RAFAEL TORRES DE SOUZA RODRIGUES

UNDERSTANDING THE DIFFERENCE IN BEEF QUALITY BETWEEN ANGUS AND NELLORE CATTLE THROUGH A PROTEOMIC AND PHOSPHOPROTEOMIC APPROACH

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Ruminantes, para a obtenção do título de Doutor

Prof. Dr. Mario Luiz Chizzotti
Orientador

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo próprio autor.

Rodrigues, Rafael Torres de Souza.
Understanding the difference in beef quality between Angus and Nellore cattle through a proteomic and phosphoproteomic approach / Rafael Torres de Souza Rodrigues. – Lavras : UFLA, 2016.
99 p. : il.

Tese(doutorado)–Universidade Federal de Lavras, 2016.
Orientador(a): Mario Luiz Chizzotti.
Bibliografia.

1. Apoptosis. 2. Fiber type. 3. Meat tenderness. 4. Phosphorylation. 5. Postmortem glycolysis. I. Universidade Federal de Lavras. II. Título.

RAFAEL TORRES DE SOUZA RODRIGUES

UNDERSTANDING THE DIFFERENCE IN BEEF QUALITY BETWEEN ANGUS AND NELLORE CATTLE THROUGH A PROTEOMIC AND PHOSPHOPROTEOMIC APPROACH (ENTENDENDO AS DIFERENÇAS NA QUALIDADE DE CARNE ENTRE BOVINOS ANGUS E NELORE ATRAVÉS DE UMA ANÁLISE PROTEÔMICA E FOSFOPROTEÔMICA)

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Ruminantes, para a obtenção do título de Doutor

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LAVRAS – MG
2016

Ao Nordeste brasileiro e seu povo.
Dedico

AGRADECIMENTOS

À Universidade Federal de Lavras (UFLA), principalmente, ao Programa de Pós Graduação em Zootecnia.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), pelo apoio financeiro ao projeto.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela bolsa de doutorado.

Ao professor Mario Chizzotti, por ter me dado mais uma oportunidade e por me permitir navegar pela "temida" Biologia Molecular. Foi muito engrandecedor sair da zona de conforto e encarar esse desafio.

À Universidade Federal de Viçosa (UFV), por ter me recebido como um de seus alunos.

Ao Núcleo de Análise de Biomoléculas (NuBioMol-UFV), por ter me dado todo o apoio necessário para o desenvolvimento desta pesquisa.

Aos amigos que fiz no NuBioMol, Edvaldo, Nívea, Humberto, Pedro e Carlos, por ter me recebido na sua família, e ter tornado essa jornada menos árdua. Sentirei saudades do café, da jaboticabeira e de ver os picos subirem.

Ao Camilo, pela amizade e por ter sido fundamental para o sucesso deste trabalho. A fosfoproteômica agradece.

À professora Cristina Baracat, por ter me aceito como seu orientando e por ter aberto as portas de seu laboratório para mim.

À Patrícia, por ter resolvido a Equação de Henderson-Hasselbalch comigo, viabilizando a análise de fosfoproteômica, quando eu já tinha largado mão.

À Erika Toriyama, pelas informações e dicas iniciais sobre delineamento e metodologia.

Ao Laboratório de Biotecnologia Animal (LABETEC), por ter aberto as suas portas para o inicio desta jornada.

Aos amigos do LABETEC, em especial, Walmir, Letícia e Taíane, pela paciência com uma pessoa que nem sabia pipetar.

Ao Fabiano e à Rita, por terem me ajudado bastante durante o período em que eu estava com o pé machucado.

Aos meus professores do doutorado, especialmente ao professor Marcos Neves, por ter passado seu conhecimento. Graças a Deus fiz todas as suas "temidas" disciplinas. Todas foram muito importantes para minha formação técnica e científica.

Aos amigos de doutorado, Karina Toledo, Tchotchola, Rayane, Cacau e Liza, saudades dos nossos estudos em grupo.

À minha amiga Karina Busato (lampis), pelo apoio em todos os momentos, principalmente, quando cheguei a Lavras, ajudando na minha instalação e, inclusive, me emprestando dinheiro enquanto a minha bolsa não tinha saído.

À Aline (Nega) e Rafael (chefe), pelo apoio e amizade.

Aos amigos Diego (o suíço mais brasileiro que conheci) e Gina, mais que locadores, grandes amigos. Sempre me lembrarei daqueles almoços deliciosos, dos estudos bíblicos, do piano e dos 7x1. Realmente, vocês serão inesquecíveis.

Aos meus pais, Maria José e Raimundo, pelo amor, educação, apoio e conforto. Além disso, agradeço à minha mãe, por ter cuidado de mim quando machuquei o pé. Foi muito prazeroso ter você comigo na reta final do doutorado, com aqueles almoços, sopas, bolo de milho e a novela escrava mãe.

Às minhas irmãs, Luana (lulu) e Mayane (galega), pelo amor, amizade e apoio sempre.

A Deus, pela força, inspiração, saúde e paz.

Muito obrigado!

"*Love me tender*"
(D'Alessandro et al., 2012)

ABSTRACT

Proteins are the major constituents of muscle and are key molecules regulating the metabolic changes during conversion of muscle to meat. Brazil is one of the largest exporters of beef and most of the Brazilian cattle are composed by zebu (Nellore) genotype. Zebu beef is generally leaner and tougher than taurine. The aim of this study was to compare the muscle proteomic and phosphoproteomic profile of Nellore and Angus. Seven animals of each breed previously subjected to the same growth management were confined for 84 days. Proteins were extracted from *Logissimus dorsi* samples collected immediately after slaughter and separated by two-dimensional electrophoresis. Pro-Q Diamond stain was used in phosphoproteomics. Proteins identification was performed using matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Tropomyosin alpha-1 chain, troponin-T, myosin light chain-1 fragment, cytoplasmic malate dehydrogenase, alpha-enolase and 78 kDa glucose-regulated protein were more abundant in Nellore, while myosin light chain 3, prohibitin, mitochondrial stress-70 protein and heat shock 70 kDa protein 6 were more abundant in Angus ($P<0.05$). Nellore had higher phosphorylation of myosin regulatory light chain-2, alpha actin-1, triosephosphate isomerase and 14-3-3 protein epsilon. However, Angus had greater phosphorylation of phosphoglucomutase-1 and troponin-T ($P<0.05$). Therefore, proteins involved in contraction and muscle organization, and heat shock proteins related to cell flux of calcium and apoptosis might be associated with differences in beef tenderness between Angus and Nellore. Furthermore, prohibitin might be linked to differences in intramuscular fat. Additionally, differences in phosphorylation of myofilaments and glycolytic enzymes could be involved with differences in muscle contraction force, susceptibility to calpain, apoptosis and postmortem glycolysis, which might also be related to differences in beef tenderness among Angus and Nellore.

Keywords: Apoptosis. Fiber type. Meat tenderness. Phosphorylation. Postmortem glycolysis.

