027087.1

[1]Log2(FC)= Fold change (log2)
*Acession number of predicted gene

3.3.3 Functional enrichment analysis

For functional analysis list of genes related to the differentially expressed mRNAs isoforms previously detected in this study considering both phenotypes, was used. A total of four significant pathways were identified (Table 6), which were related to muscle mechanisms and process of transformation of muscle into meat. In the functional classification of GO terms (i.e., molecular function, biological process and cellular component), the most significant terms were associated with structural constituents of muscle such as myosin and actin proteins, and muscle system processes such as contraction and sliding (Supplementary Table S4).

Table 6. Pathways associated with tenderness in *Longissumus thoracis* muscle of Nellore cattle

ID	Pathway Description	Source	p-value	FDR	GFI*
1269869	Striated Muscle Contraction	Reactome	$6.76E^{-10}$	$2.97E^{-07}$	*TPM2, MYL1, MYL3, TMOD4, NEB, MYBPC2*
1269868	Muscle contraction	Reactome	$1.45E^{-07}$	$3.20\ E^{-05}$	*TPM2, MYL1, MYL3, TMOD4, NEB, MYBPC2, MYL6, DMPK*
695200	HIF-1 signaling pathway	KEGG	$1.29E^{-05}$	$1.27\ E^{-03}$	*ELOB, GAPDH, ALDOA, LDHA, PGK1*
1270158	Metabolism of amino acids and derivatives	Reactome	$4.88E^{-10}$	$4.57\ E^{-02}$	*SEM1, RPS7, RPS15A, RPS29*

*Number of genes from input.

3.4 DISCUSSION

Beef tenderness is the consumer's most preferred sensory attribute. However, the great proportion of Zebu breeds in Brazil reduces substantially meat tenderness (Ferguson et al., 2000). Understanding and measuring beef tenderness in a cheaper and accurate way has been a great challenge to researchers (Aroeira et al., 2020).

Gene expression analysis has being an excellent strategy to identify positional and functional candidate genes related to economically important traits (Tizioto et al., 2013). In

this study, two methods for measuring tenderness were used (i.e., WBSF and MFI), in order to improve the identification of mRNA isoforms associated to tender beef. Both measurements have been successfully reported in the literature as indicators of meat tenderness, due to their high direct correlation with this trait (Olson & Parrish, 1977; Culler et al., 1978; Malheiros et al., 2015). Moreover, RNA-Seq is an accurate technique used in transcriptomic analyses, especially for quantification of mRNA isoforms using the *large gap mapping* approach. The *large gap mapping* approach allowed the identification of a large number of differentially expressed mRNA isoforms between groups. Additionally, this approach was essential to identify novel mRNA isoforms from known and unknown genes.

For WBSF analysis, the myosin encoders (e.g., *MYL1, MYL3, MYL6, MYH1* and *MYBPC2*) were the most abundant genes related to the differentially expressed mRNA isoforms detected. Myosin is a major contractile protein, which converts chemical energy into mechanical energy through the hydrolysis of ATP (Cooper et al., 2000), which could likely play important roles on beef tenderness. The differential expressions of myosin's mRNA isoforms were highly up-regulated in the *Longissimus thoracis* muscle of tough beef bulls in relation to the tender beef bulls (except for the mRNA isoform related to the *MYL6* gene [the MFI analysis (Table 3)]. For the *MYL6* gene, mRNA isoforms were differentially expressed in the tender beef group compared to the tough beef group (log2 (FC) =2.49). This Fold Change (FC) suggests that the tender beef group had 2.49 times more expression of *MYL6* than the tough beef, which is low compared to the expression level of other myosin (FC=> 30) detected using phenotypes from the WBSF method in this study (Table 2).

Meat tenderness is conditioned to two main muscle components: quantity of connective tissue, and the *postmortem* loss of structural integrity within the sarcomeres (myosin degradation; Sawdy et al., 2004). Muscle fiber type forms the sarcomeres' structure, which is a conditioning factor to beef tenderness since it determines the speed and intensity of muscle contraction. In this context, both speed and intensity of muscle contraction are directly related to the intensity of each type of myosin is composing the muscle (Duris et al., 2000). As described by Sazali et al. (2005), slow and fast heavy chain myosins are correlated with the percentage of type I and type II fibers, respectively. These authors also reported correlation between slow myosin heavy chain and the positive calpastatin activity ($r2 = 0.725$) across different muscle types. In this study we observed differentially expressed mRNA isoforms related to myosin encoders genes, such as light chain (*MYL1*) and binding myosin

(*MYBPC2*), which are usually expressed in fast skeletal muscles (McNamara & Sabayappan et al., 2018). Moreover, we have also identified myosin light chain (*MYL3*) gene, which is expressed in the slow skeletal muscles (Deng et al., 2019); and the *MYL6* (light chain) and *MYH1* (heavy chain) genes, which are expressed in smooth muscle and muscle of slow and automatic contraction (Park et a., 2011). In general, the mRNAs isoforms related to the myosin encoder genes identified in this study were up-expressed in tough beef animals compared to tender beef animals, using WBSF phenotypes and down-regulated when we used MFI phenotypes.

In the current study, we identified mRNA isoforms related to genes that affect the speed of degradation fibers during the meat aging process (Tajsharghi et al., 2012; D` Adamo et al., 2016; Puz et al., 2017), such as *MYOT, CASQ1*, *TPM2* and *SYNM* [(WBSF phenotypes (Table 2))] and *MB, NEB* and *FGFRL1* [(MFI phenotypes (Table 2))]. These genes can be used as candidate genes to select animals in order to improve meat tenderness. Some of the mRNA isoforms reported here have been up-expressed in tough beef animals for WBSF analysis (mRNA isoforms associated with *MYOT, CASQ1*, *TPM2, MB* and *SYNPO-201,* annotated mRNA isoforms*)*. In this context, the *MYOT* gene encodes a cytoskeletal protein responsible for the stability of thin filaments during muscle contraction, maintaining the Z-disk integrity of sarcomere (Puz et al., 2017). The supra-expression of this protein might contribute to increase muscle hardness, because this protein can protect the structural muscle proteins during the degradation process.

The *CASQ1* protein is usually expressed in fast skeletal muscles, because it is a calcium-binding protein that acts as a calcium buffer within the sarcoplasmic reticulum (Wang et al., 1998). Thus, the *CASQ1* protein helps keeping calcium in the cisterna of the sarcoplasmic reticulum after a muscle contraction (D'Adamo et al., 2016). In human studies, the up-regulation and some mutation of *CASQ1* gene are related to myopathy, a disease that causes locomotion restriction due to muscle atrophy (Simplicini et al., 2018; Bohm et al., 2018). These results corroborate with the up-expression pattern of this gene in tough beef animals identified in our study.

The *TPM2* (Tropomyosin 2) gene is a member of the actin filament binding protein family and is expressed mainly in type 1 muscle fibers that present slow contraction (Tajsharghi et al., 2012). Nucleotide sequences encoding an entire coding region for bovine tropomyosin (TPM) isoforms were determined by Oe et al., (2007). These authors also related

that three TPM isoforms were expressed in bovine skeletal muscle, and that *TPM2* corresponded to about 50% of the total TPM. In addition, other studies (Oe et al., 2009; Polati et al., 2012) have showed that *TPM2* gene was expressed in fast type fibers muscle, and that had a coordinated expression between TPM and myosin heavy chain isoforms. These findings can be one important factor to determine contractile properties of muscle fibers (Oe et al., 2016; Martilla et al., 2014). Malheiros et al. (2019) related a significant increasing in troponin (a protein attached to protein tropomyosin) degradation in tender groups compared to tough beef groups formed by Angus cattle. The troponins undergo enzymatic proteolysis during the *post-mortem* period through the calpain-1, cathepsins and caspase systems, which can be associated with meat tenderness (Polati et al., 2012).

The *MB* is a hemoprotein present in cardiac, skeletal and smooth muscle, and it is primarily responsible for the intracellular oxygen storage and transcellular facilitated diffusion of oxygen from cell's membrane to mitochondria (Rayner et al., 2009). The low supply of oxygen due to *MB* reduced efficiency affects the mitochondrial function, and decreases cell viability through inducing apoptosis (Hall et al., 2014). This fact contributes to the meat maturation process. The *SYNPO-201* (a mRNA isoform of the SYNPO gene) was up-regulated in the tough group compared to the tender group. As described by Longo et al. (2015), this gene is involved in the apoptotic behavior of Piedmontese beef muscle cells undergoing the muscle maturation process.

The mRNAs related to *NEB, FGFRL1* and *SYNM* genes were up-regulated in the tender group compared to the tough group. The *NEB* gene is a component of the cytoskeletal matrix that coexists with the thick and thin filaments within the sarcomeres of skeletal muscle (Kiss et al., 2018). This gene was related to muscle weakness in respiratory and peripheral skeletal muscles using a mouse model (Joureau et al., 2017). The weakness happens due to reduced capacity of muscle contraction, occurred due changes in cross-bridge cycling kinetics.

The *FGFRL1* gene is a fibroblast growth factor receptor highly conserved throughout evolution (Kähkönen et al., 2018). According with Amann et al. (2014), the expression of this gene is directly associated with the *MYL3* gene expression. The mentioned authors used qPCR and in situ hybridization to shown the diaphragm of *FGFRL1* knockout mouse lacks and slow muscle fibers, due to the absence of slow fiber markers (i.e., *MYH7*, *MYL2* and *MYL3)*. These myosins coded for typical constituents of slow muscle fibers that are not usually found in fast

fibers, being used as marker in the identification of muscle fiber type. We have identified similar results in our study, as the *MYL3* was down-regulated in the tender group compared to the tough group (Table 2), and the *FGFRL1* gene was up-regulated in the tender group (Table 3). Together these results suggest that animals with tender meat might have more fast muscle fibers than animals with tough meat.

The *SYNM* gene is an intermediate filament, and its expression is positively correlated to typical smooth muscle cells (Perisic et al., 2016). This gene rivalled smooth muscle myosin (MYH11) for smooth muscle cell specificity (Sward et al., 2019), perhaps could also play roles in striated muscle. Garcia-Pelagio et al. (2015) studied the roles of *SYNM* in skeletal muscle of synemin in murine skeletal muscle. Their results indicate that the myofiber surface is more elastic in muscle who's this gene is down-regulated, and that the sarcolemma can be separated from the contractile structures. Therefore, the authors suggested a possible role for *SYNM* in the stabilization of costameres and promoting its function at the sarcolemma.

The *ANKRD23-202* mRNA isoform is one splice form of the *ANKRD* gene, which is the most abundant Muscle ankyrin repeat proteins (MARPs) in human skeletal muscle (Wette et al., 2017). Wang et al. (2017), related a negative role of this gene in the myoblast differentiation, suggesting the need of further studies approaching muscle development. Genes that play important roles in the mitochondrial respiration, such as *UQCRC2*, *LDHA*, *CKMT2, ATP5F1E, ATP5ME, PGK1, MARCH6, ATOX1* and *ALDOA* genes (Boudreau et al., 2016; Hofmann et al., 2018; El-Sheikh et al., 2019), were also found in our study. In general, the mRNA isoforms associated to these genes were up-regulated in the tough group compared to the tender group.

