Spliced genes in muscle from Nelore Cattle and their association with carcass and meat quality

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Transcript data obtained by RNA-Seq were used to identify differentially expressed alternatively spliced genes in ribeye muscle tissue between Nelore cattle that differed in their ribeye area (REA) or intramuscular fat content (IF). A total of 166 alternatively spliced transcripts from 125 genes were significantly differentially expressed in ribeye muscle between the highest and lowest REA groups (p ≤ 0.05). For animals selected on their IF content, 269 alternatively spliced transcripts from 219 genes were differentially expressed in ribeye muscle between the highest and lowest IF animals. Cassette exons and alternative 3′ splice sites were the most frequently found alternatively spliced transcripts for REA and IF content. For both traits, some differentially expressed alternatively spliced transcripts belonged to myosin and myotilin gene families. The hub transcripts were identified for REA (LRRFIP1, RCAN1 and RHOBTB1) and IF (TRIP12, HSPE1 and MAP2K6) have an important role to play in muscle cell degradation, development and motility. In general, transcripts were found for both traits with biological process GO terms that were involved in pathways related to protein ubiquitination, muscle differentiation, lipids and hormonal systems. Our results reinforce the biological importance of these known processes but also reveal new insights into the complexity of the whole cell muscle mRNA of Nelore cattle.

The alternative processing of messenger RNAs (mRNAs) produces several transcripts and protein isoforms that have similar or quite different roles in mammalian tissues1,2. RNA sequencing (RNA-Seq), is an accurate method for measuring gene expression and can measure all genes in the transcriptome at the same time. This technology could be used to screen the expression of functional candidate genes and identifying essential molecular processes creating a diversity in pathways leading to different tissue phenotypes. One of the usages of RNA-Seq is the identification of alternative splicing events3,4.

The spliceosomal mechanism may produce several isoforms by interpreting the limits between exon and intron in many different ways. For the most part, these distinct interpretations, fall into subgroups of alternative splicing events5 which may be classified into: cassette, mutually exclusive and coordinate cassette exons; alternative 5′ and 3′ splice sites; intron retention; or alternative first and last exon events6,7. The most common alternative splicing events in mammals are cassette exon and 3′ or 5′ alternative splice sites. Intron retention is a rare alternative splicing event in mammals5,8.

Whole transcriptomics analysis through splicing-sensitive identified from RNA-Seq or microarrays dataset, provides the capacity to gain an accurate and deep understanding of the contribution of distinct alternative splicing mechanisms to the developmental specific gene expression programs or establishment of tissue5. Several studies have reported the frequencies of different types of alternative splicing events in different species8,9,10.

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Table 1. Descriptive statistics for the overall alignment percentage for RNA-Seq data from Nelore cattle samples. The coverage refers to the transcriptomics. Phred score = $-10 \times \log_{10}$. REA Ribeye muscle area, IF intramuscular fat content, Mb megabase pair, Mean sample Coverage mean of number of unique reads that include a given nucleotide in the reconstructed sequence.

| Animals group | Mean of raw reads pairs (Mb) | Mean trimmed reads pairs (Mb) | Overall alignment rate (%) | Mean sample Coverage (X) | Mean mapping quality (Phred score) | Uniquely mapped (%) |
|---------------|-------------------------------|-------------------------------|---------------------------|--------------------------|-----------------------------------|---------------------|
| REA (N = 30)  | 25,798,305                    | 22,468,708                    | 96.5                      | 65.85                    | 58.7                              | 88                  |
| IF (N = 30)   | 24,911,474                    | 21,976,535                    | 96.4                      | 65.42                    |                                   |                     |

In bovine, alternative splicing events have been associated with variation in mastitis susceptibility\(^{9}\), embryonic development\(^{11}\) and muscle development\(^{12}\). Currently, knowledge concerning the molecular mechanisms regulated by alternative splicing events, especially in the context of individual differences in carcass and meat traits in bovine, is limited.

All along the growth process, the animal’s weight and body size increase, and the proportion of tissue, mainly the muscle tissue proportion, changes. Ribeye muscle area (REA) in conjunction with carcass weight provides an indication of carcass muscularity and meat yield\(^{13}\). Identifying and selecting animals with REA genetic advantages could improve carcass quality, thereby increasing the production of high commercial value meat cut. One of the meat quality traits is the intramuscular fat (IF) content, it is representing the sum of triglycerides and phospholipids; it is, the amount of fat stored between inside muscle cells or muscle fibers. The extent of IF content of the ribeye muscle is a primary determinant of meat palatability and consumer satisfaction with the eating experience. Muscle lipid traits, which determine meat flavor and contribute to beef color, can influence the juiciness and tenderness of the meat\(^{14}\).

REA development and IF content are polygenic traits controlled by multiple genes directly (or not) related in muscle and fat biological. However, some genes and biological process that have yet to be elucidated, particularly alternatively spliced genes that are cell-tissue specific. It could play an essential role in overall biological systems. Most of the Brazilian herd is a composite of Zebu breeds, notably among them, the Nelore 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. The principal bovine breed in Brazil is Nelore cattle, because of its strategic importance to Brazil's GDP (Gross Domestic Product) as the one of the world's biggest exporter of beef. Nelore cattle became more commercially competitive, then cattle ranchers and researchers have been looking for novel strategies to increase and improvement production, as example, the improvement of different carcass and meat traits.