RESUMO

As proteínas são as principais moléculas constituintes do tecido muscular, sendo responsáveis pela regulação das mudanças bioquímicas que ocorrem durante a conversão de músculo em carne. O Brasil é um dos maiores exportadores de carne bovina do mundo e a maior parte do gado de corte brasileiro é composta por zebuínos da raça Nelore. A carne de zebuínos é geralmente mais magra e menos macia do que a de taurinos. Objetivou-se comparar o perfil proteômico e fosfoproteômico do músculo de bovinos da raça Nelore e Angus. Sete animais de cada raça previamente submetidos ao mesmo manejo de crescimento foram confinados por 84 dias. As proteínas foram extraídas de amostras do músculo *Logissimus dorsi* coletadas imediatamente após o abate e separadas por eletroforese bidimensional. O corante Pro-Q Diamond foi usado na análise de fosfoproteômica. A identificação das proteínas foi realizada através de espectrometria de massas com ionização/dessorção à laser assistida por matriz acoplada a analisador por tempo de vôo (MALDI-TOF/TOF). Alfa tropomiosina, troponina T, fragmento de miosina de cadeia leve 1, malato desidrogenase citoplasmática, alfa enolase e heat shock 70 kDa protein 5 foram mais abundantes em Nelore, enquanto miosina de cadeia leve 3, proibitina, heat shock 70 kDa protein 9 e heat shock 70 kDa protein 6 foram mais abundantes em Angus (P<0,05). Nelore teve maior fosforilação de miosina regulatória de cadeia leve 2, alfa actina 1, triose-fosfato isomerase e 14-3-3 protein epsilon. Por outro lado, Angus teve maior fosforilação de fosfoglicomutase 1 e troponina T (P<0,05). Foi concluído que proteínas envolvidas com a regulação e contração muscular e heat shock proteins relacionadas com o fluxo celular de cálcio e apoptose poderiam ser associadas com as diferenças na maciez de carne entre Angus e Nelore. Além disso, proibitina poderia ser relacionada com as diferenças no conteúdo de gordura intramuscular. Em adição, diferenças nos níveis de fosforilação de miofilamentos e enzimas envolvidas com o metabolismo de glicose poderiam ser relacionada à regulação da força de contração muscular, susceptibilidade à calpaína, apoptose e glicólise *post-mortem* e, consequentemente, às diferenças na maciez de carne entre Angus e Nelore.

Palavras-chave: Apoptose. Fosforilação. Glicólise *post-mortem*. Maciez de carne. Tipo de fibra.

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FIRST PART

1 INTRODUCTION

Beef is one of the main sources of animal protein consumed in the world. Thus, it is very important to know the factors related to its quality. Muscle tissue is composed primarily of proteins that are highly organized to form the contractile apparatus. In addition, biochemical and structural changes occurring during conversion of muscle to meat and aging are controlled by enzymes. Therefore, the study of proteins is critical to understanding the mechanisms involved in meat quality differences.

Proteomics is a molecular biology tool that can be used to study protein abundance levels at a given time. The main advantage of this technique is its ability to study hundreds of proteins at once. Moreover, as proteins are the "final products" of gene expression, differences in their abundances could indicate changes in gene expression. In addition, unlike transcriptomics, proteomics can also identify post-translational changes responsible for alterations in the protein structure and function. Proteomics has allowed a better understanding of the processes involved with quality meat, confirming and deepening previous concepts and identifying new mechanisms (CARVALHO et al., 2014; LAVILLE et al., 2009a; MURGIANO et al., 2010; POLATI et al., 2012).

Phosphorylation is a major post-translational modification responsible for changes in the protein structure and function and is characterized by reversible covalent binding of a phosphate group to an amino acid residue (serine, threonine or tyrosine). Phosphoproteomics is a branch of proteomics to study phosphorylated proteins. Pro-Q Diamond phosphoprotein stain (Pro-Q DPS) is one of the main technologies used to detect phosphorylated proteins. Pro-Q DPS binds directly to the phosphate moiety and allows sensitive

fluorescence detection of phosphoproteins in polyacrylamide gels (STEINBERG et al., 2003). Phosphorylation could change the myofilaments structure affecting rigor mortis and proteolysis during meat aging and regulate the glycolytic enzymes activity responsible for the post-mortem muscle pH drop (D'ALESSANDRO et al., 2012a, 2012b; MUROYA et al., 2007a, 2007b).

The zebu (*Bos taurus indicus*) cattle, mainly the Nellore breed, provide most of the beef exported by Brazil, which is the world's largest exporter (UNITED STATES OF AMERICA, 2016). However, Nellore beef is generally tougher and leaner than taurine (*Bos taurus taurus*), like Angus (DUARTE et al., 2013; MARTINS et al., 2015). Several studies have shown that the lowest tenderness of zebu beef could be due to its greatest calpastatin activity, inhibiting the proteolytic enzyme calpain (DUARTE et al., 2013; RUBENSAM; FELÍCIO; TERMIGNONI, 1998; WHIPPLE et al., 1990), which is reported as one of the main responsible for beef tenderization (GEESINK et al., 2006). However, other proteolytic systems beyond calpain/calpastatin have also been suggested to be important such as cathepsins, caspase and proteasome (KEMP et al., 2010). Furthermore, apoptosis, which is defined as programmed cell death, has also been proposed to be involved in the conversion of muscle to meat and tenderization (OUALI et al., 2006, 2013). In addition, muscle fiber type could also be related to meat quality differences (CHOI; KIM, 2009; LEFAUCHEUR, 2010).

Proteomic studies comparing animals with different meat quality characteristics have shown differences in the abundance of myofilaments expressed in fast or slow-twitch fibers and enzymes involved in the glycolytic and oxidative metabolism (D'ALESSANDRO et al., 2012b; KEADY et al., 2013; MA et al., 2015). Furthermore, proteomics and phosphoproteomics have also indicated the involvement of apoptosis with meat tenderness (LAVILLE et al., 2009b, LONGO et al., 2015). Although studies comparing fresh or aged

muscle of different breeds of cattle have been performed (KEADY et al., 2013; MARINO et al., 2013), proteomics and phosphoproteomics studies have not been conducted to compare zebu muscle with taurine. Thus, the aim of this study was to compare the muscle proteomic and phosphoproteomic profile of Nellore and Angus cattle. It is believed that this study will contribute for Meat Science increasing the understanding of the molecular mechanisms related to differences in beef quality between taurine and zebu.

2 LITERATURE REVIEW

2.1 Molecular mechanisms involved in the differences in beef quality between taurine and zebu cattle

Zebu beef is generally leaner and tougher than taurine (DUARTE et al., 2012; LAGE et al., 2012; MARTINS et al., 2015; PEREIRA et al., 2015). The highest calpastatin activity has been reported as the major mechanism responsible for lower zebu beef tenderness, because calpastatin is the natural inhibitor of calpains, which are considered the main responsible for proteolysis related to meat tenderization (GEESINK et al., 2006; WHIPPLE et al., 1990). Indeed, it was observed higher calpastatin activity in muscle of Nellore than Angus. Furthermore, Nellore had lower myofibrillar fragmentation index than Angus (DUARTE et al., 2013). Nonetheless, the mechanism responsible for reduced calpastatin activity in taurine has not been elucidated. Interestingly, it has been suggested that other proteases could hydrolyze calpastatin more intensely in taurine than Zebu (WHIPPLE et al., 1990). In this way, it has recently been reported that caspase-3, an effector of apoptosis, can degrade calpastatin (HUANG et al., 2014). Thus, it has been proposed that caspase-3 would inhibit the calpastatin activity and promote the calpain activity (LANA; ZOLLA, 2016). These findings could indicate that differences in beef tenderness between taurine and zebu could also be related to apoptosis.

There is little knowledge about the molecular mechanisms related to the difference in the intramuscular fat content between taurine and zebu. In one of the few studies available, it was reported that genes associated with fat deposition (diacylglycerol acyltransferase 1, thyroglobulin protein and leptin) were not differentially expressed among muscle of Nellore and Canchim (3/8 Zebu×5/8 Charolais) (GIUSTI et al., 2013). It was suggested that other factors

such as translation could be involved in the difference in intramuscular fat content between taurine and zebu. Furthermore, it was also proposed that other genes could be related to this difference. In another study, the expression of genes related to lipogenesis (acetyl-CoA carboxylase, fatty acid synthase, fatty acid binding protein-4 and sterol regulatory element-binding protein 1), lipolysis (carnitine palmitoyltransferase-2, lipoprotein lipase and acyl-CoA oxidase) and adipogenesis (CCAAT enhancer binding protein alpha, peroxissome proliferator activated-receptor gamma and zinc finger protein 423) did not differ between Angus and Nellore muscle (MARTINS et al., 2015). However, Angus had greater abundance of the protein peroxissome proliferator activated receptor-gamma, suggesting an enhancement of adipogenic differentiation of progenitor cells in Angus muscle. This result could also indicate that the difference in intramuscular fat content among Nellore and Angus would be related to differences in protein level rather than mRNA.