It is important to highlight that some of these genes catalyze the conversion of L-lactate and NAD to pyruvate, ATP synthesis, energy transport, and copper homeostasis (Melouane et al., 2018). Zhang et al. (2018) identified a lower expression of the *LDHA* gene in *Longissimus dorsi* muscle of Yunling cattle (a Zebu breed), which was related to the glycometabolism pathway. These authors revealed that Yunling cattle presented lower intramuscular muscular fat content, lower proportion of monounsaturated fatty acids (MUFA), and more short-chain fatty acids (sc-FA) when compared to Chinese Simmental cattle. The *CKMT2* gene was associated to spinal muscular atrophy in previous studies (e.g., Richard et al., 1993). In our study, the *CKMT2* gene was up-regulated in tender beef animals. The *GAPDH* gene mediates cell death under oxidative stress, and the overexpression of this

gene can increase mitochondrial dysfunction (Nakajima et al., 2017). Malheiros et al. (2019) observed a decrease in oxidative damage of the metabolic enzymes (GAPDH) in a group of tender meat Angus crossbred cattle. Therefore, this gene is possibly playing roles in the beef tenderness of Nellore cattle, suggesting the need of a depth investigation.

In addition, *RPS29, RPS15A, EEF1G, SEM1, MSRB1, HMGA1, YBX3, ELOB, TMEM160* and *TEAD1* genes had novel mRNA isoforms related to them. The *RPS7-201* and *SRGN-201* mRNA isoforms were up-regulated in tough beef animals compared to tender beef animals. The novel mRNA isoforms related to *PITX1*, *FAM204A*, *NUMA1* genes, and the *RGMB-201*, *ZBTB18-202*, *DCTN2-203* and *NKD2-201* specific known mRNA isoforms, were up-regulated in tender beef animals compared to tough beef animals. The *RPS29*, *RPS15A*, and *RPS7-201* genes are related to ribosomal complex building (Kishore et al., 2013; Mirabello et al., 2014). The *EEF1G* encodes a subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome (Corcoran et al., 2007). The *ELOB* gene encodes the protein elongin B, which is a subunit of the transcription factor B (SIII) complex. The *TEAD1* gene encodes a ubiquitous transcriptional enhancer factor (Liu et al., 2019). In this context, Wen et al. (2019) revealed that a deletion of *TEAD1* in cardiomyocyte and/or vascular smooth muscle cell down-regulate the expression of muscle contractile genes and key transcription factors including Pitx2c and myocardin genes.

The *MSRB1, HMGA1* (Corcoran et al., 2007) and *YBX3* (Cooke et al., 2019) genes work on the regulation of transcription processes, splicing product export and repairing proteins that correct fail transcriptional factors in oxidative stress cells. Hjorth et al. (2015) revealed that the *SRGN* gene increased expression after acute, as well as long-term exercise in humans. These authors classified the *SRGN* gene as exercise-regulated proteoglycan in skeletal muscle, with a potential role in exercise adaptation. The *SEM1* gene has a proteasome-independent role in the mRNA export as a functional component of the Sac3-Thp1 complex (Wilmes et al., 2008).

The *PITX1* gene is related to differentiation of *Longissimus thoracis* and *Semimembranosus* muscles in Hanwoo calves cattle, and it seems to modulate the muscle of satellite cells during myogenesis through a differential expression profile. In addition, this gene also controls the expression of some myogenic regulatory factors (e.g., *MYOD* and *MYF5*) (de Las-Heras- Saldane et al., 2019). In this study, this gene seems to play important

roles in the differentiation of tender and tough beef animals. The *SLC7A8* gene was down-regulated in tender beef compared to tough beef animals. This gene is involved in transport of glucose and other sugars, bile salts and ions, and amine compounds requested by cells (Xie et al., 2015).

The novel mRNA isoforms, *Gene_3524_4* (Table 5) was associated with *COL4A2* gene. This gene encodes one of six subunits of type IV collagen, the major structural component of basement membranes (Cunningham et al., 2019). Thus, collagen is an important protein to meat tenderness, because it plays an important role in the composition of connective tissue (Listrat et al., 2016).

The gene profiles found for WBFS phenotypes (Supplementary Table 5) were strictly related to muscle components (e.g., sarcomere, Z-disc and I band) and muscle system process, such as muscle contraction. The pathways detected for WBSF were associated with striated muscle contraction, and HIF-1 signaling pathway, which is a master regulator of oxygen homeostasis (Ivan & Kaelin, 2017). For the MFI phenotypes (Supplementary Table 6), we observed a gene expression profile related to cytoskeletal protein, supramolecullar complex cell component, and muscle organ and structure development biological processes. The pathways detected for MFI were strictly associated with muscle contraction. These results suggest more accuracy of functional analysis when this was performed for the integrated gene expression profiles (WBFS and MFI analysis).

These results awaken our thinking to the possibility of theses novel mRNA isoforms identified in this study, would be gene forms uniquely expressed in *Longissumis thoracis* muscle of Nellore breed. This would contribute to abridge future researches, providing candidate regions related to the tender beef. Thus, could be used in SNP`s identification studies, contributing to assembly SNP panels, including tenderness score to select Nellore cattle. However, if these would be common to different bovine breeds, these regions could be used to identify universal markers that could be useful for animals' selection for meat tenderness.

3.5 CONCLUSION

Different mRNA expression profiles were observed using the two methods to measure tenderness (i.e., WBSF and MFI). The WBSF had a profile related to oxygen homeostasis

(i.e., HIF-1 signaling pathway) and muscle components. The MFI showed a profile strictly related to muscle contraction. Thus, results indicate that the observed mRNA isoforms for both traits are acting into muscle composition and contraction mechanisms, which suggests that these regions have played important roles for meat tenderness in this Nellore cattle population.

The differentially expressed mRNA isoforms are transcripted by genes with biological functions related to oxidative process, energy production and striated muscle contraction. Potential positional candidate mRNA isoforms for beef tenderness selection are the mRNA isoforms related to the myosin encoders, *MYOT*, *CASQ1*, *TPM2*, *MB*, *SYNM* genes, and *SYNPO-201* mRNA isoform, that were identified using WBSF phenotypes, and the mRNA isoform associated with the *NEB* and *FGFRL1* genes, as well as the *ANKRD23-202* mRNA isoform (detected by MFI analysis).

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3.7 SUPPLEMENTARY MATERIAL

Table S1. Descriptive statistics of read's alignment of Nellore animals divergent for measurements collected using the Shear Force device.

Phenotype[1]	Read count	Map pairs %	Broken pairs %	Non-mapped %	Map to genes %	Mapped to intergenic %
55HShear[2]	29,476,234	88.03	10.38	1.59	95.10	4.90
34HShear	55,439,038	88.23	10.78	1.00	93.22	6.78
82HShear[2]	57,331,896	87.69	10.52	1.80	90.92	9.08
38 HShear	26,267,694	90.16	8.25	1.59	94.28	5.72
50 HShear	57,446,470	87.29	11.41	1.30	91.75	8.25
61 HShear	85,477,828	87.64	10.56	1.81	94.34	5.66
88 HShear	55,135,382	91.10	7.57	1.33	93.90	6.10
92 HShear	70,524,556	92.77	6.48	0.75	94.97	5.03
111 HShear	609,100	80.65	13.81	5.54	94.53	5.47
119 HShear[2]	73,081,816	90.52	8.39	1.09	91.69	8.31
Means	**510,241,824**	**88.40**	**9.82**	**1.78**	**93.47**	**6.53**
1Lshear[2]	35,050,386	8937	8.73	1.90	92.74	7.26
103Lshear[2]	66,166,214	85.07	13.69	1.23	88.16	11.84
52 Lshear[2]	64,221,404	88.26	10.78	0.97	91.59	8.41
72Lshear[2]	28,266,514	86.21	11.82	1.97	91.55	8.45
54Lshear[2]	64,342,082	88.03	10.91	1.06	92.41	7.59
18Lshear	61,714,992	89.21	9.70	1.10	91.99	8.01
19Lshear	62,252,910	90.61	8.27	1.12	91.68	8.32
45Lshear[2]	54,345,410	90.14	8.75	1.11	92.46	7.54
46Lshear[2]	73,922,046	89.82	8.98	1.20	91.36	8.64
117Lshear	64,168,186	89.29	9.46	1.25	91.56	8.44
Means	**574,450,144**	**88.60**	**10.10**	**1.30**	**91.55**	**8.45**

[1]Tender meat group; tough= tough meat group.
[2]Samples were selected to both traits (Warner-Bratzler shear force (WBSF) and myofibrillar fragmentation index (MFI)).

Table S2. Descriptive statistics of read's alignment of Nellore animals divergent for measurements collected using the myofibrillar fragmentation index

Phenotype[1]	Read count	Map pairs %	Broken pairs %	Non-mapped %	Map to genes %	Mapped to intergenic %
20HMFI	57,446,134	90.94	8.07	0.99	93.80	6.20
39HMFI	66,137,704	91.38	7.80	0.81	94.68	5.32
83HMFI	23,559,418	86.98	10.02	2.99	91.80	8.20
46HMFI[2]	73,922,046	89.88	8.91	1.21	91.34	8.66
54HMFI	64,342,082	88.03	10.91	1.06	92.41	7.59
1HMFI[2]	35,050,386	89.37	8.73	1.90	92.74	7.26
45HMFI [2]	54,345,410	90.14	8.75	1.11	92.46	7.54
52HMFI [2]	64,221,404	88.26	10.78	0.97	91.59	8.41
72HMFI[2]	28,266,514	86.21	11.82	1.97	91.55	8.45
103HMFI[2]	66,166,214	85.07	13.69	1.23	88,16	11.84
Means	**533,457,312**	**88.62**	**9.94**	**1.44**	**92.05**	**7.95**
11LMFI	51,620,850	89.14	9.17	1.68	89.33	10.67
122LMFI	29,908,598	85.92	12.17	1.90	92.05	7.95
36LMFI	27,797,902	88.89	9.36	1.75	86.16	13.84
60LMFI	30,193,346	87.90	10.12	1.97	92.45	7.55
56LMFI	31,690,996	89.14	9.41	1.45	94.12	5.88
64LMFI	75,213,976	90.33	8.56	1.11	90.42	9.58
119LMFI[2]	73,081,816	90.52	8.39	1.09	91.69	8.31
55LMFI[2]	29,476,234	88.03	10.38	1.59	95.10	4.90
82LMFI[2]	57,331,896	87.69	10.52	1.80	90.92	9.08
94 LMFI	20,367,498	87.42	10.89	1.69	94.84	5.16
Means	**426,683,112**	**88.69**	**9.89**	**1.60**	**91.70**	**8.30**

[1]HMFI= high myofibrillar fragmentation index group; LMFI= low myofibrillar fragmentation index group.
[2]Samples were selected to both traits (Warner-Bratzler shear force (WBSF) and myofibrillar fragmentation index (MFI).

Table S3. Descriptive statistics, heritability (h^2) and genetic correlation estimates of tenderness measured by Warner-Bratzler shear force (WBSF) and myofibrillar fragmentation index (MFI)) traits in Nellore cattle, using the total data set.

Traits	N	Mean	SD	h^2	R
Shear force (kg)	5,053	6.36	1.92	0.11	-0.76
MFI	2,449	35.56	16.09	0.02	

N= number of observations; SD= standard deviation; h2= heritability; r= genetic correlation.

Table S4. Functional classification of mRNAs isoforms differentially expressed (FDR<0.05), in the *Longissimus thoracic* muscle of distinct groups for tenderness traits measure by WBSF and MFI in the beef cattle.