Zebu animals, as Nelore breed, show a higher frequency of genes/alleles that are unfavorable to carcass and beef quality traits, such as REA development and IF content, when compared to Taurine breeds. Hence, understanding the functional and biological process from spliced genes that may control REA development and IF content in Nelore cattle is a compelling issue in meat science because this knowledge is not yet clear. Wherefore, we used RNA-Seq approach to find candidate genes that produced alternatively spliced transcripts that were differentially expressed in the Nelore’s ribeye muscle that differed for REA and IF content.

Results
RNA-seq quality control. Table 1 shows the descriptive statistics for the mean alignment rates for the samples by trait (REA and IF), for details, see Supplementary Figures S1 and S2. In summary, after filtering, the mean number of paired-end reads each sample was approximately 25 million reads. 88% of the trimmed reads, it was uniquely mapped to either the bovine reference genome (UMD3.1.1 + Chr Y). The overall alignment rate was 96.5% (approximately 21.6 million reads mapped in pairs).

The details about quality control indicators and bias estimations (transcript coverage, junction analysis, reads genome origin, and 5’–3’ bias computation) which are specific for RNA-Seq data were summarized in Supplementary Figures S3, S4 and S5. In accordance with Qualimap\(^{15}\) reports, on average, 63% and 22% of reads were mapped within the exonic and intronic regions of the genes, respectively. Another 13% of reads were mapped to intergenic regions. Mean of number of reads with splice junctions was approximately 16.8 million reads. Splicing junction analysis showed, approximately, 24% novel, 66% known and 10% partly known splicing junctions (for details, see Supplementary Figures S3 and S4).

Ribeye area (REA). There were 166 alternatively spliced transcripts produced from 125 genes that were differentially expressed between the high ribeye area (HREA) and low ribeye muscle area (LREA) groups (the comprehensive list of the differentially expressed alternatively spliced transcripts associated with REA is provided in Supplementary Table S1). For REA, cassette exon was the most frequently found alternative splicing event (Fig. 1). Of the differentially expressed alternatively spliced transcripts (DAŠ), 54 were known and 112 were novel transcripts.

Some DAS transcripts belong to important gene families, such as myosin (MYBPC1 and MYH1), myotilin (MYOT) and myomesin (MYOM2) have an important role in the stability of thin filaments along muscle contraction. The transcript isoforms produced by these gene families differ by cassette exon, intron retention, alternative 3’ and 5’ splice sites events (see Supplementary Table S1). The solute carrier (SLC) gene family (SLC25A2, SLC25A25, SLC29A1 and SLC29A2) produced DAS transcripts with alternative 3’ splice sites. Solute carrier gene family play roles as transporters\(^{16}\). Among DAS transcripts, we found BOLA-DOA (alternative 3’ splice site).
In cattle, **BOLA** is a major histocompatibility complex class II gene that exhibits very little sequence variation, especially at the protein level. For bulls selected on REA, the biological process Gene Ontology (GO) terms for the DAS genes identified in the pairwise comparison is summarized in Supplementary Table S2. We observed GO terms and pathways related to muscle growth and muscle protein ubiquitination, for example: regulation of protein stability (GO:0031647), smooth muscle tissue development (GO:0048745), protein ubiquitination involved in ubiquitin-dependent protein catabolic process (GO:0042787), cAMP (3',5'-cyclic adenosine monophosphate) signaling (bta04024), focal adhesion (bta04510) and ECM-receptor (extracellular matrix-receptor) interaction (bta04512), among others. The analysis also found biological GO terms and pathways related to complex immunological processes, neurological and hormonal systems, such as: adrenergic signaling in cardiomyocytes (bta04261); oxytocin signaling (bta04921) and inflammatory mediator regulation of TRP channels (bta04750). Calcium/Calmodulin Dependent Protein Kinase II Beta (**CAMK2B**)—represented by a novel cassette exon transcript isoform—and Adenylate Cyclase 2 (**ADCY2**)—represented by novel alternative 3′ splice site isoform—were present in the pathways mentioned above and also in six other pathways (Supplementary Table S2).

Co-expression network analyses found 61 significantly correlated DAS genes pairs (edges) involving 65 genes (nodes) for REA, density = 0.030 and clustering coefficient = 0.130 for REA (Fig. 2A). Networks with clustering and density values close to 1 may contain many edges and nodes connected. Clustering and density values of the network were close to zero, it means that some genes (nodes) are isolated, that is, at that moment they are not being co-expressed with the set of genes. From co-expression network the hub DAS genes were predicted and then, the top three hub DAS genes were chosen to define the subnetwork (Fig. 2B). The subnetwork was mapped onto biological processes, which had clear associated: biological regulation (GO:0065007), among others (Fig. 3A).

We predicted three top hub DAS genes: LRR Binding FLII Interacting Protein 1 (**LRRFIP1**), Regulator of Calcineurin 1 (**RCAN1**) and Rho Related BTB Domain Containing 1 (**RHOBTB1**). The high interconnection between hub genes and nodes within network, have been shown to be biologically significant. The hub DAS genes identified, could have putative functions as regulators on genes that play a role in development and growth of muscle cells. In general, **LRRFIP1**, **RCAN1** and **RHOBTB1** have functions in the proliferation of smooth muscle cells and interact with the calcineurin A and actin filament systems, respectively.