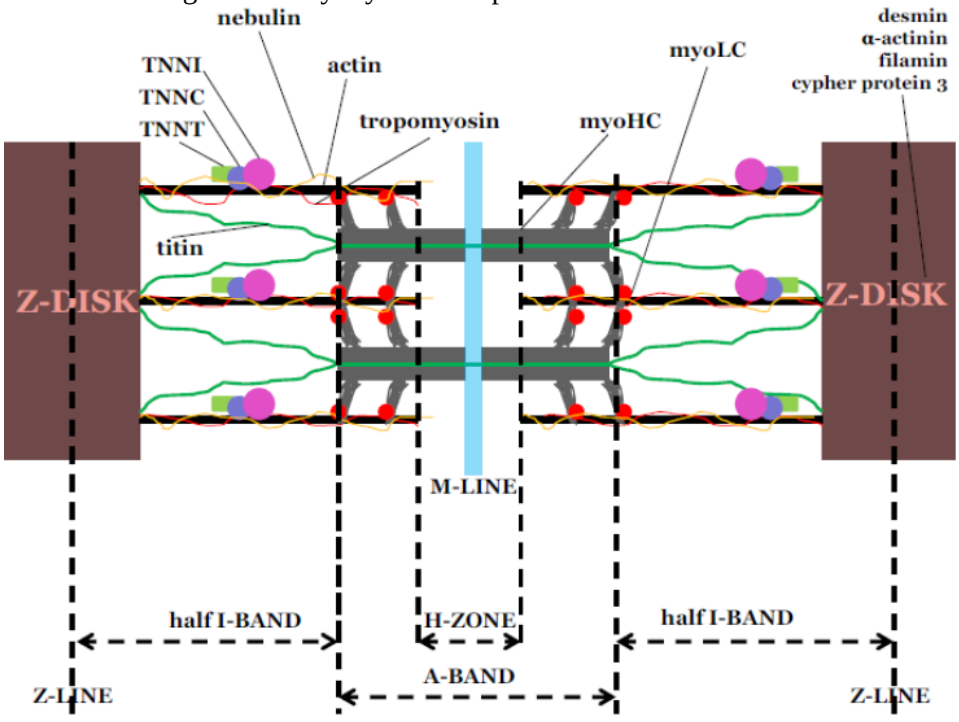
Thus, it is noted that the molecular mechanisms responsible for the differences in beef quality between taurine and zebu have not yet fully understood. Proteomics could provide new insights about these mechanisms.

2.2 Myofibrillar proteins

As this review will show results of proteomics and phosphoproteomics studies related to meat quality, we will provide a brief description of the main myofibrillar proteins because they occupy most of the muscle cell volume and are responsible for major reactions linked to conversion of muscle to meat, and are the main substrates for proteolytic enzymes during meat aging (HUFF-LONERGAN; ZHANG; LONERGAN, 2010). The myofibrillar proteins are part of the myofibril, which is a specialized organelle found in the muscle fiber responsible for its structural rigidity and contractile property. These proteins are

organized into thick and thin filaments called myofilaments, which repeat along the length of the myofibril in sections called sarcomeres. In general, myofibrillar proteins can be classified as follows (FIGURE 1): 1) contractile proteins (myosin and actin); 2) regulatory proteins (tropomyosin and troponin) and cytoskeletal proteins (α-actinin, filamin, nebulin, desmin, titin and vinculin, among others) (GOMIDE; RAMOS; FONTES, 2013; KEMP et al., 2010; LANA; ZOLLA, 2016).

Figure 1 – Key myofibrillar proteins of the sarcomere.



Captions:Troponin-I (TNNI), Troponin-C (TNNC), Troponin-T (TNNT), Myosin heavy chain (myoHC) and Myosin light chain (myoLC).
Source: Lana and Zolla (2016).

Myosin is the major myofibrillar protein and is characterized by two functional parts: two globular heads and an alpha-helical coiled-coil rod,

forming the thick filament of myofibril. The myosin molecule in skeletal muscle consists of two myosin heavy chains, two essential myosin light chains and two regulatory myosin light chains (REGGIANI; BOTTINELLI; STIENEN, 2000). F-actin is the main thin filament of myofibril and is formed by the polymerization of G-actin monomers. Two F-actin molecules form two twisted alpha helices that associate with the regulatory proteins tropomyosin and the troponins (CLARK et al., 2002). Tropomyosin is a two-stranded alpha-helical coiled coil protein which lies along the long double helical array of G-actin monomers on thin filaments. Tropomyosin is also associated at specific points with the troponin complex, which is composed for three globular subunits: troponin-T, troponin-I and troponin-C. Tropomyosin and the troponin complex regulate the interaction of actin filaments with myosin (LEHMAN et al., 2009).

Titin is the third most abundant muscle protein and is the largest known protein. It forms the third filament system of striated muscle connecting the Z-line to the thick filaments and helps in maintaining sarcomeric alignment of myofibrils during contraction, functioning as a kind of molecular spring regulating the myofibrillar stiffness. Nebulin is part of the fourth system of muscle filaments extending from the Z-line to the pointed ends of the F-actin filament. Nebulin is considered a kind of molecular rule due to its high inextensibility defining the length of the thin filaments. Desmin is the predominant intermediate filament protein of striated muscle and is a component of the Z-line region, costameres, the myotendinous junction and intercalated discs. Desmin connects adjacent myofibrils at the level of their Z-lines and myofibrils with the sarcolemma, nuclei and mitochondria (CLARK et al., 2002).

2.3 A proteomic and phosphoproteomic view of the main factors affecting meat quality

Meat is one of the main sources of animal protein consumed in the world. However, consumer expectations concerning the meat quality are often not met and this is a great problem for industry that seeks to understand the mechanisms related to meat quality, in order to develop management and handling strategies before and after slaughter to provide a product with more consistent quality (HOCQUETTE et al., 2012; PICARD et al., 2015; TROY; KERRY, 2010).

Tenderness, color and marbling are the major meat quality attributes considered by consumers during their purchase decision (FONT-I-FURNOLS; GUERRERO, 2014). Several factors may affect these attributes and they can be classified in pre and post-slaughter. The main pre-slaughter factors are: individual variation, genetic group, nutrition and feeding management, slaughter age, sex class and pre-slaughter stress. On the other hand, we can highlight carcass electrical stimulation and meat aging as the major post-slaughter factors (GUERRERO et al., 2013).

Proteomics is a molecular biology technique that studies proteins and has been heavily used in Meat Science to promote a better understanding on the mechanisms linked to meat quality, because proteins are the main constituents of muscle tissue and biochemical changes related to conversion of muscle to meat and structural alterations that occur during meat aging are controlled by enzymes (D'ALESSANDRO; ZOLLA, 2013; LANA; ZOLLA, 2016; PAREDI et al., 2012). Furthermore, as proteomics can compare the abundance of many proteins at the same time, it has been possible to identify new proteins linked to meat quality, which had not previously been studied with targeted methods (JIA et al., 2009; MORZEL et al., 2008). In addition, as proteins are the "end products" of genes, differences in their abundance could reflect differences in gene expression (GOBERT et al., 2014).