GO: Molecular Function				
Name	Description	p-value	FDR	GFI*
GO:0008307	structural constituent of muscle	1.65 e^{-13}	4.94 e^{-11}	8
GO:0008092	cytoskeletal protein binding	1.94 e^{-09}	2.92 e^{-07}	16
GO:0005198	structural molecule activity	2.79 e^{-08}	2.79 e^{-06}	13
GO:0003779	actin binding	1.08 e^{-07}	8.10 e^{-06}	10
GO:0044877	protein-containing complex binding	7.43 e^{-05}	2.23 e^{-03}	12
GO:0016835	carbon-oxygen lyase activity	1.39 e^{-04}	3.37 e^{-03}	5
GO:0051015	actin filament binding	1.74 e^{-04}	3.48 e^{-03}	5
GO: Cellular Component				
Name	Description	p-value	FDR	GFI*
GO:0030017	Sarcomere	2.55 e^{-15}	6.89 e^{-13}	13
GO:0030016	Myofibril	7.24 e^{-15}	9.78 e^{-13}	13
GO:0043292	Contractile fiber	1.41 e^{-14}	1.27 e^{-12}	13
GO:0031674	I band	7.38 e^{-11}	4.98 e^{-09}	9
GO:0015629	Actin cytoskeleton	2.04 e^{-09}	1.10 e^{-07}	14
GO:0099512	Supramolecular fiber	1.73 e^{-08}	6.38 e^{-07}	17
GO:0099081	Supramolecular polymer	1.85 e^{-08}	6.38 e^{-07}	17
GO:0099080	Supramolecular complex	1.89 e^{-08}	6.38 e^{-07}	17
GO:0030018	Z disc	3.70 e^{-08}	1.11 e^{-06}	7
GO:0016459	Myosin complex	4.67 e^{-07}	1.26 e^{-05}	5
GO:0005739	Mitochondrion	2.67 e^{-03}	2.89 e^{-02}	11
GO:0030054	Cell junction	3.12 e^{-03}	3.24 e^{-02}	9
GO: Biological Process				
Name	Description	p-value	FDR	GFI*
GO:0006936	Muscle contraction	6.10e^{-15}	8.37 e^{-12}	15
GO:0003012	Muscle system process	2.43 e^{-14}	1.67 e^{-11}	16
GO:0030049	Muscle filament sliding	4.19 e^{-10}	1.68 e^{-07}	6
GO:0033275	Actin-myosin filament sliding	4.90 e^{-10}	1.68 e^{-07}	6
GO:0061061	Muscle structure development	1.56 e^{-07}	4.29 e^{-05}	12
GO:0030029	Actin filament-based process	3.01 e^{-07}	5.92 e^{-05}	12
GO:0006941	Striated muscle contraction	3.02 e^{-07}	5.92 e^{-05}	7
GO:0070252	Actin-mediated cell contraction	6.36 e^{-07}	1.09 e^{-04}	6
GO:0030239	Myofibril assembly	1.17 e^{-06}	1.79 e^{-04}	5
GO:0007517	Muscle organ development	1.40 e^{-06}	1.92 e^{-04}	9
GO:0030048	Actin filament-based movement	1.74 e^{-06}	1.99 e^{-04}	6
GO:0097435	Supramolecular fiber organization	5.44 e^{-06}	4.97 e^{-04}	10
GO:0060538	Skeletal muscle organ development	8.74 e^{-06}	7.05 e^{-04}	6
GO:0046034	ATP metabolic process	1.04e^{-05}	7.19 e^{-04}	7
GO:0031032	Actomyosin structure organization	1.11 e^{-05}	7.19 e^{-04}	6
GO:0010927	Cellular component assembly involved in morphogenesis	1.19 e^{-05}	7.19 e^{-04}	5

GO:0014706	Striated muscle tissue development	1.21 e^{-05}	7.19 e^{-04}	8
GO:0007010	Cytoskeleton organization	1.63 e^{-05}	8.51 e^{-04}	14
GO:0060537	Muscle tissue development	1.68 e^{-05}	8.51 e^{-04}	8
GO:0007015	Actin filament organization	5.53 e^{-05}	2.11 e^{-03}	7
GO:0006754	ATP biosynthetic process	6.26 e^{-05}	2.32 e^{-03}	5
GO:0009145	Purine nucleoside triphosphate biosynthetic process	8.69 e^{-05}	3.06 e^{-03}	5
GO:0007519	Skeletal muscle tissue development	9.56 e^{-05}	3.28 e^{-03}	5
GO:0009201	Ribonucleoside triphosphate biosynthetic process	9.79 e^{-05}	3.28 e^{-03}	5
GO:0009205	Purine ribonucleoside triphosphate metabolic process	1.15 e^{-04}	3.76 e^{-03}	5
GO:0009142	Nucleoside triphosphate biosynthetic process	1.26 e^{-04}	4.02 e^{-03}	5
GO:0055002	Striated muscle cell development	1.35 e^{-04}	4.11 e^{-03}	5
GO:0009199	Ribonucleoside triphosphate metabolic process	1.35 e^{-04}	4.11 e^{-03}	5
GO:0009144	Purine nucleoside triphosphate metabolic process	1.35 e^{-04}	4.11 e^{-03}	5
GO:0051146	Striated muscle cell differentiation	1.65 e^{-04}	4.62 e^{-03}	6
GO:0055001	Muscle cell development	1.85 e^{-04}	5.08 e^{-03}	5

GFI*=Genes from Input

Table S5. Functional classification of mRNAs isoforms differentially expressed (FDR<0.05) in the *Longissimus thoracic* muscle of distinct Nellore beef cattle groups for tenderness traits measured using the WBSF methods.

GO: Molecular Function				
Name	Description	p-value	FDR	GFI*
GO:0008307	structural constituent of muscle	6.06 $^{E-11}$	1.34 $^{E-8}$	6
GO:0003779	actin binding	1.30 $^{E-08}$	1.44 $^{E-06}$	9
GO:0005198	structural molecule activity	1.11 $^{E-07}$	8.22 $^{E-06}$	10
GO:0008092	cytoskeletal protein binding	1.94 $^{E-09}$	2.92 $^{E-07}$	16
GO: Cellular Component				
Name	Description	p-value	FDR	GFI*
GO:0030017	Sarcomere	6.68 $^{E-15}$	1.32 $^{E-12}$	11
GO:0030016	Myofibril	1.62 $^{E-14}$	1.60 $^{E-12}$	11
GO:0043292	Contractile fiber	2.86 $^{E-14}$	1.89 $^{E-12}$	11
GO:0031674	I band	1.78 $^{E-11}$	8.81 $^{E-07}$	7
GO:0030018	Z disc	4.57 $^{E-08}$	1.81 $^{E-06}$	6
GO: Biological Process				
Name	Description	p-value	FDR	GFI*
GO:0006936	Muscle contraction	8.49 $^{E-14}$	8.03 $^{E-11}$	12
GO:0003012	Muscle system process	2.87 $^{E-12}$	1.35 $^{E-9}$	12
GO:0006941	Striated muscle contraction	2.82^{E-07}	4.56 $^{E-05}$	6
Pathway				
ID	Pathway Description	Source	p-value	FDR

1269869	Striated Muscle Contraction	Reactome	5.70 $^{E-9}$	1.98 $^{E-06}$
695200	HIF-1 signaling pathway	KEGG	1.48 $^{E-06}$	2.33 $^{E-04}$

GFI*=Genes from Input ; WBSF= Warner-Bratzler shear force

Table S6. Functional classification of mRNAs isoforms differentially expressed (FDR<0.05) in the *Longissimus thoracic* muscle of distinct Nellore beef cattle groups for tenderness traits measured using the MFI methods.

GO: Molecular Function				
Name	Description	p-value	FDR	GFI*
GO:0008092	Cytoskeletal protein binding	5.5 $^{E-4}$	4.34 $^{E-2}$	6
GO: Cellular Component				
Name	Description	p-value	FDR	GFI*
GO:0099081	Supramolecular polymer	4.01 $^{E-4}$	1.77 $^{E-2}$	7
GO:0099080	Supramolecular complex	4.02 $^{E-4}$	1.77 $^{E-2}$	7
GO: Biological Process				
Name	Description	p-value	FDR	GFI*
GO:0061061	Muscle structure development	4.05 $^{E-7}$	3.19 $^{E-4}$	8
GO:0007517	Muscle organ development	5.17 $^{E-05}$	1.71 $^{E-3}$	6
Pathway				
ID	Pathway Description	Source	p-value	FDR
1269868	Muscle contraction	Reactome	8.29 $^{E-5}$	1.22 $^{E-2}$

GFI*=Genes from Input; MFI= Myofibrillar fragmentation index

Figure S1. Differentially expressed novel mRNA isoform transcribed by the *MYL1* gene (Table 2) in animals with high WBSF in relation to low WBSF. 1) cow genome reference (ARS.UCD 1.2); 2) cow reference genome (gene track); 3) predicted transcripts track using the large gap read mapping approach; 4) the reads coverage for high WBSF group; 5) the reads coverage for low WBSF group.

Figure S2. Differentially expressed novel mRNA isoform transcribed by the *FGRL* gene (Table 3) in animals with high MFI in relation to low MFI. 1) cow genome reference (ARS.UCD 1.2); 2) cow reference genome (gene track) ; 3) predicted transcripts track using the large gap read mapping approach; 4) the reads coverage for high MFI group; 5) the reads coverage for low MFI group.

Figure S3. Differentially expressed novel mRNA isoform "Gene_3916_3"(Table 4) in animals with high WBSF in relation to low WBSF. 1) cow genome reference (ARS.UCD 1.2); 2) cow reference genome (gene track) ; 3) Predicted transcripts track using the large gap read mapping approach; 4) the reads coverage for high WBSF group; 5) the reads coverage for low WBSF group.

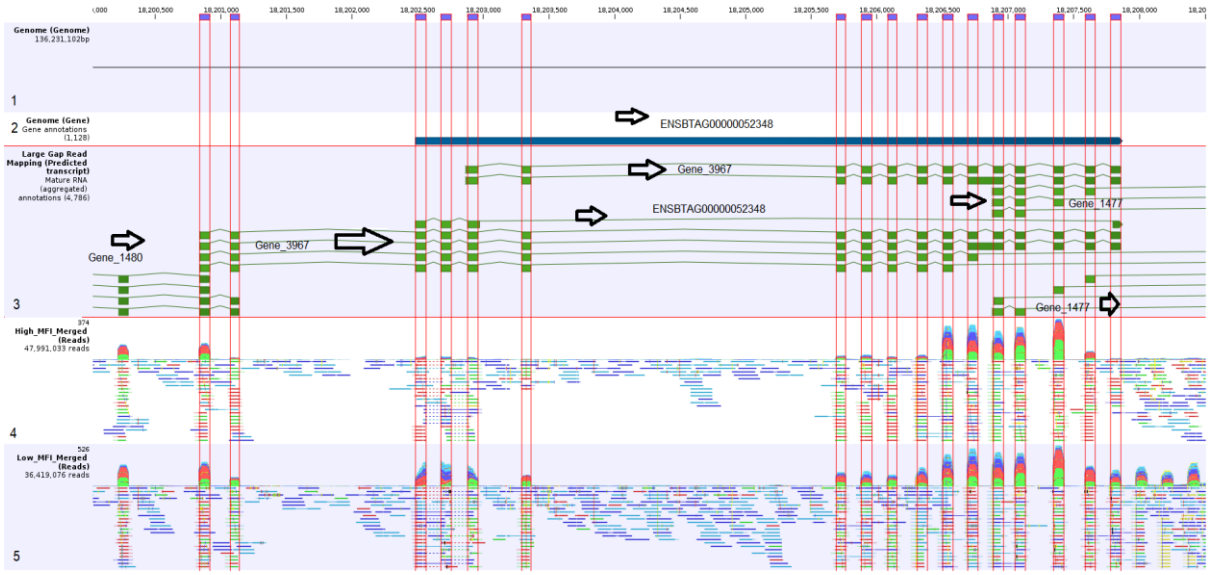

Figure S4. Differentially expressed novel mRNA isoform "Gene_3967_2"(Table 5) in animals with high MFI in relation to low MFI. 1) cow genome reference (ARS.UCD 1.2); 2) cow reference genome (gene track); 3) Predicted transcripts track using the large gap read mapping approach; 4) the reads coverage for high MFI group; 5) the reads coverage for low MFI group.