Figure 4 showed the different DAS transcripts for hub genes associated with ribeye area in Nelore bulls. For the **LRRFIP1** three known DAS transcripts: alternative last exon, cassette exon and coordinate cassette exon. Cassette exon transcripts were more abundant in the HREA animals (see Supplementary Table S1), whereas alternative last exon and coordinate cassette exon transcripts were more abundant in the LREA animals. In accordance with annotation produced by the Ensembl, ten different transcript variants were produced by **LRRFIP1**, previously, in cattle.

**RCAN1** has two known transcript variants annotated in cattle, we found one **RCAN1** produce transcript that differ by known alternative first exons, which were more abundant in the LREA animals (see Supplementary Table S1). Eight transcript isoforms were produced by **RHOBTB1** in accordance with annotation produced by the Ensembl. This study identifies a novel alternative 3′ splice site for this gene, predicted a putative novel transcript. The alternative 3′ splice site usage in **RHOBTB1** was more abundant in the LREA animals (see Supplementary Table S1).

**Intramuscular fat content (IF).** A total of 269 (78 known and 191 novel) alternatively spliced transcripts from 219 genes were differentially expressed between highest intramuscular fat (HIF) and lowest intramuscular fat (LIF) animals (see Supplementary Table S3). Alternative 3′ splice site was the most frequently observed alternative splicing event found in the IF content analysis (Fig. 1).
**Figure 2.** Alternatively spliced genes co-expression networks. (A,C) The nodes represent the differentially expressed alternatively spliced genes and the lines represent the interaction between nodes. (B,D) Genes with the most interactions in the network (highest degree), were identified as hub genes for each trait (ribeye area and intramuscular fat content). The top three hub genes were chosen to define the subnetwork (top modules), as this represents the most functional elements of the network for each trait. The more forward ranking is represented by a redder color.

**Figure 3.** Biological process from alternatively spliced genes present in subnetwork. (A) Ribeye area. (B) Intramuscular fat content.
Members of myosin (MYH7B, MYLK2 and MYO18A) and myotilin (MYOT) gene families were among the DAS transcripts. These genes, produced transcripts that differed by alternative first exons, alternative 3′ splice sites, alternative 5′ splice sites and retained introns. In addition to these, members of the myozenin (MYOZ1 and MYOZ3) family were found to produce DAS transcripts: alternative first exons, alternative 3′ and 5′ splice sites. These genes play an important role in the modulation of calcineurin signaling. Another DAS transcripts belong ubiquitin family (UBAC2 and UBE3C). These genes produced transcripts with cassette exons, alternative 3′ splice sites and alternative 5′ splice sites. The ubiquitin–proteasome system plays an important role in muscle loss23.

Most biological terms found for animals selected to differ for IF content were associated with growth, regulation and proteolysis of muscle cells (Supplementary Table S4), for example: myofibril assembly (GO:0030239); skeletal muscle cell differentiation (GO:0035914) and ubiquitin-dependent protein catabolic process (GO:0006511). The analysis found GO terms and pathways related to lipid metabolism, such as PI3K-Akt (bta04151), mechanistic target of rapamycin (mTOR) signaling (bta04150) and negative regulation of TORC1 signaling (GO:1904262). Serine/Threonine Kinase 11 (STK11) and Eukaryotic Translation Initiation Factor 4B (EIF4B) produce transcript isoforms with alternative 3′ splice sites and were present in these pathways. All of the GO terms and pathways mentioned above play important roles in muscle and lipid metabolism.

For IF, there were 92 significantly correlated gene pairs (edges) involving 101 genes (nodes), density = 0.018 and a clustering coefficient = 0.152 (Fig. 2C). Clustering and density values of the network were close to zero, it means that some nodes are isolated, that is, at that moment they are not being co-expressed with the set of genes18. Hub DAS genes within the co-expression network were predicted. From the first three hub DAS genes were chosen to define the subnetwork18 (Fig. 2D). The three hub DAS genes were: Thyroid Hormone Receptor Interactor 12 (TRIP12), Heat Shock Protein Family E member 1 (HSPE1) and Mitogen-Activated Protein Kinase Kinase 6 (MAP2K6). TRIP12, HSPE1 and MAP2K6 genes are involved in ubiquitin mediated proteolysis24, facilitates the correct folding of imported proteins25, the regulation of the mitogen-activated protein kinase pathway26, respectively. The subnetwork genes (Fig. 2D) were mapped onto biological processes, which include biological regulation (GO:0065007) and cellular processes (GO:0009987), among others (Fig. 3B).

The Fig. 5 showed the different DAS transcripts for hub genes associated with IF content. For the TRIP12 one known DAS transcript that differed by their use of alternative 3′ splice sites. This transcript was more abundant in the HIF animals (see Supplementary Table S3). For HSPE1 a novel DAS transcript: alternative 5′ splice. In accordance with annotation produced by the Ensembl, three different transcript variants were produced by MAP2K6, previously, in cattle. In this study, one of these transcripts was differentially expressed: alternative first exons, being more abundant in the LIF animals (see Supplementary Table S3).
Differentially expressed alternatively spliced transcripts and their associated genes were identified in this study, using RNA-Seq data from the ribeye muscle of Nelore bulls phenotypically divergent for REA or IF content. In cattle, most genes have multiple isoforms characterized by different event types. The cassette exon and alternative 3′ splice site events were frequently responsible for the transcript isoforms found to be differentially expressed in the ribeye muscle of bulls with the highest and lowest REA or IF content.