Two-dimensional electrophoresis coupled to mass spectrometry (2DE/MS) has been the main proteomics technique used in Meat Science (PAREDI et al., 2012). Briefly, the proteins are first separated by their isoelectric point (isoelectric focusing or first dimension) using an immobilized pH gradient gel strip. Then, the strip is placed on a polyacrylamide gel (SDS-PAGE) and the focused proteins are separated by their molecular weight (second dimension). Subsequently, the differentially abundant protein spots are identified using mass spectrometry (RABILLOUD; LELONG, 2011).

However, 2DE/MS is generally limited to study low abundance and high molecular weight proteins. Thus, differences in abundance of proteolytic enzymes such as calpain, caspases, cathepsins and proteasome, and contractile and structural proteins of high molecular weight like myosin heavy chain and titin have not usually been identified (D'ALESSANDRO et al., 2012a; HUANG; LAMETSCH, 2013a). Nevertheless, 2DE/MS allow the large-scale study of protein abundance at a lower cost than other most advanced techniques (HUANG; LAMETSCH, 2013a).

Proteins may undergo post-translational modifications that can change their structure and function (MANN; JENSEN, 2003). Phosphorylation stands out between the major post-translational modifications. Phosphoproteomics is a branch of proteomics to study phosphorylated proteins. Many of the metabolic and structural changes that take place during the conversion of muscle to meat are regulated by protein phosphorylation (HUANG; LAMETSCH, 2013b). Nevertheless, phosphoproteomic studies in Meat Science are still scarce (CHEN et al., 2016; HUANG et al., 2011; HUANG; LARSEN; LAMETSCH, 2012).

The aim of this review was to show results of proteomic studies evaluating the main factors affecting the meat quality. We have focused our attention on the following factors: individual variation, genetic group, nutrition and feeding management, sex class, slaughter age, pre-slaughter stress, carcass

electrical stimulation and meat aging. In addition, when available, we will also present results of phosphoproteomic approaches evaluating these factors. Moreover, as the identification of several proteins belonging to the family of heat shock proteins (HSPs) linked to differences in meat tenderness has been one of the most important contributions of proteomics and phosphoproteomics to Science Meat, we will start this review by addressing the relationship between HSPs and meat tenderness because it is a very controversial issue.

2.3.1 Heat shock proteins and meat tenderness

When caspase-dependent apoptosis was suggested as a major responsible for meat tenderization (OUALI et al., 2006), the study of HSPs became of great interest to Meat Science. HSPs are members of a great family of proteins expressed in different tissues and located in various cellular compartments and can be classified into 5 classes according to their approximate molecular weights (kDa): HSP40 (DNAJ), HSP70 (HSPA), HSP90 (HSPC), HSP100 (HSPH) and small HSPs (HSPB), which are formed by different proteins that differ in structure, location, type of stressor and function (KAMPINGA et al., 2009). For example, within the family of HSP70, HSP72 is localized in the cytoplasm and nucleus, while HSP75 (HSPA9 or GRP75) is located in the mitochondria and HSP78 (HSPA5 or BIP) in the endoplasmic reticulum (KREGEL, 2002). Although HSPs have been initially associated with the heat stress, they are not all stimulated by it (KREGEL, 2002).

Several studies have found a relationship between meat tenderness and HSPs abundance (CARVALHO et al, 2014; GUILLEMIN et al., 2012; KIM et al., 2008). Due to chaperone activity is highly conserved among the HSPs, many research groups have discussed the involvement of HSPs in the meat tenderizing process, without considering their singularities. In general, when the association

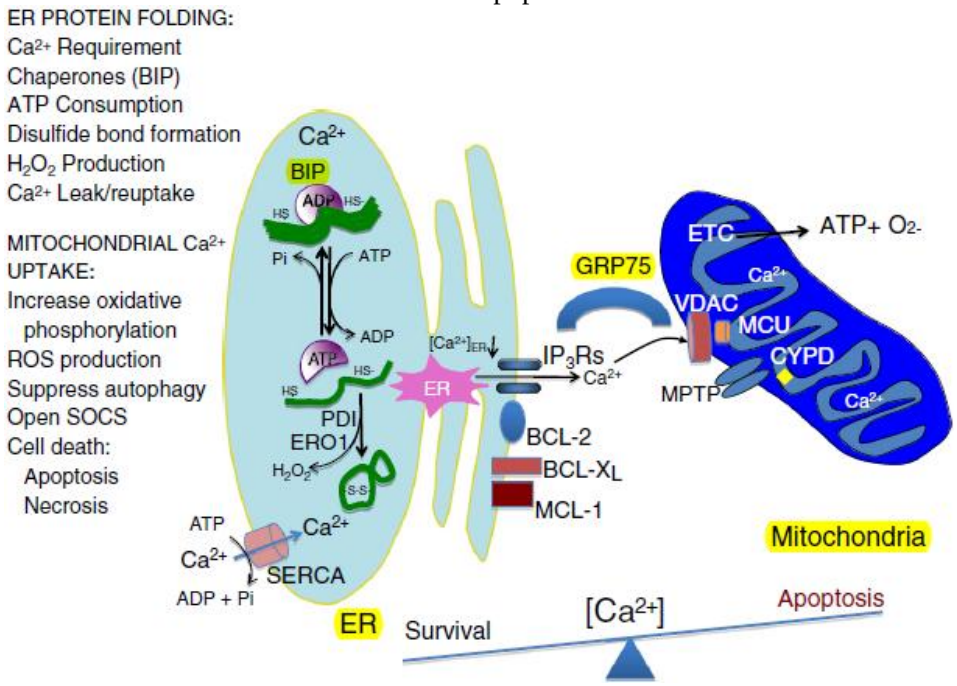
between HSPs abundance and meat tenderness is positive, it has been suggested that HSPs would prevent the formation of protein aggregates immediately after slaughter and this would facilitate the activity of proteolytic enzymes during meat aging (MORZEL et al., 2008). Conversely, other studies have reported a negative correlation between HSPs abundance and meat tenderness and, in these cases, it has been proposed that the anti-apoptotic effect of HSPs could prevent or delay the meat tenderizing by apoptosis (PICARD et al., 2010). These contradictions about the effect of HSPs on meat tenderness might be related to their different cell locations, stressor and activities. Thus, it has been recommended that the role of HSPs on meat tenderness should be evaluated by considering their singularities (OUALI et al., 2013).

It was initially proposed that HSPs would affect meat tenderness by their anti-apoptotic role as follows: 1) Forming complex with active caspases (initiators and effectors) preventing their activity; 2) Protecting caspase substrates preventing or delaying their degradation; 3) Reinstating damaged protein (OUALI et al., 2006). Later, other mechanisms of how HSPs could affect meat tenderness were elucidated. For example, it was reported that αβ-crystallin, which belongs to the class of small HSPs and is also known as heat shock protein beta-5, can be an alternate substrate for μ-calpain, preventing the degradation of structural proteins such as titin and desmin (LOMIWES et al., 2014a). Furthermore, it was observed that the levels of heat shock protein beta-5 in the sarcoplasmic fraction had greater reduction during the meat aging at muscle with the lowest ultimate pH, suggesting that HSPs could migrate from the sarcoplasmic fraction to myofibrillar during muscle acidification to associate with myofilaments protecting them against denaturation and calpain/caspase-mediated proteolysis (LOMIWES et al., 2014a; PULFORD et al., 2009).