CHAPTER 4- STRUCTURAL VARIANTS AFFECTING mRNA ISOFORMS ASSOCIATED WITH MARBLING AND COLOR TRAIT IN CATTLE

ABSTRACT - The identification of mRNA isoforms and the associated splice variants affecting marbling and beef color traits in Nellore Cattle, could allow to better understanding the genetic architecture of complex traits such as meat quality. *Longissimus thoracis* muscle samples from the 20 most extreme bulls (out of 80 bulls set) for marbling (high (n=10) and low (n=10) groups) and beef color (desirable (n=10) and undesirable (n=9) group) traits were used to perform transcriptomic analysis using RNA-Sequencing. The CLC Genomics Workbench, was used to align the fragments of each sample to the bovine reference genome ARS.UCD1.2, and to perform the differentially gene expression analysis. An average of 37 thousand transcripts were detected in the Nellore muscle transcriptomic analysis. A total of 14 and 15 mRNA isoforms were differentially expressed (DE) (p-value ≤0.001) between the two groups of divergent bulls for marbling and color of meat traits, respectively. The *HBB-201* mRNA isoform was the only isoform differentially expressed in common for both traits. The DE mRNA for marbling trait (*COL4A2-202, RPL30-203, MAPKAPK2-204* and *NEURL1-201*) and color trait (*SCL16A6-201* and *JSP.1-204*) shown sites of splicing modified by structural variants as Single Nucleotide Variant (SNV), insertion or deletion (Indels). Enrichment analysis using the list of significant differentially expressed mRNA isoforms identified common metabolic pathways, such as O2/CO2 exchange in erythrocytes, tyrosine biosynthesis and phenylalanine degradation. The interaction mechanism among them, suggested that, mitochondrial lipid oxidation was closely related to ferric myoglobin formation, molecule important for maintaining fresh red meat color. The results obtained suggest potential key regulatory genes associated with economically important traits for the beef industry and for the consumer.

KEYWORDS: mRNA isoforms, Nellore beef cattle, RNA-Sequencing

CAPÍTULO 4 - VARIANTES ESTRUTURAIS AFETANDO ISOFORMAS DE mRNA ASSOCIADAS AS CARACTERÍSTICAS DE MARMOREIO E COLORAÇÃO DA CARNE EM BOVINOS

RESUMO - A identificação de isoformas de mRNA e a detecção de pequenas variantes estruturais em *splice sites* associadas as características de marmoreio e coloração da carne em bovinos da raça Nelore, proporcionará um melhor dessas características. Nesse estudo foram utilizadas 20 amostras de músculo *Longissimus thoracis* de animais (selecionados a partir de uma amostra de 80 animais) fenotipicamente extremos para as características de marmoreio (alto (n = 10) e baixo (n = 10)) e coloração da carne (desejável (n = 10) e indesejável (n = 9) grupos) e dados de RNA-Sequencing (RNA-Seq). O software CLC Genomics Workbench foi utilizado para alinhar os fragmentos de cada amostra ao genoma bovino de referência (ARS.UCD1.2), bem como para realizar as análises de expressão gênica diferencial. Uma média de 37 mil transcritos foram detectados sendo expressos no músculo de bovinos Nelore. Um total de 14 e 15 isoformas de mRNA foram diferencialmente expressas (P-valor ≤ 0,001) entre o grupo alto marmoreio em relação ao grupo de baixo marmoreio, e entre o grupo de coloração desejável em relação ao grupo de coloração indesejável, respectivamente. A *HBB-201* mRNA foi a única isoforma diferencialmente expressa em comum entre essas duas características. As isoformas de mRNAs: *COL4A2-202, RPL30-203, MAPKAPK2-204, NEURL1-201, SCL16A6-201* e *JSP.1-204* apresentaram variantes estruturais (variantes de nucleotídeo único, inserção ou exclusão), as quais foram responsáveis pela a interrupção de *sites de splicing* nessas regiões. A análise de enriquecimento, realizada para a lista de isoformas de mRNA diferencialmente expressas para marmoreio e para coloração da carne, identificou vias metabólicas comuns entre essas características, como por exemplo, a via de troca de O2/CO2 nos eritrócitos, biossíntese de tirosina e a via de degradação de fenilalanina. Possivelmente, o mecanismo de interação entre essas características dá-se através da oxidação lipídica mitocondrial que está intimamente relacionada à formação de mioglobina férrica, molécula importante para manter a cor fresca da carne vermelha. Os resultados obtidos nesse estudo mostram que as isoformas de mRNAs encontrados podem ser potenciais genes reguladores das características de coloração e marmoreio da carne bovina, as quais são características economicamente importantes para a indústria de carne.

Palavras- chave: isoformas de mRNA, Nelore, RNA-Sequencing, qualidade de carne

4.1 INTRODUCTION

Meat quality traits such as meat color and marbling have an increasing importance because of their strong impact on consumer acceptance as visual indicators of meat freshness and juiciness (González-Prendes et al., 2019; Fonseca et al., 2019). Marbling is an indicator of intramuscular fat (IMF) (Williams, 2008). The IMF content and composition is a desirable trait associated with nutritional and organoleptic properties such as tenderness, flavour and juiciness in both beef and pork meat (Wood et al., 2004; Hausman & Cánovas, 2017). As describe by Jung et al. (2016) there is a high correlation between IMF content and juiciness in steer`s Hanwoo beef breed. Studies in beef cattle have identified that high values of IMF content were associated with meat sensorial properties such as tenderness, flavor and juiciness in Spanish cattle breeds and beef cattle from the Washington State Department of Agriculture (WSDA), respectively (Insausti et al., 2004; Hale et al., 2013; Hunt et al., 2016). In addition, marbling helps to compensate the effects of increased cooking temperatures on beef juiciness (Drey et al., 2019).

The color is another of the main aspect determining the meat quality. Market studies described the consumers' preference is bright red color as indicator of fresh meat (Guerrero et al., 2013). Biochemically, the meat color results from myoglobin (Mb) and hemoglobin (Hb) concentration, as well as the state these proteins and muscle structure (Listrat et al., 2016). Some studies have characterized the biochemical patterns of beef color, trying to explain the multitude of exogenous and endogenous factors interactions with the redox biochemistry of myoglobin in post-mortem skeletal muscles (Suman et al., 2014; McKeith et al., 2016). Gagaoua et al. (2017) studied the relationship between 21 biomarkers and meat color traits in the *Longissimus thoracis* muscle of young Aberdeen Angus and Limousin bulls. They suggested the possibility of using biomarkers to classify meat cuts sampled at early post-mortem. As described by Macini et al. (2018), the mitochondrial oxygen consumption rate and the mitochondrial metmyoglobin reducing activity are muscle-specific, and they could contribute partially to the variations in meat color.

Currently, one of the research's challenges in the field of beef genomics is to find ways to increase reliability and accuracy of selection of individuals with favorable genes associated with meat quality traits. Although molecular and physiological understanding of

complex traits such as beef color and marbling is growing, little is known about the genes determining these traits and their functions, and a significant unexplained source of variation of phenotypes remains in beef cattle (Cánovas et al., 2017; Cao et al., 2019).

The RNA-Sequencing (RNA-Seq) technology contributes to identification of different patterns of candidate genes, and genetic regulation of important regions for the expression of complex traits (César et al., 2015; Berton et al., 2016; Fonseca et al., 2019; Silva et al., 2020). Cardoso et al. (2018) investigated the mRNAs isoforms associated with distinct lipid profiles using skeletal muscle samples from Duroc pigs. The authors concluded that 10.9% of transcripts expressed in muscle were produced through splicing alternative process, creating multiform transcripts from a genome region. Beef quality studies have identified structural variants, such as copy number variants (CNVs) associated with IMF content (de Lemos et al., 2018) and meat tenderness (Lines et al., 2009; Braz et al., 2019). In addition, single nucleotide polymorphisms (SNPs) have been associated to other meat quality traits such as marbling (Tong et al., 2015; Enriquez-Valencia et al., 2017).

Identification of mRNA isoforms regulating marbling and meat color, and detection of structural variants affecting splice sites of these mRNA isoforms, become an important way to integrate structural and functional genomic data. The use of these different methodologies could increase the prediction accuracy of regulator mechanisms of complex traits in beef cattle.

4.2 MATERIAL AND METHODS

4.2.1 Sample collection and phenotype measurements

The most 20 divergent Nellore bulls for each trait (marbling and meat color) were selected from a population of 80 Nellore bulls from the Nellore Qualitas breeding program. The animals belonged to the same contemporary group, i.e. these animals were born in the same year and herd and they were kept in the same management group from birth to yearling (Fonseca et al., 2017). The animals were slaughtered in a commercial slaughterhouse with average of 24 months old.

Samples from *Longissimus thoracis* muscle were collected between the 12th and 13th ribs of the left half carcass. Two samples were obtained at two different times: one sample at

the time of slaughter for RNA extraction, and another sample 24 hours after slaughter to perform marbling and color standard measurements (Fonseca et al., 2017).

The visual marbling scores were evaluated according to USDA Quality Grade methodology (2000), which use a marbling classification scale values ranged from 0 to 10, being zero values applying to absent marbling and 10 corresponding to excessive marbling. According to these scores, the samples were ranked and divided in two groups with 10 animals in each marbling group (HIGH (n=10) and LOW (n=10) marbling values). The LOW marbling group had average of 2.26 ± 0.05 and the HIGH marbling group had average of 3.26 ± 0.12. The groups were statistically different by the *t*-test (p-value < 0.001).

The meat color phenotypes were measured using the CR-400 Colorimeters (Minolta Co. Ltd.). The Renerre methodology (1982), was used to the colorimeter calibration, and the variables *l* a* b** corresponding to blue, green and red colors respectively, were collected. For the meat color evaluation, a system that transform the reflectance and transmittance spectrum of the object, which emit in the primary colors: blue, green and red, then convert them in variables *l* a** and *b** respectively, of a three-dimensional graphic was used. Thus, any color was localized by measuring it in a colorimeter with standardized adjustments of the observer's lamp (MacDougall, 1982).

From these results, the set of variables (*l* a* b**) of color per sample were clustered in two groups, using the k-means clustering algorithm, implemented in the R environment. Then, 10 extreme sample from each group were selected and classified as: desirable group with average (l*=30.5± 0.89; a*=13.68± 0.79; b*=6.35± 0.48) which suit the red bright color; and the undesirable color with average (l*=27.2± 1.6; a*=10.97± 0.58; b*=6.35± 0.38) which trend to a dark color. The groups were statistically different by the *t*-test (p-value < 0.05).

4.2.2 RNA isolation and library construction

The RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia-CA, USA) was used to extract the total RNA from muscle samples, according to the manufacturer's recommendation. The purity of the RNA was determined at A260/230nm and A260/280nm ratios using the NanoDrop 1000 Spectrophotomether (Thermo Fisher Scientific, Santa Clara, CA, USA, 2007). The integrity of the RNA samples was assessed in the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA, 2009) and the RNA concentration and genomic DNA

contamination were determined by Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA, 2010) (Fonseca et al., 2020). The RNA integrity number values ranged from 7.0 to 9.0 in all muscle samples, indicating good RNA quality (Cánovas et al., 2013).

The total RNA, from each sample, was used to prepare the cDNA libraries using the TruSeq RNA Sample Preparation kit® v2 guide (Illumina, San Diego, CA, USA) following the manufacturer's recommendation. Sequencing libraries were quantified using a KAPA Library Quantification kit® (KAPA Biosystems, Foster City, CA, USA), according to Illumina's library quantification protocol. Paired-end (2 x 100 pb) reads were sequence using the HiSeq 2500 sequencer (Illumina, San Diego, CA, USA).