Cassette exon and alternative 3′ splice site are the most common alternative splicing events in mammalian pre-mRNAs. This case, the exons are flanked on two side (constitutive and competing alternative splice sites). The exons are included or excluded from the mature RNA leading to extended or shortened transcripts variants. Bland et al. studied mice myogenic differentiation and found 95 alternative splicing events. Most of the splicing events found for Bland et al. was cassette exons (86%).

The co-expression analyses were performed with gene sets that contained the isoforms that were differentially expressed between the highest and lowest REA and IF content groups. Through co-expression analyses hub DAS genes for each trait were predicted that may be the primary regulators of the other genes with differentially expressed transcript isoforms found in this study. Hub genes are expected to play significant functions in organism’s. In addition, a functional enrichment analysis was performed for the gene sets found for each trait. In general, for both traits, the results indicated that hub DAS genes had functions in protein ubiquitination, muscle differentiation and regulation of lipolysis in adipocytes.

This study also found biological terms and pathways related to immune function systems, indicating the pleiotropic nature of the genes and their isoforms in several phenotypes. Several of the genes that were found to produce differentially expressed splice transcripts in this study; have not previously been reported to be associated with carcass or meat traits of beef cattle. For example, CAMK2B (differentially expressed in animals phenotypically divergent for REA and represented by novel cassette exon events) was present in nine pathways (Supplementary Table S2) related to the neurological, hormonal, and immune systems, including: adrenergic signaling in cardiomyocytes (bta04261); oxytocin signaling (bta04921) and inflammatory mediator regulation of TRP channels (bta04750).

CAMK2B has been associated with neurodevelopmental disorders in humans. CAMK2B is a serine/threonine kinase that is highly abundant in brain, especially in the post synaptic density. Different isoforms of this gene have distinct cellular localizations and interact differently with calmodulin. Calmodulin mediates many key processes, such as, metabolism, smooth muscle contraction, apoptosis, short-term memory, long-term memory, intracellular movement and the immune response.

Another gene that has not previously been reported to be associated with carcass or meat traits of beef cattle is Serine/Threonine Kinase 11 (STK11). This gene was differentially expressed between animals phenotypically...
differential for IF content and by a known isoform with an alternative 3’ splice site, regulates cell polarity and functions as a tumor suppressor. STK11 is involved in TORC1 signaling (GO:1904262); mTOR signaling (bta04150) and PI3K-Akt signaling (bta04151) (see Supplementary Table S4). All of these processes have important roles in the growth, proliferation, motility and survival of cells.

Several other genes that were predicted to produce alternatively spliced isoforms that were differentially expressed have also been implicated in muscle differentiation and growth; protein ubiquitination in muscle; and lipid metabolism. All of these processes could promote changes in ribeye muscle area and intramuscular fat content phenotypes.

**Ribeye area.** Several differentially expressed alternatively spliced transcripts were produced by myosin (MYRPC1 and MYH1), myotilin (MYOT), myomesin (MYOM2), solute carrier (SLC25A2, SLC25A25, SLC29A1 and SLC29A2) and BOLA (BOLA-DOA) genes families. Myosin, myotilin and myomesin proteins have a significant role in the stability of thin filaments during muscle contraction and are the main components of myofilaments. SLC gene family play roles as transporters and have been associated with REA in Nelore cattle. BOLA-DOA belongs to the major histocompatibility complex and has effects in the immune system. Members of the BOLA gene family have previously been associated with tenderness in Nelore cattle and growth traits in Limousin, Simmental Charolais, Holstein, Angus and Hereford, beef cattle.

Three hub genes (LRRFIP1, RCAN1 and RHOBTB1) with alternatively spliced transcripts were differentially expressed between Nelore bulls selected to differ for REA. The differences in transcript splicing event usage for LRRFIP1, RCAN1 and RHOBTB1 found here may be due to the tuning of the spliceosome machinery. The spliceosome is a complex snRNA. The introns are removed from pre-mRNAs through two consecutive phosphoryl transfer reactions. The spliceosome is assembled de novo on a immature mRNA for each splicing event and a complex series of assembly steps result to the active conformation.

Hub genes LRRFIP1 and RHOBTB1 play roles in muscle cell development and regulation in humans and mice. LRRFIP1 performs the function of transcriptional repressor who has binding affinity with GC-rich consensus sequence and can control the proliferation of smooth muscle cells. We found known alternative last, cassette and coordinate cassette exons transcripts produced by LRRFIP1. For LRRFIP1, alternative last and coordinate cassette exons transcripts were more abundant in the LREA animals, this result showed, these transcripts could be playing a role as repressor of some genes that function in proliferation of smooth muscle cells, whereas, cassette exon transcript was more abundant in HREA animals. This result could be explained by the fact that for some genes, to initiate transcription, the presence of an activator is required. For others, it will be avoiding the connection of a repressor. These cases are referred to as down-regulation because it is the absence of a protein that allows transcription to occur.