Interestingly, it has been reported that under conditions of acute stress such as those found in muscle after slaughter (interruption in the supply of

nutrients and oxygen and pH drop), HSPs could also trigger apoptosis (D'ALESSANDRO et al., 2012a). Moreover, some HSPs are stimulated by glucose deprivation and Ca^{2+} influx, such as 78 kDa glucose-regulated protein and mitochondrial stress-70 protein (LIÈVREMONT et al., 1997; LONDONO et al., 2012), conditions that occur in muscle after slaughter. Mitochondrial stress-70 protein is the main mitochondrial HSP and is part of the communication system between endoplasmic reticulum and mitochondria, which leads Ca^{2+} from the former to the latter (KAUFMAN; MALHOTRA, 2014). Accumulation of Ca^{2+} in mitochondria can cause mitochondrial membrane depolarization with the release of pro-apoptotic factors inducing caspase-dependent apoptosis (OUALI et al., 2013). Mitochondrial stress-70 protein can also contribute to apoptosis by forming complexes with mitochondrial calpain and pro-apoptotic factors allowing their transport into the mitochondria, which may also induce mitochondrial membrane depolarization and subsequent release of pro-apoptotic factors in the cytoplasm (FENG et al., 2005; OZAKI; YAMASHITA; ISHIGURO, 2011). This could explain why the mitochondrial stress-70 protein abundance had negative correlation with both pH at 3 hours and ultimate pH in cattle muscle and positive correlation with calpain (GAGAOUA et al., 2015a, 2015b). Moreover, it could also explain why muscle classified as tender beef had higher mitochondrial stress-70 protein abundance (GUILLEMIN et al., 2011a; PICARD et al., 2014). Figure 2 shows a communication model between endoplasmic reticulum and mitochondria, wherein 78 kDa glucose-regulated protein participates in maintaining Ca^{2+} in the endoplasmic reticulum, while mitochondrial stress-70 protein forms part of the communication system that leads Ca^{2+} from the endoplasmic reticulum to mitochondrion, which promotes apoptosis (KAUFMAN; MALHOTRA, 2014).

Figure 2 – Model showing the importance of the 78 kDa glucose-regulated protein and of the mitochondrial stress-70 protein for calcium flux between endoplasmic reticulum and mitochondria, and consequent cell fate toward survival or apoptosis.



Captions: Endoplasmic reticulum (ER), Reactive oxygen species (ROS), 78 kDa glucose-regulated protein (BIP), Protein disulfide isomerase (PDI), ER oxidase 1 (ERO1), Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), Inositol-1,4,5-triphosphate receptors (IP_3Rs), Anti-apoptotic proteins of Bcl-2 (B cell lymphoma 2) family members (BCL-2 and BCL-XL), Induced myeloid leukemia cell differentiation protein MCL-1 (MCL-1), Mitochondrial stress-70 protein (GRP75), Electron transport chain (ETC), Voltage-dependent anion-selective channel (VDAC), Mitochondrial inner membrane permeability transition pore (MPTP), Ruthenium red-sensitive mitochondrial Ca2+ uniporter (MCU) and Cyclophilin D (CYPD).
Source: Kaufman and Malhotra (2014).

Post-translational modifications such as phosphorylation could also affect the activity of small HSPs and their relationship with meat tenderness (LOMIWES et al., 2014b). It was observed that muscle classified as tough beef had greater phosphorylation of heat shock protein beta-1, which would be associated with the dissociation of the oligomeric state of heat shock protein

beta-1 into smaller oligomers and monomers that would be more effective against apoptosis (D'ALESSANDRO et al., 2012b). On the other hand, the phosphorylation effect of small HSPs and its relationship with meat tenderness is still controversial and appears to be a more complex process. For example, another study reported that heat shock protein beta-1 was more phosphorylated in muscle classified as tender beef, which could be related to dissociation of the complex heat shock protein beta-1 with serine/threonine kinase triggering apoptosis (D'ALESSANDRO et al., 2012a). In addition, it was observed that the phosphorylation level of heat shock protein beta-6 decreased during beef aging, which would indicate a reduction in the inhibitory effect of it on the initiators and effectors caspases (LONGO et al., 2015). However, the phosphorylation level of heat shock protein beta-5 increased during beef aging, which might be linked to progress of apoptosis.

2.3.2 Individual variation

Individual variation is the difference in meat quality between animals of the same genetic group raised under identical management conditions. This is one of the main factors studied by proteomics aiming to identify differentially abundant proteins or post-translational modifications related to meat quality. One of these studies has compared the muscle proteomic profile of Korean native cattle classified for high or low-quality beef (color score, tenderness and intramuscular fat) (KIM et al., 2008). The high-quality beef group had higher abundance of alpha actin, which would be associated with a decrease in the myofilament integrity and an increase in the myofibril fragmentation, because alpha actin is the major thin myofilament forming the muscle structure. On the other hand, the low-quality beef group had greater abundance of T-complex protein-1 and heat shock protein beta-1, which are proteins involved in the

recovery of denatured proteins and protecting the myofibrillar structures integrity. Thus, it was proposed that increased abundance of these proteins could delay the beef tenderization in the low-quality beef group.

More recently, it was compared the muscle protein profile of Nellore cattle classified for tough or tender beef (CARVALHO et al., 2014). Among the findings, we can highlight differences in the abundance of heat shock proteins and structural proteins. The tender beef group had greater abundance of heat shock protein beta-1 (two spots) than the tough beef group (one spot). However, the tough beef group had greater abundance of heat shock protein 70 kDa 1A and structural and contractile proteins (myosin light chain-1/3, myosin regulatory light chain-2 and tropomyosin alpha-1 chain). These contradictory relationships between heat shock proteins abundance and meat tenderness could be related to different activities of heat shock proteins and post-translational modifications.

Proteomics and phosphoproteomics approaches have provided new insights to help explain the effect of individual variation on beef quality. Maremmana cattle muscle classified as tender beef had greater glycolytic enzymes abundance (alpha enolase-1, phosphoglucomutase-1 and triosephosphate isomerase), which were less phosphorylated (alpha enolase-1, triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase) compared to tough beef (D'ALESSANDRO et al., 2012a). Based on additional metabolomics data and literature, it was suggested that glycolytic enzymes phosphorylation could inhibit their activity, which would have delayed the muscle pH drop in the tough beef group.

In a similar study conducted by the same group (D'ALESSANDRO et al., 2012b), Chianina cattle muscle classified as tough beef had greater actin phosphorylation compared to tender beef, which could be related to its lower susceptibility to apoptosis, which is defined as programmed cell death and has

been proposed to be one of the events responsible for meat tenderization through the caspase proteolytic system (OUALI et al., 2013). Furthermore, the tough beef group had greater abundance of myofilaments related to fast-twitch fiber (fast-twitch myosin light chain-1) and lower abundance of myofilaments expressed in slow-twitch fiber (slow skeletal troponina-T) (D'ALESSANDRO et al., 2012b). Although this issue is still controversial, especially in cattle, it has been reported that muscle fiber type may affect meat quality due to morphological and biochemical differences (LEFAUCHEUR, 2010).

In both studies (D'ALESSANDRO et al., 2012a, 2012b), differences were observed in heat shock proteins, which were generally more abundant in the tender beef group. Because apoptosis has been considered one of the main mechanisms involved in the meat tenderization and as heat shock proteins are important in this process, it was suggested that heat shock proteins might contribute to beef tenderization by triggering apoptosis. Similarly, another study reported a greater abundance of proteins of the outer and inner mitochondrial membranes (prohibitin, mitofilin, elongation factor Tu, two voltage-dependent anion-selective channel protein-1 and protein-2 and NADH-ubiquinone oxidoreductase) in Charolais cattle muscle classified for tender beef compared to tough beef group (LAVILLE et al., 2009b). It was proposed that this result could indicate a greater deterioration of the mitochondrial membrane in the tender beef group, which could induce apoptosis.