4.2.3 Sequence assembly and quantification

Quality control analysis was performed using the CLC Genomics Workbench software 12.0 (CLC Bio, Aarhus, Denmark). Quality control analysis included GC content, ambiguous base content, phred quality score, base coverage, nucleotide contributions, and over-represented sequence parameters. All samples passed the quality control analysis showing same length (100bp), 100% coverage in all bases, 25% of A, T, G, and C nucleotide contributions, 50% GC base content, and less than 0.1% over-represented sequences (Cánovas et al., 2014).

Paired sequence reads were aligned to the annotated reference genome ARS.UCD1.2 (ftp://ftp.ensembl.org/pub/release-95/genbank/bos_taurus/) using the CLC Genomics Workbench software 12.0 (CLC Bio, Aarhus, Denmark). The parameters used for the assembly included global alignments with 0.8 length fraction and 0.8 similarity fraction; two mismatches; three insertions and three deletions were allowed per read. Transcript levels were quantified in reads per kilobase per million mapped reads (RPKM) and transformed to log10. By normalizing the data for transcript length and total reads in each sample, the RPKM measure facilitated comparisons of transcript levels between both groups (Allison et al., 2006; Mortazavi et al., 2008).

4.2.4 Differential mRNA isoform expression and splice variant effects detection

Differential mRNA isoform expression analysis was performed using CLC Genomics Workbench. The empirical statistical analysis included the following parameters: estimate tag-wise dispersions; comparisons against the genome reference, considering the higher and desirable groups as a reference group in relation to the lower and undesirable group, respectively; and FDR correction to p-value. The common dispersion estimate was $3.38e^{-02}$ and the coefficient of biological variation was $1.84e^{-01}$ for the experiments.

The reads mapping merged by groups, for each trait, were used to identify the structural variants in relation to the bovine genome reference. For it, the tool "fixed ploidy variant detection" implemented in CLC Genomics Workbench environment was used considering the following parameters: minimum frequency (20%); minimum coverage (10 bp); minimum central quality (25 bp); minimum read length (20 bp); minimum neighborhood quality (25 bp); ignore broken pairs reads were allowed (Lam et al., 2020). Then, the "functional consequences" tool was used to predict variants effect into splice site of differential expressed mRNA isoforms (Shirley et al., 2013). This function take into account all variants detected by "fixed ploidy variant detection" as input, and to analyze whether the variants fall within potential splice sites. If a variant fell within two base pairs of an intron-exon boundary, it was annotated as a possible splice site disruption.

4.2.5 Functional annotation and metabolic pathway analysis

The "PANTHER - statistical enrichment test" algorithm with Reactome annotation (version 65) set, was used to perform the Gene Ontology Resource (GO) enrichment analysis of the list of differential mRNA isoforms between high and low marbling values and desirable and undesirable meat color groups (Ashburner et al., 2000). The GO analysis were performed taking into account the three GO categories (biological process, molecular function and cellular component) using Panther (Canovas et al., 2012; Cardoso et al., 2018).

Metabolic pathway analysis were performed using Ingenuity Pathway Analysis software (QIAGEN Bioinformatics). Thresholds of p-value ≤ 0.01, FDR ≤ 0.05, and FC $> \pm 2$ were applied to filter significant genes for function, pathway, and network analyses effects (Cardoso et al., 2017).

4.3 RESULTS

4.3.1 Assembly and quantification of sequences

The sets up used to perform the differential mRNA isoform expression analyses and the variants discovery were: meat color groups = desirable (N=10) and undesirable (N=9); marbling groups= higher (N=10) and lower (N=10). After performing the quality control analysis, one sample from undesirable group was discarded for further analysis.

An average of 47.8 million paired ends per sample, and 84.49% of reads mapped in pairs. Among them, 83.29% and 16.68% mapped reads were located into genic and intergenic regions, respectively (Supplementary table S1).

4.3.2 Differentially expressed mRNA isoforms

A total of 27.525 genes were expressed in *Longissimus thoracis* muscle of Nellore cattle with divergent marbling profiles, producing 37.525 different transcripts. Among the expressed transcripts, 14 mRNA isoforms were differentially expressed between high and low marbling (p-value ≤ 0.001), including 11 down regulated and 3 up regulated (Table 1).

The *HBA-201* mRNA isoform, encodes one form of hemoglobin (hemoglobin, alpha 2), it was differentially expressed only when high and low marbling samples were compared. The *COL4A2-202* (collagen type IV alpha 2 chain), collagen encoder, is related to extracellular matrix structural constituent (Paiva et al., 2018). Also, the *RPL14-202* (ribosomal protein L14), *RPL30-203* (ribosomal protein L30) and *RPS6KA3-203* (ribosomal protein S6 kinase A3), ribosomal protein encoders, are related to structural constituent of ribosome (Huang et al., 2006). The transcripts of the solute carrier (*SLC25A25- 202, SLC25A25- 204* and *SLC16A6-201*), a membrane transport proteins group, are involved to the pyrimidine nucleotide transmembrane transporter activity (Colas et al., 2016), and the *RPS6KA3-203* gene was previously identified differentially expressed between high and low intramuscular fat content (Silva et al., 2020).

Table1. Differentially expressed mRNA isoforms (p-value≤0.001) between extreme groups of marbling in *Longissimus thoracis* muscle from Nellore beef cattle

Gene ID	transcript ID	Transcript Name	Positon	Length (bp*)	p-value	Log2 (FC*)
ENSBTAG00000037644.3	ENSBTAT00000057323.3	HBB-201	15:48362236-48363996	733	$4.2e^{-07}$	-8.92
ENSBTAG00000051412.1	ENSBTAT00000037545.2	HBA-201	25:216448-217264	612	$1.9e^{-05}$	-6.99
ENSBTAG00000002038.5	ENSBTAT00000084955.1	RPL14-202	22:13293116-13296057	642	0.0001	-37.19
ENSBTAG00000013356.6	ENSBTAT00000026750.5	CATHL1-201	22:51579579-51581082	658	0.0001	-6.27
ENSBTAG00000054972.1	ENSBTAT00000070459.1	---	9:4621622-4621918	297	0.0002	1.68
ENSBTAG00000053016.1	ENSBTAT00000044788.3	CATHL4-201	22:51592817-51594193	554	0.0004	-5.42
ENSBTAG00000038107.3	ENSBTAT00000015530.6	MAPKAPK2-204	16:4468141-4514607	1104	0.0007	-1.44
ENSBTAG00000011866.5	ENSBTAT00000015750.5	PCBD1-201	28:27067751-27072908	1131	0.0008	-8.2
ENSBTAG00000016278.6	ENSBTAT00000021651.6	RPL30-203	14:66176419-66194695	439	0.0010	-1.67
ENSBTAG00000003741.6	ENSBTAT00000046515.4	NEURL1-202	26:24126973-24160165	3788	0.0010	-1.38
ENSBTAG00000012710.6	ENSBTAT00000016891.5	GHRH-201	13:66207181-66216486	408	0.0012	19.65
ENSBTAG00000025210.4	ENSBTAT00000005916.5	COL4A2-202	12:85040120-85153241	4590	0.0012	1.66
ENSBTAG00000002635.4	ENSBTAT00000003414.4	PGLYRP1-201	18:53513706-53515925	844	0.0014	-4.82
ENSBTAG00000026848.2	ENSBTAT00000038384.2	---	3:11350806-11351297	492	0.0019	-4.24

*bp: base pair; FC: Fold Change.

A total of 27.571 genes were expressed in the *Longissimus thoracis* muscle of animals with color variant from bright red to dark red, generating 37.526 transcripts that produce an average of 1.36 mRNA isoforms for each gene. Comparing the desirable and undesirable color groups, 15 mRNA isoforms were differentially expressed (p-value ≤ 0.001), including 8 down regulated and 7 up regulated (Table 2).

The *ALDOA-201* (transcripts aldolase, fructose-bisphosphate), *MAPKAPK2-204* (mitogen-activated protein kinase-activated protein kinase 2) and *PCBD1-201* (pterin-4 alpha-carbinolamine dehydratase 1) genes are involved in the glucose, kinase, serine and tetrahydrobiopterin biosynthesis metabolism, respectively. The transcripts *PITX1-201* (paired like homeodomain 1), *FOSB-201* (FosB proto-oncogene, AP-1 transcription factor subunit), *FOXO1-201* (forkhead box O1), *ATP1A2-201* (ATPase Na+/K+ transporting subunit alpha 2) and *GOLGA4-205* (golgin A4) are related to DNA and nucleotide binding (Liu et al., 2019; Wang et al., 2019; Fittal et al., 2018; Schwarz et al., 2018; Sohda et al., 2015). Silva et al. (2019) identified an association of the *FOXO1* gene with intramuscular fat content.

The *NEURL1-202* (neuralized E3 ubiquitin protein ligase 1) mRNA isoform is related to ligase activity and translation factor activity (Taal et al., 2019). The *PGLYRP1-201* (peptidoglycan recognition protein 1), *JSP.1-204* (Bos taurus uncharacterized protein 100125016 (LOC100125916), *CATHL1-201* (cathelicidin 1), and *CATHL4-201* (cathelicidin 4) are involved to innate immune system and defenses activity (Whelehan et al., 2014). The *SPARCL1-201* transcript is related to calcium binding (Viloria et al., 2016), and *BMP5-201* (bone morphogenetic protein 5) is related to cytokine activity and transforming growth factor beta receptor binding, such as *GHRH-201* (growth hormone releasing hormone) is include to the hormone activity and growth hormone-releasing hormone receptor binding (Ricci et al., 2010; Leone et al., 2019). The *NREP-201* transcript (neuronal regeneration related protein) has functions in cellular differentiation (Yang et al., 2015).

The *HBB-201* (Hemoglobin, beta) is the only mRNA isoform differently expressed for marbling and color traits, having been down regulated in both of them.

Table 2. Differentially expressed mRNA isoforms (p-value≤0.001) between extreme groups of beef color in *Longissimus thoracis* muscle from Nellore beef cattle

Feature ID	Feature transcript ID	Transcript Name	Position	Length (bp*)	p-value	Log2 (FC*)
ENSBTAG00000020116.6	ENSBTAT00000082227.1	JSP.1-204	23:28664115-28667367	1179	$1.39e^{-05}$	-26.83
ENSBTAG00000003074.6	ENSBTAT00000003997.6	SLC16A6-201	19:61867803-61877805	1896	0.0002	3.02
ENSBTAG00000011912.6	ENSBTAT00000032751.5	SLC25A25-202	11:98712579-98748930	3403	0.0002	1.75
ENSBTAG00000011912.6	ENSBTAT00000015802.5	SLC25A25-204	11:98712579-98748930	3343	0.0004	1.84
ENSBTAG00000011338.4	ENSBTAT00000015066.4	NREP-201	10:2213062-2245525	1943	0.0004	-2.10
ENSBTAG00000037644.3	ENSBTAT00000057323.3	HBB-201	15:48362236-48363996	733	0.0005	-6.65
ENSBTAG00000012927.5	ENSBTAT00000067155.1	ALDOA-201	25:26212553-26218476	1098	0.0006	3.38
ENSBTAG00000016563.6	ENSBTAT00000076004.1	GOLGA4-205	22:10776195-10885548	6849	0.0006	1.82
ENSBTAG00000004602.6	ENSBTAT00000006039.6	PITX1-201	7: 46474432-46481011	2334	0.0009	-6.44
ENSBTAG00000012870.6	ENSBTAT00000017102.6	BMP5-201	23:4470532- 4614742	3044	0.0010	-1.94
ENSBTAG00000004094.3	ENSBTAT00000005348.3	SPARCL1-201	6:102370630-102423199	2612	0.0012	-1.43
ENSBTAG00000008182.6	ENSBTAT00000010762.6	FOSB-201	18:53074888-53081288	3218	0.0012	-3.01
ENSBTAG00000010551.5	ENSBTAT00000049161.3	ATP1A2-201	3:9528257-9552859	5089	0.0013	-1.61
ENSBTAG00000044105.3	ENSBTAT00000061127.3	FOXO1-201	12:21900230-21991693	3306	0.0013	1.91
ENSBTAG00000017639.5	ENSBTAT00000023465.5	RPS6KA3-203	X:122949194-123056196	3353	0.0017	1.63

*bp: base pair; FC: Fold Change.