The protein encoded by RHOBTB1 has function in actin filament system organization and small and small GTPase-mediated signal transduction. For this gene, a putative novel transcript (3’ splice sites) was predicted. The alternative 3’ splice sites usage in RHOB1 was more abundant in the LREA animals, which could result in the likely morphological changes that occur in the muscle structure of these animals. RCAN1 has previously been associated with REA in a Wagyu × Angus F1 population. RCAN1 produces a protein that inhibits the transcriptional responses of the calcineurin-dependent mediated by the binding to the catalytic domain of calcineurin A.

It is known that calcineurin A signaling is important for the normal function of cardiac and skeletal muscle. In skeletal muscle, calcineurin A is involved in different processes, as dystrophic muscle damage, fiber type specific responses of the calcineurin-dependent mediated by the binding to the catalytic domain of calcineurin A.

The results show the biological importance of cAMP signaling, focal adhesion and ECM-receptor signaling in determining the composition and organization of bovine muscle tissue. Pathways related to hormonal systems were also found, among them: oxytocin (bta04510) and glucagon signaling (bta04922). RCAN1 is a member of the oxytocin signaling pathway is fundamental for the maintenance of homeostasis and regeneration of skeletal muscle tissue in mice. Glucagon signals liver and muscle cells to change stored glycogen into glucose, which is then released into the bloodstream and used by other cells for energy. The protein turnover (synthesis and breakdown) is continuous and ensures the skeletal muscle quality and functional integrity. In addition, the hormones are essential regulators of this remodeling process.

In contrast to muscle growth, muscle loss is an undermining consequence of a range of pathology and metabolic conditions, for example, an increase in protein degradation. We found processes related to the proteolytic system (protein ubiquitination involved in ubiquitin-dependent protein catalytic process—GO:0042787, ubiquitin mediated proteolysis—bta04120 and protein polyubiquitination—GO:0000209), some of which were involved in...
in muscle degradation. Among these, the ubiquitin–proteasome system is very conserved across vertebrates, and works as a degrader of the main proteins of the contractile skeletal muscle, that is, it has an important role in muscle loss.

Genes encoding differentially expressed alternatively spliced transcripts associated with REA were also related to immune function, such as the Adenylate Cyclase 2 (ADCY2) gene which also is annotated with several other GO terms and is a member of other pathways including those related to the neural and hormonal systems. There are complex interactions between the skeletal muscle and the immune systems that regulates muscle regeneration. ADCY2 is a membrane-associated enzyme that catalyzes the formation of cyclic adenosine monophosphate, a secondary messenger. An alternative 3′ splice site event was found for this gene which is a member of the cAMP signaling pathway (bta04024). ADCY2 may be related in different biological processes, and has previously been associated with meat tenderness in Yambian cattle.

**Intramuscular fat content.** We found differentially expressed alternatively spliced transcripts belonging to members of myosin (MYH7B, MYLK2 and MYO18A), myotilin (MYOT), myozin (MYOZ1 and MYOZ3) and ubiquitin (UBAC2 and UBE3C) families. Skeletal muscle fiber metabolism is a factor that may affect intramuscular fat deposition. There is a positive correlation between the extent of intramuscular fat and oxidative muscle fiber percentage. When the rate of protein deposition decreases (possibly due to ubiquition processes), lipid deposition becomes the major component of weight gain and the energy request to fat tissues increases.

TRIP12, HSPE1 and MAP2K6 which have functions related to cell regulation and maintenance were predicted to be hub genes associated with IF content. TRIP12 encoded transcript with known alternative 3′ splice site, which was more abundant in the HIF animals. TRIP12 is a protein coding gene that is a key regulator of the response to DNA damage. The related pathways include Class I MHC mediated antigen processing and presentation, and ubiquitin mediated proteolysis. TRIP12 transcripts were expressed in the skeletal muscle of Holstein–Friesian, Limousin, Hereford and Polish Red bulls. In humans, TRIP12 plays a role in the ubiquitin fusion degradation pathway, which is a proteolytic system that is conserved in mammals. Therefore, the TRIP12 transcripts more expressed in HIF animals could play role in the immune systems and protein degradation.

This study predicted a putative novel HSPE1 transcript with alternative 3′ splice site more expressed in HIF animals. SNPs (Single nucleotide polymorphisms) found in HSPE family genes were associated with thermal tolerance traits in Chinese Holstein and Angus cattle. Heat shock proteins provide subsidies for cells to identify and promote the refolding of damaged proteins or target them to appropriate proteolytic systems, thereby helping to eliminate proteins whose damage is not suitable for recovery. As long as the animal is under stress, HS levels will be high. This high expression increases the synthesis and maturation of new proteins that will replace those affected by stress. The increase of HSP in damaged cells not only contributes to protein repair, but also plays an important role in maintaining viability because it inhibits apoptosis. Therefore, the HSPE1 transcript found in this study could be aiding in the repair of proteins in HIF group.