Moreover, differences in phosphorylation of glycolytic enzymes and myofilaments have also been reported in sheep muscle (fat-tailed sheep×tail sheep) classified for tough or tender meat (CHEN et al., 2016). The tough meat group had greater phosphorylation of glycolytic enzymes (glycogen phosphorylase, 6-phosphofructokinase, enolase, phosphoglycerate kinase, lactate dehydrogenase-A and aldolase-A) and myosin light chain-2. It was suggested that increased phosphorylation of glycolytic enzymes could be related to their

lower activity, while phosphorylation of myosin light chain-2 could be a type of biochemical memory enriching the muscle contraction force.

In view of these results, we could suggest that the individual variation in meat quality could be related to differences in the abundance of structural proteins and myofilaments isoforms expressed in slow or fast-twitch fibers. Moreover, differences in the heat shock proteins abundance indicate a possible involvement of apoptosis in the regulation of these differences. In addition, phosphoproteomic data suggest that differences in phosphorylation of glycolytic enzymes and myofilaments could also be involved in this process.

2.3.3 Genetic group

Genetic group is one of the main factors affecting carcass characteristics and meat quality attributes, and proteomics studies have allowed a better understanding of this effect. For example, a study was conducted with cattle to evaluate the effect of genetic group for different purposes, Friesian (dairy), Romagnola×Podolian crossbreed (beef), and Podolian (rustic), on the proteolytic pattern of myofibrillar protein (MARINO et al. 2013). Podolian cattle had lower intramuscular fat content and tougher beef at 1 and 7 days of aging. Furthermore, Podolian cattle beef had greater number of spots on 2DE gels at 21 days of aging. This result would be due to higher proteolysis in Podolian beef, highlighted by greater amount of myosin heavy chain fragments spots, which could explain the lack of difference in the beef tenderness among the three genotypes at 14 and 21 days of aging. In addition, Podolian beef had higher amount of troponin spots. It has been showed that troponin-T degradation is related to beef tenderization during aging (CRUZEN et al., 2014).

In another study, it was compared the muscle protein profile between Japanese Black (high marbling, tenderness and beef flavor) and Holstein cattle

(beef quality lower than Japanese Black) (OHSAKI et al., 2007). Holstein muscle had greater abundance of skeletal muscle ankyrin repeat protein-2, myosin light chain-2 and OGN protein (mimecan). The higher abundance of myosin light chain-2 in Holstein muscle could be related to its greater fast-twitch fibers proportion, because this protein is expressed in fast-twitch fiber, while the greater abundance of OGN protein might be associated with its increased connective tissue content, since OGN protein would have an important role in collagen fibrillogenesis and be expressed in connective tissue.

Differences in the abundance of proteins related to fiber type were also shown in a study comparing genetic groups of cattle with different body maturity and intramuscular fat content (KEADY et al., 2013). It was reported that Belgian Blue crossbred muscle (late body maturity genotype and high lean muscle tissue deposition potential) had greater abundance of fast-twitch myosin light chain-1 and phosphoglucomutase-1 (enzyme responsible to drive glucose into glycolysis or glycogenesis) than Angus crossbred muscle (early body maturity genotype and high intramuscular fat deposition potential), which could reflect a greater fast-twitch glycolytic fibers proportion and increased glycogen content in the Belgian Blue crossbred muscle. However, Angus crossbred muscle had greater abundance of aconitase-2 and 2-oxoglutarate dehydrogenase. This result was consistent with the highest intramuscular fat content in Angus crossbred, because these enzymes are linked to production of NADPH necessary for fatty acid synthesis via the pentose phosphate pathway.

Similar approaches have also been conducted with pigs. Casertana muscle (fat carcass breed) had greater abundance of L-lactate dehydrogenase A chain, triosephosphate isomerase-1, enolase-3 and cytosolic glycerol-3-phosphate dehydrogenase than Large White muscle (lean carcass breed) (MURGIANO et al., 2010). Thus, it was suggested that these enzymes could contribute to providing intermediates for lipogenesis, connecting glycolytic

pathway to lipogenesis, which would be related to higher fat content in Casertana carcass. Conversely, Large White muscle had greater abundance of fast skeletal muscle myosin light chain-1 and actin, which would be associated with a higher fast-twitch glycolytic fiber proportion and protein mass deposition in Large White muscle.

The abundance of glycolytic enzymes (triosephosphate isomerase-1 and phosphoglucomutase-1) and myosin binding protein-H (regulatory protein of the thick filament that is mainly found in fast skeletal muscle) were higher in Large White pigs muscle than Meishan pigs muscle (breed with high intramuscular fat content) (XU et al., 2009). However, Meishan pigs muscle had greater abundance of ATPase β chain (enzyme involved in mitochondrial energy metabolism) and myosin light chain-1 slow-twitch muscle-A and myosin light chain-1 slow-twitch muscle-B. Given these results, it was suggested that animals selected for increased lean meat deposition would have higher fast-twitch fiber proportion and greater glycolytic energy metabolism, while animals with higher intramuscular fat content would have higher slow-twitch fiber proportion and greater oxidative energy metabolism.

Taken together, these findings suggest that one of the main effects of genetic group on meat quality would be related to differences in muscle fiber type. Generally, animals with higher intramuscular fat content have greater slow-twitch oxidative fibers proportion, while animals with leaner meat have higher fast-twitch glycolytic fibers proportion, resulting in differences in the abundance of myofilaments expressed in fast or slow-twitch fibers and glycolytic and oxidative enzymes. Nevertheless, higher abundance of some glycolytic enzymes also occurs in muscle of animals selected for greater intramuscular fat content, which would be associated with the supply of glycolytic intermediates required for lipogenesis. However, fewer studies have compared genetic groups differing in meat tenderness. It has been observed

greater abundance of target myofilaments for proteolysis in genotypes with tougher beef. On the other hand, although the zebu beef is generally considered to be tougher and leaner than taurine, the muscle protein profile between them have not been compared. Furthermore, phosphoproteomics studies comparing fresh or aged muscle of different genetic groups have not been performed.

2.3.4 Sex class

Sex class is one of the largest sources of variation in meat quality. Steer beef is usually more tender, fatter and less dark than bull beef (MARTI et al., 2013). Generally, bull has greater insoluble collagen content in muscle, which is related to its lower meat tenderness (BOCCARD et al., 1979). Nevertheless, to the best of our knowledge, only one large-scale proteomics study assessing the effect of sex class on the muscle protein profile has been conducted with cattle (ZHANG et al., 2012). Bulls had higher abundance of myosin light chain-1, ankyrin repeat domain-containing protein-1 and heat shock protein beta-1, and lower abundance of cofilin-2 than steers. It was proposed that the greater abundance of ankyrin repeat domain-containing protein-1 and heat shock protein beta-1 in bull would be related to its higher muscle hypertrophy, while the greater abundance of cofilin-2 in steers would be negatively related to muscle hypertrophy due to actin filaments depolymerization. This result could indicate that bulls would have lower muscle protein turnover than steers, which might reduce its beef tenderness. The lowest muscle protein turnover in bull would be linked to its increased calpastatin expression, because it inhibits calpain, which in turn is responsible for the breakdown of structural proteins related to meat tenderization (MORGAN et al., 1993a, 1993b; GEESINK et al., 2006).

However, it has been proposed that differences in meat quality among intact and castrated male could also be partly explained by differences in muscle

fiber type. Castrated male would have higher fast-twitch glycolytic fiber proportion and lower slow-twitch oxidative fiber than intact males (SCHREURS et al., 2008). Fast-twitch glycolytic fiber generally has greater volume and sectional area than slow-twitch oxidative fiber. Although controversial, it has been proposed that animals with higher fast-twitch glycolytic fiber content would have tougher meat (CHOI; KIM, 2009).