4.3.3 Splice variant effecting differential expressed mRNAs

For marbling, 4,785 variants (high group) and 4,782 (low group) were identified. A total of 2,109 variants (desirable color) and 1,792 (undesirable group) were identified for color trait (supplementary table 2). The total number of variants identified in each group and those overlapping between groups for both marbling (fig. 1A) and color (fig. 1B) traits were shown in Figure 1. The structural variants identified include single nucleotide variants (SNV), insertion, and deletion variant type.

Table 3 and 4 shown the structural variants with disruption effect on splicing sites of differentially expressed mRNA isoform between high and low marbling groups, and desirable and undesirable color groups, respectively. A total of 19 and 6 structural variants were identified for marbling and color groups, respectively.

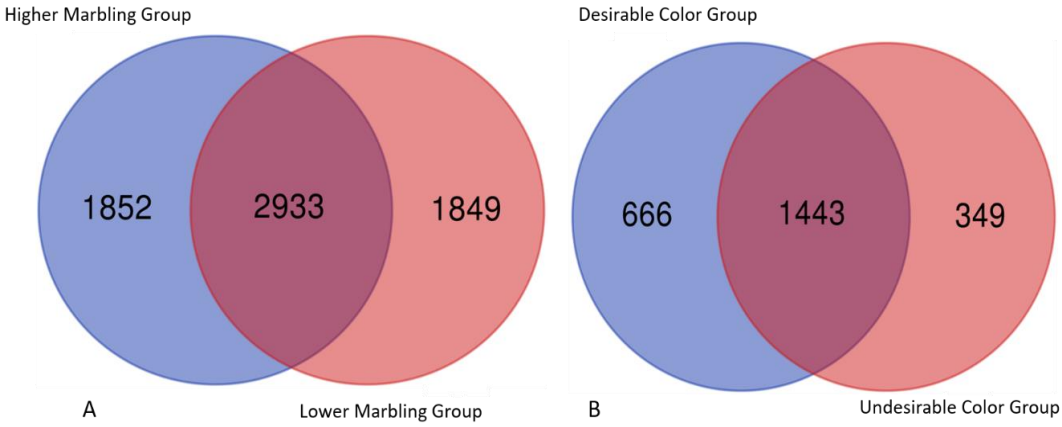

Figure 1. Total number of structural variants (SNVs, Indels and replacement) causing possible splice site disruption in several genes across whole genome of in phenotypically distinct animals for the (A) marbling trait and (B) color trait groups.

For marbling trait, 9 and 9 unique structural variants were identified in high marbling and low marbling groups, respectively. The SNV located on chromosome 12 (85,104,867 bp) was the only one common (non-synonyms) structural variant between these groups, which had been causing an amido acid changes [ENSBTAP00000005916.5:p.His149Gln; ENSBTAP00000063030.1:p.His147Gln; ENSBTAP00000072665.1:p.His180Gln; ENSBTAP00000073525.1:p.His149Gln; ENSBTAP00000063657.1:p.His158Gln]. Those variants were SNV, deletion or insertion type, and were located in chromosomes 12, 13, 14, 16, 18, 22 and 26 (Table 3). The differential expressed isoforms *COL4A2-202* presented

several variants (deletion, insertion and SNV) in different sites, which were unique between the distinct marbling groups (Table 3).

In the higher marbling group, beyond *COL4A2-202* isoform with three SNV [(G) 85,104,867 bp, (C) 85,119,139 bp and (C) 85,145,937 bp], three more mRNA isoforms are having variants, as *RPL30-203* with three SNV located in chromosome 14 [(T) 66,176,417 bp, (A) 66,177,009 bp and (T) 66,178,624 bp], *MAPKAPK2-204* with two insertions located in chromosome 16 [(G) 4510675^4510676 bp and (GT) 4,511,320^4,511,321bp] and *NEURL1-202*, which had thymine (T) insertion in chromosome 26 (24,157,812^24,157,813 bp) and one deletion (24,127,007…24,127,008) in chromosome 26. In addition, the SNV (C) located on chromosome 12 (85145937 bp) produced a possible splice site disruption effect in the *COL4A2* gene in animals form the high marbling group. This SNV is a non-synonyms mutation, which caused an amino acid change [ENSBTAP00000005916.5:p.Glu1088Asp] of Collagen alpha-2(IV) chain protein, such another proteins [ENSBTAP00000072665.1:p.Glu1086Asp; ENSBTAP00000063030.1:p.Glu1089Asp].

For low marbling group, beyond *COL4A2-202* isoform, another five more mRNA isoforms are having variants, as *GHRH-201*, which had a deletion *(*66,216,424 bp), *RPL30-203* with one SNV located in chromosome 14 [(T) 66,176,417 bp], *MAPKAPK2-204* with one deletion located in chromosome 16 (4,468,904 bp), *PGLYRP1-201* with one insertion of three base (GCA) located in chromosome 18 (53,511,935^53,511,936 bp) and *RPL14-202*, which had adenine (A) insertion in chromosome 22 (13,295,507^13,295,508 bp). In addition, we observed that animals from the low marbling group presented a non-synonyms deletion (85,100,930..85,100,931pb) in the *COL4A2* gene, which had caused an amino acid change in the COL4A2 protein [ENSBTAP00000005916.5:p.Pro113fs], and in the another proteins [ENSBTAP00000063030.1:p.Pro111fs; ENSBTAP00000072665.1:p.Pro144fs; ENSBTAP00000063657.1:p.Pro122fs].

Table 3. Structural variants affecting splice sites of differentially expressed mRNA in high or low marbling group.

High marbling group									
mRNA	Chr[1]	Region	Type	Ref[2]	Allele	Genotype	Freq[3]	Prob[4]	Non-synonymous
COL4A2-202	12	85104867	SNV*	C	G	G/G	99.42	1.00	Yes
		85119139	SNV[5]	G	C	C/G	50.00	0.99	-
		85145937	SNV	G	C	C/G	30.00	0.98	Yes
RPL30-203	14	66176417*	SNV	A	T	T/A	92.02	1.00	-
		66177009	SNV	G	A	A/G	42.35	1.00	
		66178624	SNV	A	T	T/A	54.71	1.00	
MAPKAPK2-204	16	4510675^4510676	Insertion	-	G	-/G	26.66	0.99	-
		4511320^4511321	Insertion	-	GT	GT/GT	84.21	1.00	
NEURL1-202	26	24127007..24127008	Deletion	GT	-	-/-	71.74	1.00	-
		24157812^24157813	Insertion	-	T	-/T	23.08	0.98	
Low marbling group									
mRNA	Chr[1]	Region	Type	Ref[2]	Allele	Genotype	Freq[3]	Prob[4]	Non-synonymous
COL4A2-202	12	85042926	SNV	C	G	G/G	98.90	1.00	-
		85044980^85044981	Insertion	-	G	-/G	45.45	0.99	
		85100930..85100931	Deletion	CT	-	complex	35.56	1.00	Yes
		85104867	SNV*	C	G	G/G	98.46	1.00	Yes
		85150772^85150773	Insertion	-	A	-/A	22.85	0.99	-
GHRH-201	13	66216424	Deletion	T	-	-/-	91.66	0.99	-
RPL30-203	14	66176417*	SNV	A	T	T/T	86.16	1.00	-
MAPKAPK2-204	16	4468904	Deletion	T	-	-/T	26.31	0.98	-
PGLYRP1-201	18	53511935^53511936	Insertion	-	GCA	Complex	49.05	1.00	-
RPL14-202	22	13295507^13295508	Insertion	-	A	-/A	30.0	0.99	-

[1]Chromosome; [2] Genome Reference; [3]Frequency (the number of paired reads supporting the allele divided by the number of paired reads covering the position of the variant); [4] Probability (probability of the different possible configurations at each site); [5] single nucleotide variant;

*Structural variant common between high and low marbling groups.

For color trait, 1 structural variants common between desirable and undesirable color groups and 4 unique structural variants were identified for the undesirable color groups. Those variants were deletion, insertion and SNV type and were located in chromosomes 6, 19, 22, 23 and 25 (Table 4). The *JSP.1-204* differentially expressed mRNA isoform between desirable and undesirable color (Table 4), presented the SNV in chromosome 23 (28,664,247 bp) in animals with desirable and undesirable meat color, respectively. This SNV is a non-synonyms mutation that had been causing an amino acid changes [ENSBTAP00000071621.1:p.Trp346Cys] of JSP.1 protein as well as has been changed the proteins [ENSBTAP00000070120.1:p.Trp349Cys; ENSBTAP00000011795.3:p.Arg346Ser].

For undesirable color group, beyond *JSP.1-204* isoform, 4 another mRNA isoforms are having variants, as *SLC16A6-201* isoform had a thymine (T) insertion in chromosome 19 (61,867,891^61,867,892 bp). The *ALDOA-201* and *GOLGA4-205* isoforms had an insertion in the chromosomes 25[(T) 26,218,110^26,218,111 bp) and 22 [(C) 10,776,267^10,776,268 bp] in animals with undesirable meat color, respectively.

All those variants detected for marbling and beef color showed a possible splice site disruption effect (Supplementary Table 2) in the statistical enrichment test (p-value < 0.01).

Table 4. Structural variants affecting splice sites of differentially expressed mRNA in desirable or undesirable color group.

Desirable color group									
mRNA	Chr[1]	Region	Type	Ref[2]	Allele	Genotype	Freq[3]	Prob[4]	Non-synonymous
JSP.1-204	23	28664247	SNV*	C	G	G/G	78.29	1.00	Yes
Undesirable color Group									
mRNA	Chr[1]	Region	Type	Ref[2]	Allele	Genotype	Freq[3]	Prob[4]	Non-synonymous
SLC16A6-201	19	61867891^61867892	Insertion	-	T	T/T	86.01	1.00	-
JSP.1-204	23	28664247	SNV*	C	G	G/G	92.11	1.00	Yes
ALDOA-201	25	26218110^26218111	Insertion	-	T	-/T	70.65	1.00	-
GOLGA4-205	22	10776267^10776268	Insertion	-	C	-/C	28.57	0.99	-
SPARCL1-201	6	102379112	Deletion	A	-	A/-	25.0	0.93	-
		102387648	SNV[5]	A	G	A/G	25	0.96	

[1]Chromosome; [2] Genome Reference; [3]Frequency (the number of paired reads supporting the allele divided by the number of paired reads covering the position of the variant); [4] Probability (probability of the different possible configurations at each site); [5]single nucleotide variant;

*Structural variant common between high and low marbling groups

4.3.4 Functional annotation and pathways analysis

Functional analysis using the list of 29 differently expressed mRNA isoforms (Table 1) were performed using Gene Ontology Resource (http://geneontology.org/), showing 21 mapped ID genes, 4 unmapped (*JSP, NREP, ENSBTAG00000054972* and *ENSBTAG00000026848*) and 2 multiple mapping information (*RPL14* and *COL4A2*). The functional classification of differentially expressed mRNA isoforms for the three main GO categories such as biological process, molecular function and cellular component were shown in figure 2. Biological regulation, cell proliferation and cell process were the most enriched GO terms in molecular function category (Figure 2A). Among the biological process category, binding, catalytic activity and structural molecule activity were the most enriched GO terms (Figure 2B). Regarding the cellular component, cell, extracellular region and organelle were the most enriched GO terms (Figure 2C).