MAP2K6 produces three known transcripts one off them differ by known alternative first exons, which were found in this study and more abundant in the LIF animals. Single nucleotide polymorphisms in MAP2K6 have previously been associated with marbling score, back fat thickness and carcass weight in Hanwoo cattle. MAP2K6 is a member of the dual specificity protein kinase family and plays a role in the regulation of the mitogen–activated protein kinase pathway. Mitogen-activated protein kinase (MAPK or MAP kinase) is a protein kinase that is specific to the serine and threonine aminoacids. Mapk protein is involved in directing the response of cells to a variety of stimulus, such as osmotic stress, mitogens, proinflammatory cytokines and heat shock. They regulate cell functions including apoptosis, gene expression, proliferation, mitosis, differentiation, and cell survival. Therefore, the MAP2K6 transcript, as well as the HSPE1 transcript, found this study, could be aiding in the repair of proteins in LIF animals.

The enrichment analysis suggested that the most relevant function of the genes with significant isoform expression shifts for IF was related to the growth, regulation and proteolysis of muscle cells (Supplementary Table S4), for example: myofibril assembly (GO:0030239); skeletal muscle cell differentiation (GO:0035914) and ubiquitin-dependent protein catabolic process (GO:0006511). This suggests that variation in IF content may be due to a balance between the absorption, synthesis and degradation of intramyocellular and extramyocellular lipids, which involves many metabolic pathways in myofibers. Intramuscular fat accumulates both within (intramyocellular) and externally (extramyocellular) of muscle fibers. The intramyocellular lipids are stored as spherical droplets in muscle cells and are reportedly associated with aerobic metabolism, whereas the extramyocellular lipids are located in long fatty septa of laminar shape, along muscle fiber bundles. Intramyocellular lipids play an important role in muscle metabolism.

The PI3K-Akt (bta04151), mTOR signaling (bta04150) and negative regulation of TORC1 signaling (GO:1904262) pathways were overrepresented in this study. EIF4B which is a member of these pathways, produces a transcript with a novel alternative 3′-splice site, that was found to be more abundant in the LIF animals. EIF4B is a protein coding gene that is required for the binding of mRNA to ribosomes. The activity of EIF4 family genes is controlled by mTOR serine threonine kinase which forms the mTORC1 and mTORC2 complexes. mTORC1 may play a role in regulating lipid synthesis, which is required for cell growth and proliferation, but this is not completely clear yet. It has been demonstrated that mTORC1 positively regulates the transcription factors (sterol regulatory element binding protein 1—SREBP1 and peroxisome proliferator-activated receptor γ—PPARG) that control the expression of genes encoding proteins involved in lipid and cholesterol homeostasis.

The PI3K-AKT pathway is an intracellular signaling pathway essential in regulating the cell cycle. PI3K activation phosphorylates and activates AKT. AKT can have a number of downstream effects such as activating mTOR.

We found candidate genes that encode differentially expressed alternatively spliced transcripts associated with IF content in Nelore cattle. Previous studies have identified differentially expressed genes in Simmental-Luxi
described by Fonseca et al.35) and again at 24 h post-slaughter for the evaluation of REA and determination of IF of each carcass, at two time points: immediately following slaughter for the RNA-Seq analysis (details previously used to estimate metrics and bias, including reads genomic origin, junction analysis, transcript coverage and downstream analyses were performed on the trimmed data with high-quality reads. HISAT2 (v.2.0.5)75 was used to align the paired-end trimmed data to the bovine reference genome (UMD3.1.1) and chrY (Bos taurus) merged both, UMC3.1.1 and chrY 4.6.1), both deposited in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The parameters used were: − p 12, − dta, − known-splicesite-infile, − x, − 1, − 2, − S. The annotation was extracted from the samples was processed using a TruSeq RNA Sample Preparation Kit® (Illumina, San Diego, CA) according to the manufacturer’s protocol. Differently bar-coded libraries were pooled to enable multiplexed sequencing and generated, on average, approximately 25 M read-pairs per sample. RNA-Seq was performed using a HiSeq 2500 System (Illumina) that generated 100 bp paired-end reads.

The quality of RNA-Seq reads (quality scores, GC content, N content, length distributions, duplication, overrepresented sequences and K-mer content) was checked using FastQC (v.0.11.4) software73. Trimmed reads data were obtained using Trimomatic (v.0.36)74, with following parameters: PE ILLUMINACLIP: TruSeq3-PE. fa:2:40:15 LEADING:20 TRAILING:20 CROP:100 SLIDINGWINDOW:4:20 AVGQUAL:20 MINLEN:50. All overrepresented sequences and K-mer content) was checked using FastQC (v.0.11.4) software73. Trimmed reads data were obtained using Trimomatic (v.0.36)74, with following parameters: PE ILLUMINACLIP: TruSeq3-PE. fa:2:40:15 LEADING:20 TRAILING:20 CROP:100 SLIDINGWINDOW:4:20 AVGQUAL:20 MINLEN:50. All downstream analyses were performed on the trimmed data with high-quality reads. HISAT2 (v.2.0.5)75 was used to align the paired-end trimmed data to the bovine reference genome (UMD3.1.1 Bos taurus) and chrY (Btau 4.6.1), both deposited in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The parameters used were: − p 12, − dta, − known-splicesite-infile, − x, − 1, − 2, − S. The annotation was merged both, UMC3.1.1 and chrY (Btau 4.6.1), Table 1 shows the descriptive statistics for the mean alignment rates for the samples by trait (REA and IF), (for detail, see “Supplementary figures”). Qualimap (v.2.2.1)15 was used to estimates metrics and bias, including reads genomic origin, junction analysis, transcript coverage and 5′–3′ bias computation.