Interestingly, a study has been conducted to compare the abundance of 24 protein biomarkers of beef tenderness among bulls and steers muscles by western blot (GUILLEMIN et al., 2011b). As previously suggested, steers had greater abundance of fast skeletal muscle myosin light chain-1, myosin heavy chain IIx (isoform expressed in fast-twitch fiber) and glycolytic enzymes (enolase-1 and enolase-3). Furthermore, steers had higher abundance of m-calpain and μ-calpain. In addition, differences in the heat shock proteins abundance were also observed. Bulls had higher abundance of alpha-crystallin B chain and heat shock protein beta-1, which are generally known to inhibit apoptosis. However, steers had higher abundance of heat shock protein family A (Hsp70) member 8 (HSPA8) and heat shock protein family A (Hsp70) member 9 (HSPA9). Intriguingly, although HSPA9 (also known as mortalin and GRP75) is associated with cellular protection due to its chaperone activity, it has also been implicated in triggering apoptosis (FENG et al., 2005; LONDONO et al., 2012; OZAKI; YAMASHITA; ISHIGURO, 2011). Therefore, differences in beef tenderness between bulls and steers could also be related to apoptosis. Given these findings, we could suggest that differences in beef tenderness between bulls and steers might be related to differences in protein turnover and apoptosis. Fiber type could also be involved in differences of beef quality among bulls and steers.

2.3.5 Nutrition and feeding management

Although nutrition and feeding management are key factors affecting meat quality, there are few studies that have evaluated their effect on changes in muscle proteome. In one of these studies, Japanese Black cattle previously raised in feedlot with a concentrated-based diet were finished on pasture or kept in feedlot (SHIBATA et al., 2009). Pasture-finished cattle muscle had greater abundance of slow-twitch myosin light chain-2 and proteins related to oxidative metabolism (myoglobin and adenylate kinase-1). Conversely, feedlot-finished cattle muscle had greater abundance of glycolytic enzymes (β-enolase-3, fructose-1,6-bisphosphate aldolase-A and triosephosphate isomerase). It was suggested that these results could indicate a conversion in muscle fiber type associated with metabolic changes when the animals moved from feedlot to pasture. Although beef quality characteristics have not been evaluated, this result was interesting because muscle fiber type (slow-twitch or fast-twitch) and predominant energy metabolism (glycolytic or oxidative) may be related to meat quality (CHOI; KIM, 2009; LEFAUCHEUR, 2010).

In another study, the effect of compensatory growth on protein abundance in pigs muscle at 0 and 48 h after slaughter was evaluated (LAMETSCH et al., 2006). Animals that had compensatory growth were feed-restricted (60% of *ad libitum* intake) from 28 to 80 days and then fed *ad libitum* until 140 days when were slaughtered. Two members of the heat shock protein family (heat shock cognate 70 kDa protein and heat shock protein beta-1) were more abundant in pigs muscle that exhibited compensatory growth. Because these proteins regulate the fate of damaged proteins and participate in the myofilaments stabilization, it was proposed that this result would be linked to greater muscle protein turnover in response to compensatory growth. Furthermore, pig showing compensatory growth had higher abundance of the mitochondrial protein sulfite oxidase and two nuclear proteins (elongin-B and

intracellular chloride channel-1) at 48 h after slaughter, which could be related to greater number of mitochondria and satellite cells and/or lower mitochondrial and nuclear membranes stability in response to compensatory growth. So, it was suggested that higher muscular turnover would be associated with greater meat tenderness in pigs exhibiting compensatory growth.

Besides the studies evaluating the effect of compensatory growth and finishing system, proteomics has also been used to evaluate the effect of dietary supplementation on meat quality. For example, the effect of conjugated linoleic acid (CLA) supplementation on changes in muscle protein profile linked to meat quality in pigs has been evaluated (ZHONG et al., 2011). Pigs fed CLA-supplemented diet (25 g of CLA/kg of diet) had higher intramuscular fat content (approximately 54%) and greater abundance of muscle proteins involved in the energy expenditure (creatine kinase M-type and creatine kinase), fatty acids oxidation (medium-chain acyl-CoA dehydrogenase specific and 5'-AMP-activated protein kinase γ3 subunit) and amino acid metabolism and long chain fatty acids transport (mitochondrial aspartate aminotransferase precursor), which would be associated with greater macronutrients degradation to provide the energy needed for intramuscular fat synthesis. Moreover, a positive correlation was observed between carbonic anhydrase-3 abundance and intramuscular fat content, which would be related to higher bicarbonate and hydrogen supply for fat synthesis.

Furthermore, the effect of high-fat diet (10% lard-supplemented) on meat quality and changes in muscle proteome has also been reported in pigs (LIU et al., 2014). High fat-fed pigs had greater intramuscular fat content than control group. Interestingly, high fat-fed pigs muscle had lower abundance of myofilaments (myosin heavy chain, fast skeletal muscle troponin T and tropomyosin) and greater abundance of energy metabolism enzymes (phosphoglucomutase-1 and adenylate kinase-1) and stress response and

apoptosis-related proteins (heat shock protein, α-crystallin-related, B-6, calreticulin, excision repair protein and calmodulin). Therefore, it was suggested that high-fat diet could modify the muscle structure, energy metabolism, stress response and apoptosis, which could affect meat quality (D'ALESSANDRO; ZOLLA, 2013).

More recently, it was shown that 1% L-arginine-supplemented pigs had higher intramuscular fat content (approximately 32%) compared to a control group (MA et al., 2015). Moreover, L-arginine-supplemented pigs muscle had greater abundance of slow-twitch skeletal troponina-I and lower abundance of myosin heavy chain-2b (fast-twitch isoform) and glucose-related enzymes (phosphorylase glycogen, phosphoglycerate kinase-1 and muscle-specific enolase beta subunit). The higher intramuscular fat content in the supplemented animals would be associated with a higher proportion of slow-twitch oxidative fibers. It has been suggested that the intramuscular fat content is positively related to the oxidative muscle fibers proportion (KEADY et al., 2013; MURGIANO et al., 2010).

Although there are few studies that have evaluated the effect of nutrition and feeding management on changes in the muscle protein profile linked to meat quality, it has been observed differences in the abundance of myofilaments expressed in fast or slow-twitch fibers and enzymes involved in energy metabolism. In addition, these results suggest that the large variation observed for the relationship between a given biomarker and meat quality might be due to differences in nutrition and feeding management, because a single dietary ingredient could change the muscle protein profile.

2.3.6 Slaughter age

Slaughter age may be considered one of the major management factors affecting meat quality, because animals slaughtered at a later stage generally have tougher meat, mainly due to an increase in insoluble collagen content (PURSLOW; ARCHILE-CONTRERAS; CHA, 2012). Nevertheless, few studies have evaluated changes in muscle protein profile related to meat quality in response to slaughter age. In one such study, Charolais cattle slaughtered at 18 months had higher abundance of alpha-actin and glycolytic enzymes (alpha-enolase and triosephosphate isomerase) compared to those slaughtered at 14.5 months. However, animals slaughtered at 14.5 months had greater abundance of heat shock protein beta-1 (GULYÁS et al., 2015). According to the authors, these results could indicate an increased glycolytic activity in muscle of older cattle. Furthermore, the greater abundance of actin in muscle of older cattle would be related to an increase in the muscle fiber size, which in turn could affect beef tenderness. In addition, it was suggested that the greater abundance of heat shock protein beta-1 in muscle of younger cattle could be positively associated with beef tenderness. Another research was conducted with pigs slaughtered at 6, 9 or 12 months (HOLLUNG et al., 2009). Muscle of older pigs had greater abundance of glycolytic enzymes (enolase-3 and triosephosphate isomerase-1). However, older pigs had lower abundance of actin, which could indicate lower myofibrils degradation.