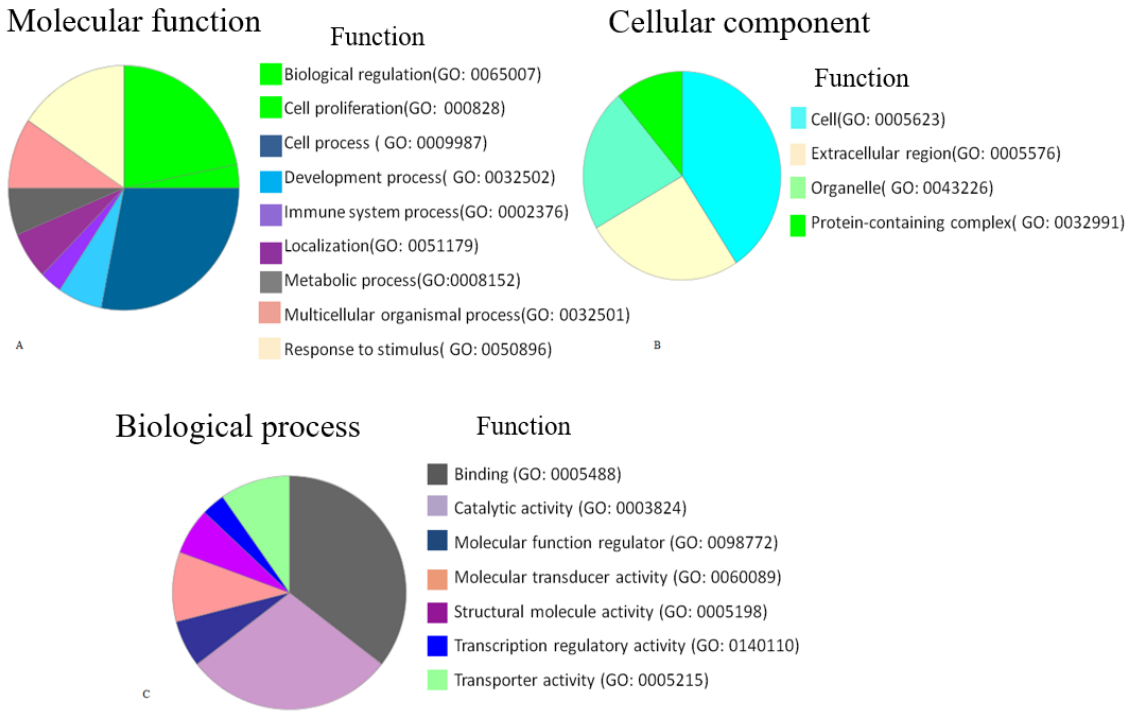

Figure 2. Functional classification of the mRNAs isoforms differentially expressed (FDR<0.05), in the *Longissimus thoracis* muscle for the three main GO categories (A) biological process, (B) molecular function and (C) cellular component.

Metabolic pathway analysis identified 34 significant pathways (p-value< 0.01; supplementary table 3). The top 4 most significant metabolic pathways were VDR/001 RXR Activation, tyrosine biosynthesis IV, phenylalanine degradation I (Aerobic) and p38 MARK signaling (p-value< 0.001). These pathways have functions related to skeletal and muscular disorders, connective tissue disorders, and cellular development and, cardio and hepatic functions.

4.4 DISCUSSION

The average of mRNAs isoforms expressed in the *Longissimus thoracis* muscle was 1.36 per gene, showing a lower mRNA diversity expression in muscle tissue compared to other species (Cardoso et al., 2018). Some studies reported low number of transcripts per gene in primary tissues, e.g. skeletal muscle (Gonzalez-Porta et al., 2013), that could explain the low number of differential mRNA isoforms detected and the low distribution of isoforms per gene such as, supposedly, reduced number of proteins constituent of muscle tissue. Lindholm et al. (2014) detected an average of two mRNA isoforms per gene in the human *Longissimus thoracis* muscle. Cardoso et al. (2018) detected an average of 2.9 mRNA isoforms per genes in the same muscle tissue in porcine. However, we did not find studies reporting averages of mRNAs isoforms expressed in bovine *Longissimus thoracis* muscle.

Specific mRNA isoforms of genes with key functions involved in the meat coloring mechanisms, as encoders of hemoglobin, collagen and ATPase (*ATP1A2* (ATPase Na+/K+ Transporting Subunit Alpha 2), *HBA* (hemoglobin, alpha 2), *HBB* (Hemoglobin, beta), and *COL4A2* (collagen type IV alpha 2 chain)) were identified. Some of these mRNA isoforms are potentially polymorphic and have several biochemical functions that could be used as biological markers to identify animals with adequate color and marbling patterns.

The *COL4A2-202, MAPKAPK2-204, RPL30-203* and *NEURL1-202* mRNA isoforms were differentially expressed between animals with highest intramuscular fat in relation to animals with a lowest intramuscular fat, presenting different structural variants within its encode sites. Those mRNAs isoforms are variations of collagen type IV alpha 2 (*COL4A2*) gene, mitogen-actived protein (*MAPKAPK2*), neuralized E3 ubiquitin protein ligase 1(*NEURL1*) and

ribosomal protein L30 (*RPL30*) genes, respectively. They are involved with the collagen metabolic process and angiogenesis (Karsdal et al., 2017); the regulation of protein serine/threonine kinase activity (Mogemark et al., 2019), 3`- UTR- mediated mRNA stabilization (McLoughlin et al., 2019); the regulation of long-term neuronal synaptic plasticity and synapse maturation (Taal et al., 2019). These are important functions in the cellular regulation and tissue formation that could influence the meat composition, deposition of the intramuscular fat, muscle and connective tissues, and the coloring of beef that is dependent of the accumulation of the myoglobin in the tissues.

The collagen protein are present in approximately 20 – 30 % of the total corporal protein, constituting the main structural element of tissues in vertebrate animals, especially type IV, in basal membrane (Zeugolis & Raghunath, 2011). This protein is abundant in the connective tissue (Halper & Kjaer, 2014), which could be affecting directly all the meat quality traits in beef such as color, succulence and tenderness (Ebarb et al., 2016). Here, we detected a splice donor variant of exon 4 in *COL4A2* common to low and high marbling groups. Animals in the higher marbling group were heterozygotes for two SNV (C/G genotype), one those located on chromosome 12 (85,145,937 bp) causes an amino acid changes of COL4A2 protein, such as, the animals in the lower marbling group had one SNV in the intron 5 of this gene, two insertion and one deletion (CT), this last one has been caused an amino changes in the COL4A2 protein. These SVs presented a splice site disruption effect coding the *COL4A2*. Cesar et al. (2015) associated the *COL4A2* gene with intramuscular fat content in Nellore cattle. Therefore, could be used to identify animals with high fat deposition, which probably reduce connective tissues concentration in the muscle providing the beef succulence characteristic.

In addition, animals with highest marbling score showed SNVs [A/G (66,178,624 bp) and T/A (66,177,009 bp) genotypes] in a splice donor site variant of intron and exon 3 of *RPL30* gene, respectively; a guanine insertion (genotype: -/G) in the exon 3 and a guanine (G) and thymine (T) (genotype: GT/GT) in intron 4 of the *MAPKAPK2-204* transcript, and a deletion of GT in intron 1, one T insertion in chromosome 26 (24,157,812^24,157,813) in exon 6, which is in overlapping with a CNV (p.: 26:24157343-24287577) (NCBI, 2019) in the *NEURL1-202* transcript region. Those variants are important to determine which coding regions could be used, in the future, to select animals with better meat quality in Nellore cattle.

For the color trait a smaller number of structural variants were found than for marbling trait. However, those variants were within key regulator genes in the transcriptional regulation process. Both color groups presented a SNV (G) in the exon 6 of *JSP.1-204* mRNA isoform, this SNV has been caused an amido acid changes of JSP.1 protein (ENSBTAP00000071621.1). In bovine, the *JSP.1* gene has been identified in study of signatures of selection regarding the adaptive immune responses in Muturu cattle, a West African *Bos taurus* (Tijjani et al., 2019). Fonseca et al. (2019) found that the *JSP.1* gene, as well as, the *HBB*, *PCBD1*, and *NREP* genes, also identified in this study, were associated with intramuscular fat content in Nellore cattle. However, we did not find studies that identified mRNA isoforms differentially expressed associated with meat color in beef cattle. Thus, our findings could provide new insights to better understanding the genetic architecture of meat color in beef cattle.

Functional annotation (figure 2) and pathways analysis (supplementary table 3) were performed on a list with all differentially expressed annotated mRNA isoforms (marbling and color traits). Here, several GO terms involved in cell proliferation as the main molecular function (figure1A) were identified; binding and catalytic activity like as top biological processes and cell as principal GO term in the cellular component GO category (figure1C). The top five significant metabolic pathways (supplementary table 3) using Reactome, and the top four canonical pathways using IPA software are related to scavenging of heme from plasma, the O2/CO2 exchange in erythrocytes; maintaining calcium and phosphate, tyrosine synthesis and phenylalanine degradation. These results are consistent with Cardoso et al. (2018) that identified differentially expressed mRNA isoforms in the skeletal muscle of pigs, with distinct fatness profiles. Moreover, our results (supplementary table 3) were in concordance with other authors (Berton et al., 2016; Silva et al., 2020) that identified biological pathways related to fat development, cell adhesion, cell differentiation, and immune system in Nellore cattle.

The CREB phosphorylation was one of the pathways identified in the functional analysis (supplementary table 3). The cAMP response element-binding protein that binds to certain DNA sequences called cAMP response elements (CREB), thereby increasing or decreasing the transcription of genes (Montminy & Bilezikjian, 1987), such as *FOSB* (FosB Proto-Oncogene, AP-1 Transcription Factor Subunit) and *MAPKAPK2*(mitogen-activated-protein kinase-activating protein kinase) genes. In this study, we identified differentially expressed *FOSB-201* and

MAPKAPK2-204 mRNA isoforms (Table 2). As described by Dunkley et al. (2004), the *MAPKAPK2* gene has a preference for Ser40 and Ser19 that can be provided through the CREB phosphorylation.

In this study, the *HBA-201* (hemoglobin, alpha 2) mRNA isoform was differentially expressed between desirable and undesirable color groups in beef cattle. Tang et al. (2005) related a potential interaction between myoglobin and lipid oxidation. Faustman et al. (2010) related that dissolved oxygen in meat maturation could be decreased by lipid oxidation, as well as, occur in modification of beef color (Ramanathan et al., 2011). Thus, could have an interaction between the color and intramuscular fat metabolism pathways. In addition, tyrosine biosynthesis pathways are involved to conversion of the amino acid L-tyrosine to L- DOPA and for this process are used molecular oxygen (O^2) and iron (Fe^{+2}) (Nakashima et al., 2009). The Fe^{+2} molecule is an important factor during the meat maturation process and can modify the beef color through its oxidation. Besides that, phenylalanine and tyrosine present in pigment cells have crucial roles to the animal body pigmentation (Słominski et al., 1988). The tyrosine biosynthesis and phenylalanine degradation are involved in pigmentation of skin, hair and eyes (Sabatino et al., 2013). Thus, they could be important factors in the pigmentation determination of beef color as well.