Identification of differentially expressed alternatively spliced transcripts (DAS). JuncBASE (v.0.9) software (Junction-Based Analysis of Splicing Events)7 was used to identify and classify exon-centric alternative splicing events (cassette exons, alternative 5′ splice site, alternative 3′ splice site, mutually exclusive exons, coordinate cassette exons, alternative first exons, alternative last exons and intron retention) based on splice junction reads predicted using the Cufflinks2 (v.2.1.1) suite of tools (Cufflinks2, and Cuffmerge2)15.

Table 2. Descriptive statistics for ribeye muscle area and intramuscular fat content of Nelore cattle. HREA high ribeye muscle area, LREA low ribeye muscle area, HIF high intramuscular fat content, LIF low intramuscular fat content, N number of animals. *The % data were transformed using the arcsine square root function.

| Phenotype | N  | Mean ± standard deviation | Minimum | Maximum | p-value |
|-----------|----|--------------------------|---------|---------|---------|
| HREA (cm²) | 15 | 83.66 ± 2.55            | 81      | 88      | 0.01    |
| LREA (cm²) | 15 | 65.73 ± 2.05            | 61      | 68      |         |
| HIF*      | 15 | 0.101 ± 0.0095          | 0.094   | 0.126   | 0.05    |
| LIF*      | 15 | 0.063 ± 0.0049          | 0.051   | 0.067   |         |

Cross, Wagyu and Angus, Wagyu and Holstein and Nelore, suggesting that some of these genes regulate lipid composition and deposition in intramuscular fat. These findings and our results enable a better understanding of the mechanisms underlying the gene transcription associated with IF content in Nelore cattle.

Conclusion
RNA-Seq was used to identify candidate genes that encode alternatively spliced transcripts that were associated with REA and IF content in Nelore bulls. The results suggest that REA and IF content may be influenced by sets of genes that encode alternatively spliced transcripts (both known and novel transcripts). For both traits, we found genes with biological process GO terms that are involved in pathways related to protein ubiquitination, muscle differentiation, regulation of lipolysis in adipocytes, and hormonal systems. These results provide a foundation for further research into the specific functions of candidate genes encoding alternatively spliced transcripts that are differentially expressed in animals differing for REA and IF content.

Methods
Animals and phenotypes. The intact Nelore bulls used in this study (N = 80) were from the Capivara Farm, which participates in the Nelore Qualitas Breeding Program. The animals from a single contemporary group (that remained together from birth until slaughter) were reared on Brachiaria sp. and Panicum sp. forages, and had free access to mineral salt and were finished in a feedlot for approximately 90 days. The diet was based on whole-plant silage and mix of sorghum grain, soybean meal or sunflower seeds were used as concentrate, with a concentrate/roughage ratio from 50/50 to 70/30. The animals were slaughtered at an average age of 24 months in a commercial slaughterhouse, in accordance with Brazilian Federal Inspection Service procedures.

Samples from the longissimus thoracis muscle were collected, between the 12th and 13th ribs of the left half of each carcass, at two time points: immediately following slaughter for the RNA-Seq analysis (details previously described by Fonseca et al.15) and again at 24 h post-slaughter for the evaluation of REA and determination of IF content. REA was measured by the grid method in units of centimeters squared15. The Bligh and Dyer2 method was used to determine IF content. From the 80 animals, groups of animals were selected for RNA-Seq analysis of ribeye muscle tissue: (1) REA, the 15 animals with the highest and 15 with the lowest REA (HREA and LREA); and (2) IF, 15 animals with the highest and 15 with the lowest IF content (HIF and LIF). A Student’s t-test was applied to determine if there were statistically significant differences between the selected groups (Table 2).

RNA-Seq library preparation and data processing. High-quality total RNA (approximately 500 ng) extracted from the samples was processed using a TruSeq RNA Sample Preparation Kit® (Illumina, San Diego, CA) according to the manufacturer’s protocol. Differently bar-coded libraries were pooled to enable multiplexed sequencing and generated, on average, approximately 25 M read-pairs per sample. RNA-Seq was performed using a HiSeq 2500 System (Illumina) that generated 100 bp paired-end reads.

The quality of RNA-Seq reads (quality scores, GC content, N content, length distributions, duplication, overrepresented sequences and K-mer content) was checked using FastQC (v.0.11.4) software73. Trimmed reads data were obtained using Trimomatic (v.0.36)74, with following parameters: PE ILLUMINACLIP: TruSeq3-PE. fa:2:40:15 LEADING:20 TRAILING:20 CROP:100 SLIDINGWINDOW:4:20 AVGQUAL:20 MINLEN:50. All downstream analyses were performed on the trimmed data with high-quality reads. HISAT2 (v.2.0.5)75 was used to align the paired-end trimmed data to the bovine reference genome (UMD3.1.1 Bos taurus) and chrY (Btau 4.6.1), both deposited in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The parameters used were: − p 12, − dta, − known-splicesite-infile, − x, − 1, − 2, − S. The annotation was merged both, UMC3.1.1 and chrY (Btau 4.6.1), Table 1 shows the descriptive statistics for the mean alignment rates for the samples by trait (REA and IF), (for detail, see “Supplementary figures”). Qualimap (v.2.2.1)15 was used to estimates metrics and bias, including reads genomic origin, junction analysis, transcript coverage and 5′–3′ bias computation.

Identification of differentially expressed alternatively spliced transcripts (DAS). JuncBASE (v.0.9) software (Junction-Based Analysis of Splicing Events)7 was used to identify and classify exon-centric alternative splicing events (cassette exons, alternative 5′ splice site, alternative 3′ splice site, mutually exclusive exons, coordinate cassette exons, alternative first exons, alternative last exons and intron retention) based on splice junction reads predicted using the Cufflinks2 (v.2.1.1) suite of tools (Cufflinks2, and Cuffmerge2)15.
For each RNA-Seq sample, the spliced alignment is given as input, in BAM format. Splice junctions passing an entropy score threshold were combined with exon coordinates from transcript reference annotations and optional novel transcripts to identify alternative splicing events. The inclusion and exclusion isoforms of each alternative splicing event are quantified using the RNA-Seq read alignments and Percentage Spliced Index (PSI) values are calculated. Differential splicing analysis is performed from isofrom abundances or PSI values. For more details, the following eight steps were used.

**Step 1** Building annotation database: the annotation database was used to identify all exons in the transcriptome and it was derived from a GTF file. We created two different databases. The first, was GTF file that combined Cufflinks2 and Cuffmerge2 identified transcripts with those that are annotated in a reference set, and it was used to identify all internal exons (not the first or last exon in a transcript). The second database was derived from just a reference annotation. This second database was used to define alternative first and last exons.

**Step 2** Determination of junction entropy cutoff: for all subset of samples, was built an entropy plot of annotated and novel junctions. Cutoff of 1.1 was used to reduce the number of potential false positive novel junctions.

**Step 3** BAM files processing: first, the junctions were filtered (based on entropy score obtained in step 2). From this step, for each sample, it was obtained a BED file which contained all junctions and a TXT file, which contained all the non-junction reads from the BAM file.

**Step 4** Identification of all junctions: this step created one BED file that combined junctions from all samples.

**Step 5** Building of exon–intron junction count files: this step created a counts file of the number of reads aligning to every exon–intron junction.

**Step 6** Building of pseudo/“all junction” sample: The pseudo sample was only used to define the set of all junctions and was not used for quantification. The pseudo sample was linked to the BED files created in step 4. An arbitrary sample had given in order to create other pseudo files that were not used.

**Step 7** Alternative splicing events identification and quantification of the events from each sample: this step identified, classified, and quantified alternative splicing events. The result of this step was a table with details of each alternative splicing event identified in the set of samples examined. The details of each splicing event include coordinates, and raw and length-normalized read counts to specific junctions and regions involved in the alternative splicing event. Also, we built tables of raw and length-normalized read counts of exclusion and inclusion isoforms.

**Step 8** Differential splicing analysis: this last step identified differentially spliced events between two specified groups (Highest and Lowest) of animals (REA and IF). We obtained a set of uniform transcripts for every sample in the previous steps, and then, the Percentage Spliced Index (PSI) was estimated as the ratio of the number of reads including exons to the sum of the numbers of reads including or excluding exons. The PSI value was calculated and Fisher’s exact test was used to test for differences in alternative splicing events between the high and low REA and IF groups (p < 0.05). Only alternatively spliced transcripts that were supported by at least ten reads and that had PSI differences (ΔPSI) between the respective high and low groups of greater than 5% were considered significant.

The enrichment and pathway analyses of genes set DAS transcripts associated with REA or IF content were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID v.6.8)77. Fisher’s exact test was used and p values were adjusted for multiple testing using the Benjamini–Hochberg method78. DAVID Pathway was used to map the gene enriched pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database79.

**Co-expression network analysis and prediction of hub genes DAS transcripts.** All analyses were performed with plugins or applications from Cytoscape (v.3.7)80. For co-expression network analyses, the ExpressionCorrelation plugin81 was used to identify consensus networks of gene sets in which differentially expressed alternatively spliced transcripts were associated with REA or IF content. Similarity matrices were computed using the Pearson correlations among PSI values. A histogram tool was used for the screening criteria at node score cut-offs > 0.75 and < −0.75 and employed to identify statistical significance of the pairwise correlations. We established a co-expression network between the significantly co-expressed DAS genes. The cytoHubba plugin82 was used to explore co-expression network nodes (hub genes). The maximal clique centrality (MCC) was used for analyses, this method has a better performance on the precision of predicting essential genes from the co-expression network78 and to generate a subnetwork83. The top three hub genes were chosen to define gene co-expression subnetwork and then, it was used to analyze the functional enrichment of biological processes.

**Ethics approval.** All experimental procedures were approved by Ethics Committee of the School of Agricultural and Veterinarian Sciences, São Paulo State University (UNESP), Jaboticabal, SP, Brazil (protocol number 18.340/16). The animals were provided by Qualitas Nelore breeding program company and they were slaughtered in commercial slaughterhouses. These slaughterhouses have animal welfare departments staffed by professionals trained by WAG (World Animal Protection) to ensure that the animals are killed humanely using a captive bolt pistol for stunning.

**Data availability**

The dataset utilized in this study belongs to a Qualitas Nelore breeding program company, and could be available on request. The author does not have authorization to share the data.

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**Competing interests**
The authors declare no competing interests.

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