4.5 CONCLUSION

We have identified potential candidate mRNA isoforms (*HBA-201, HBB-201, COL04A2-202, RPL30-203, MAPKAPK2-204* and *JSP.1-204*) associated with marbling and color of meat. These regions are holding structural variants that hide the splicing sites, which determine the expression and the functionality of these mRNA isoforms.

Thus, our results suggest that, if validated in another populations, the structural variants identified here, probably could be used as biological markers in the identification of genetically superior animals for marbling and beef color traits. Furthermore, the functional analysis using the list of differentially expressed mRNA isoforms, suggested that lipid oxidation and myoglobin oxidation are two process linked to both marbling and meat color traits. These traits are possibly

controlled by the same genetic mechanisms in Nellore beef cattle, which could be improved together.

These genomic areas would be highly suitable for further studies in order to validate biological markers and candidate genes associated with marbling and color of meat in Nellore cattle.

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4.7 SUPPLEMENTARY MATERIAL

Table S1. Descriptive statistics of read's alignment to animals Nellore divergent for marbling trait

Phenotype[1]	Read count	Map pairs %	Broken pairs %	Non-mapped %	Map to genes %	Mapped to intergenic %
1HMAR	35,050,386	84.29	13.70	2.02	82.09	17.91
2HMAR	28,773,506	84.18	14.00	1.82	81.73	18.27
3HMAR	29,476,234	87.25	11.05	1.70	85.55	14.45
4HMAR[2]	27,795,678	85.69	12.18	2.13	81.96	18.04
5HMAR[2]	55,135,382	86.42	12.15	1.43	84.63	15.37
6HMAR	75,881,468	86.13	12.58	1.29	80.48	19.52
7HMAR	64,168,186	85.25	13.39	1.35	81.59	18.41
8HMAR	27,348,988	84.64	12.95	2.41	80.02	19.98
9HMAR	63,702,988	89.29	9.14	1.57	86.18	13.82
10HMAR	28,340,362	84.69	12.52	2.80	83.98	16.02
Means	**43,567,317.8**	**85.78**	**12.36**	**1.85**	**82.82**	**17.17**
1LMAR	27,130,572	87.78	9.71	2.51	83.16	16.84
2LMAR	71,967,572	83.88	14.26	1.86	81.67	18.33
3LMAR	49,778,354	87.21	10.85	1.95	82.77	17.23
4LMAR	28,048,904	87.07	11.23	1.71	83.04	16.96
5LMAR	61,120,288	86.32	11.91	1.77	83.41	16.59
6LMAR	65,291,544	89.44	9.53	1.04	84.73	15.27
7LMAR	28,049,670	80.44	16.87	2.68	79.21	20.79
8LMAR	57,446,134	87.87	11.02	1.10	83.57	16.43
9LMAR	65,794,210	84.75	12.67	2.57	80.34	19.66
10LMAR[2]	63,166,744	84.76	13.86	1.38	78.98	21.02
Means	**51,779,399.2**	**85.95**	**12.19**	**1.85**	**82.08**	**17.91**

[1] HMAR= high marbling group; LMAR= low marbling group.
[2] Samples were selected to both traits (marbling and meat color).

Table S1. Descriptive statistics of read's alignment to animals Nellore divergent for meat color trait

Phenotype[1]	Read count	Map pairs %	Broken pairs %	Non-mapped %	Map to genes %	Mapped to intergenic %
1HMCO[3]	55,135,382	85.86	11.98	2.17	84.7	15.3
2HMCO	32,211,976	85.1	12.41	2.49	83.26	16.74
3HMCO	99,130,396	82.97	15.32	1.71	81.09	18.91
4HMCO	73,081,816	85.03	13.16	1.81	81.66	18.34
5HMCO	54,345,410	85.51	12.52	1.97	82.18	17.82
6HMCO	68,215,916	84.95	12.08	2.97	81.79	18.21
7HMCO	27,348,988	83.62	13.05	3.33	80.03	19.97
8HMCO	51,620,850	85.91	11.41	2.68	79.76	20.24
9HMCO[3]	63,166,744	86.14	11.83	2.02	79.01	20.99
10HMCO	71,967,572	83.88	14.26	1.86	81.67	18.33
Means	**59,622,505**	**84.9**	**12.8**	**2.3**	**81.5**	**18.5**
1LMCO	35,050,386	84.17	13.03	2.79	82.17	17.83
2LMCO	75,433,680	86.89	10.55	2.56	83.2	16.8
3LMCO	58,505,198	85.84	12.25	1.91	81.12	18.88
4LMCO	27,849,388	85.00	11.98	3.02	80.04	19.96
5LMCO	54,802,708	87.19	10.87	1.94	81.86	18.14
6LMCO	57,446,470	85.44	12.36	2.21	81.46	18.54
7LMCO[3]	27,795,678	85.08	11.98	2.94	82.04	17.96
8LMCO	22,195,258	84.44	13	2.56	86.07	13.93
9LMCO	31,085,494	86.93	10.19	2.88	82.34	17.66
10LMCO[2]	60,910,00	78.45	15.04	6.51	84.32	15.68
Means	**39,022,517**	**84.94**	**12.12**	**2.93**	**82.46**	**17.53**

[1] HMCO= high meat color group; LMCO= low meat color group.

[2] Sample excluded to the differential expression test.

[3]Samples were selected to both traits (marbling and meat color).

Table S2. Enrichement Analysis performed to DE mRNA isoforms list for Marbling and Color of meat

Reactome pathways	Bos taurus REFLIST (22008)	upload_1 (25)	upload_1 (expected)	upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw p-value)
CREB phosphorylation (R-BTA-199920)	7	2	0.01	+	> 100	4.43E-05
Erythrocytes take up oxygen and release carbon dioxide (R-BTA-1247673)	11	2	0.01	+	> 100	9.57E-05
Erythrocytes take up carbon dioxide and release oxygen (R-BTA-1237044)	15	2	0.02	+	> 100	1.66E-04
O2/CO2 exchange in erythrocytes (R-BTA-1480926)	15	2	0.02	+	> 100	1.66E-04
Scavenging of heme from plasma (R-BTA-2168880)	19	2	0.02	+	92.67	2.56E-04
Nuclear Events (kinase and transcription factor activation) (R-BTA-198725)	21	2	0.02	+	83.84	3.08E-04
MAPK targets/ Nuclear events mediated by MAP kinases (R-BTA-450282)	27	2	0.03	+	65.21	4.92E-04
Binding and Uptake of Ligands by Scavenger Receptors (R-BTA-2173782)	35	2	0.04	+	50.3	8.03E-04
MAP kinase activation (R-BTA-450294)	50	2	0.06	+	35.21	1.58E-03
Signaling by NTRK1 (TRKA) (R-BTA-187037)	56	2	0.06	+	31.44	1.97E-03
Interleukin-17 signaling (R-BTA-448424)	58	2	0.07	+	30.36	2.10E-03
Toll Like Receptor 3 (TLR3) Cascade (R-BTA-168164)	66	2	0.07	+	26.68	2.69E-03
Signaling by NTRKs (R-BTA-166520)	70	2	0.08	+	25.15	3.01E-03
Toll Like Receptor 5 (TLR5) Cascade (R-BTA-168176)	70	2	0.08	+	25.15	3.01E-03
MyD88 cascade initiated on plasma membrane (R-BTA-975871)	70	2	0.08	+	25.15	3.01E-03
Toll Like Receptor 10 (TLR10) Cascade (R-BTA-168142)	71	2	0.08	+	24.8	3.09E-03
Toll Like Receptor TLR1:TLR2 Cascade (R-BTA-	71	2	0.08	+	24.8	3.09E-03

168179)						
Toll Like Receptor TLR6:TLR2 Cascade (R-BTA-168188)	71	2	0.08	+	24.8	3.09E-03
MyD88:Mal cascade initiated on plasma membrane (R-BTA-166058)	71	2	0.08	+	24.8	3.09E-03
Toll Like Receptor 2 (TLR2) Cascade (R-BTA-181438)	71	2	0.08	+	24.8	3.09E-03
Innate Immune System (R-BTA-168249)	901	5	1.02	+	4.89	3.11E-03
TRAF6 mediated induction of NFkB and MAP kinases upon TLR7/8 or 9 activation (R-BTA-975138)	73	2	0.08	+	24.12	3.26E-03
MyD88 dependent cascade initiated on endosome (R-BTA-975155)	75	2	0.09	+	23.48	3.43E-03
Toll Like Receptor 7/8 (TLR7/8) Cascade (R-BTA-168181)	75	2	0.09	+	23.48	3.43E-03
Toll Like Receptor 9 (TLR9) Cascade (R-BTA-168138)	79	2	0.09	+	22.29	3.79E-03
MyD88-independent TLR4 cascade (R-BTA-166166)	89	2	0.1	+	19.78	4.76E-03
TRIF(TICAM1)-mediated TLR4 signaling (R-BTA-937061)	89	2	0.1	+	19.78	4.76E-03
Regulation of beta-cell development (R-BTA-186712)	4	1	0	+	> 100	5.66E-03
AKT-mediated inactivation of FOXO1A (R-BTA-211163)	4	1	0	+	> 100	5.66E-03
Regulation of gene expression in beta cells (R-BTA-210745)	4	1	0	+	> 100	5.66E-03
Toll Like Receptor 4 (TLR4) Cascade (R-BTA-166016)	110	2	0.12	+	16.01	7.12E-03
RSK activation (R-BTA-444257)	6	1	0.01	+	> 100	7.92E-03
Cellular Senescence (R-BTA-2559583)	126	2	0.14	+	13.97	9.21E-03
Signaling by Receptor Tyrosine Kinases (R-BTA-9006934)	389	3	0.44	+	6.79	9.61E-03

CHAPTER 5 – FINAL CONCLUSIONS

According with the results related in the previous chapters this study contribute to better understanding of the functional genomics behind beef quality regulation and for the identification of potential candidate genes and mRNA isoforms in beef cattle.

The differentially expressed genes and mRNA isoforms, as well as novel transcripts related to beef quality regulation in Nellore cattle were identified. Among these genes may be selected as candidate genes to beef tenderness, are myosin encoders (*MYL6* and *MYBPH*), and tripartite motif encoders (*TRIM63* and *TRIM55*), *TRIOBP* and *CHRNG* genes that were identified in the differential gene expression analysis performed to animals with high MFI (tender beef) in relation to tough beef animals (Chapter 2). The differentially expressed mRNA isoforms detected to using two methods to measure tenderness [i.e., WBSF and MFI (chapter 3)] were identified, some of those were related to the myosin encoders and other genes such as *MYOT*, *CASQ1*, *TPM2*, *MB*, *SYNM* genes, and *SYNPO*-201 mRNA isoform,those identified using WBSF phenotypes, and the mRNA isoform associated with the *NEB* and *FGFRL1* genes, as well as the *ANKRD23*-202 mRNA isoform (detected by MFI analysis). In addition, we have identified potential candidate mRNA isoforms (*COL4A2*-202, *RPL30*-203, *MAPKAPK2*-204 and *JSP.1*-204) associated with marbling and color of meat. These last ones are holding structural variants that hide the splicing sites, which determine the expression and the functionality of these mRNA isoforms.

The transcripts and structural variants here found contributed to identification of important functional information associated with biological functions related to oxidative process, energy production and striated muscle contraction, muscle components, lipid and myoglobin oxidation and oxygen homeostasis (i.e., HIF-1 signaling pathway). Thus, these results allowed us to filter specific genomic regions to better guide the studies of meat quality traits, contributing to abridge future researches, providing candidate regions related to beef quality. In addition, these results awaken our thinking to the connection among these traits, as well as for the importance of to understand how those traits wok as a trait set. The acquisition of that knowledge

could contribute to identify universal markers that could be useful for animals' selection for these trait set that composed the quality meat.