2.3.7 Pre-slaughter stress

Pre-slaughter stress is a major contributor to reduction in meat quality. The conversion of muscle to meat involves many physical and chemical changes that can be influenced by animal physiological stage prior to slaughter. In cattle, pre-slaughter stress is mainly related to depletion of muscle glycogen stores that contributes to high ultimate pH and production of dry, firm and dark (DFD)

beef. In pigs and poultry, pre-slaughter stress is mostly linked to an accelerated glycolysis immediately after slaughter that leads to a decrease in meat pH below normal, resulting in production of pale, soft and exudative (PSE) meat (ADZITEY; NURUL, 2011). Proteomic and phosphoproteomic studies have allowed a better understanding of the pre-slaughter stress effect on meat quality. For example, DFD beef had lower abundance of myosin light chain-3, myosin light chain-6B, myosin regulatory light chain-2 and troponin C type-2 than normal beef (FRANCO et al., 2015). As DFD beef was more tender than normal beef, it was suggested that the lowest abundance of myofilaments in DFD beef could be due to increased enzymatic degradation of myofibrillar protein. Moreover, DFD beef had greater abundance of cofilin-2. Because cofilin controls reversibly actin polymerization and depolymerization in a pH-dependent manner, with maximal depolymerization at pH 8 and almost abolished at pH <7, it was hypothesized that the higher pH (6.4 vs. 5.6) of DFD beef could lead to actin depolymerization, promoting tenderness.

In addition, it has been reported that muscle of pigs with HAL nn genotype (animals more susceptible to produce PSE meat) had greater abundance of glycolytic enzymes fragments (enolase-3 and phosphoglycerate kinase) and lower abundance of myofilaments (fast skeletal myosin light chain-1, myosin regulatory light chain-2 and alpha actin-1), chaperones (heat shock cognate 71 kDa protein and heat shock protein beta-1) and of the antioxidant protein mitochondrial aldehyde dehydrogenase-2 than HAL NN genotype (animals less susceptible to produce PSE meat) (LAVILLE et al., 2009a). It was proposed that nn pigs muscle could have lower antioxidant and repair capacity than NN pigs muscle. Furthermore, a faster early postmortem pH drop in nn pigs muscle could decrease the solubility of myofilaments and heat shock proteins due to increased actin-myosin bonding strength and/or heat shock proteins migration to the insoluble fraction to prevent myofilaments denaturation.

Additionally, it was proposed that myofilaments hypercontraction and protein aggregation and precipitation on myofilaments could reduce their exposure to degradation by proteolytic enzymes and contribute to lower tenderization observed in PSE meat.

In another study with pigs, it was observed that glucose-related enzymes (glycogen phosphorylase, phosphofructokinase and pyruvate kinase) had higher phosphorylation level during the conversion of muscle to meat in RN^- genotype pigs (more susceptible to PSE meat) compared to wild-type pigs (less susceptible to PSE meat) (LAMETSCH et al., 2011). According to the authors, the phosphorylation of these enzymes has been reported to make them more active, less susceptible to inhibition by lactate and more stable at lower pH values. Thus, besides higher muscle glycogen content in RN^-genotype, it was hypothesized that its greater rate and muscle pH fall extension would also be consequence of an increased glucose-related enzymes phosphorylation. Interestingly, it was also observed that myosin regulatory light chain-2 had higher phosphorylation level during the conversion of muscle into meat in wild-type pigs compared to RN^- genotype. This result was interesting because wild-type pigs were expected to have a higher meat pH than RN^- genotype pigs. Similarly, the phosphorylation level of myosin regulatory light chain-2 was greater in DFD beef than normal beef, and it was hypothesized that the higher pH in DFD beef could run against the activity of acid phosphatase, which is responsible for dephosphorylation of myosin regulatory light chain-2 (FRANCO et al., 2015). It has been reported that phosphorylation of myosin regulatory light chain-2 can increase the strength of muscle contraction (RYDER et al., 2007), which could affect meat tenderness (LANA; ZOLLA, 2016).

Taking these findings together, we could suggest that differences in the abundance of chaperones and phosphorylation of glycolytic enzymes and myofilaments could explain the effect of pre-slaughter stress on meat quality.

2.3.8 Carcass electrical stimulation

Electrical stimulation of carcass has been linked to improve beef quality promoting a faster muscle pH drop due to increased glycolysis rate that accelerates rigor mortis and prevents cold shortening. In addition, electrical stimulation could also improve meat tenderness stimulating the breakdown of myofilaments and structural proteins (HWANG; DEVINE; HOPKINS, 2003). Proteomics and phosphoproteomics have allowed a deeper understanding of how electrical stimulation improves beef quality. It has been shown that electrically stimulated muscle had lower abundance of enzymes related to energy metabolism (α-enolase and creatine kinase M chain) than unstimulated muscle, which could be related to higher glycolysis and energy metabolism due to electrical stimulation, resulting in the degradation of these enzymes (BJARNADÓTTIR et al., 2011). Furthermore, electrically stimulated muscle had lower abundance of myofilaments and structural proteins (fast troponin-T, actin and desmin), which could be associated with myofibrillar fragmentation and beef tenderization. However, electrically stimulated muscle had greater abundance of small heat shock proteins (heat shock protein family B (small) member-6, α-crystallin B chain and heat shock protein beta-1) at 1 h after stimulation, indicating a greater stress in response to electrical stimulation, and supporting the hypothesis that postmortem proteolysis is enriched in electrically stimulated beef.

Similar result was obtained in another study (SHEN et al., 2012). Electrically stimulated beef had lower abundance of the protein belonging to the contractile apparatus myosin binding protein H and energy metabolism enzymes (triosephosphate isomerase and M-type creatine kinase) than unstimulated beef,

suggesting greater proteolysis and muscle energy metabolism in response to electrical stimulation.

More recently, it was also reported that the abundance of glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase A, phosphoglycerate kinase-1 and pyruvate kinase isozyme M1) decreased in electrically stimulated beef, indicating greater glycolysis due to electrical stimulation (KIM et al., 2013). Interestingly, it was also observed that the abundance of Annexin A5 increased in electrically stimulated beef. As Annexin A5 is associated with the sarcoplasmic reticulum membrane and is involved in the calcium flux regulation, it was suggested that its higher abundance in electrically stimulated beef could be associated with the calpain system and activation of calcium-dependent proteolysis.

Electrical stimulation of carcass may affect the postmortem muscle energy metabolism promoting post-translational changes in enzymes such as phosphorylation. Electrically stimulated cattle muscle had lower phosphorylation of enzymes involved in energy metabolism and glycolysis (creatine kinase M chain, fructose-bisphosphate aldolase C-A, β-enolase and pyruvate kinase) than unstimulated muscle (LI et al., 2012). It was hypothesized that electrical stimulation would stimulate dephosphorylation of these enzymes, increasing their activity and promoting a faster ATP depletion and muscle pH drop.

Therefore, proteomics and phosphoproteomics have provided further evidence for the hypothesis that electrical stimulation would accelerate glycolysis and promote proteolysis, contributing to beef tenderness.

2.3.9 Meat aging

Meat aging is essential for resolution of rigor mortis and is mainly characterized by myofilaments and structural proteins degradation by proteases such as calpain, cathepsins, caspases and proteasome (KEMP et al., 2010). Proteomics and phosphoproteomics have confirmed previous findings and identified new insights on the mechanisms responsible for meat tenderization in response to aging. For example, it was reported that abundance of actin and myosin heavy chain fragments increased in aged pigs muscle, indicating their degradation during aging (LAMETSCH et al., 2003). Similar results were obtained in another study with pigs, in which the abundance of actin, myosin light chain-1, slow troponina-T and desmin decreased in aged meat, indicating disruption of sarcomere structure during aging (HWANG et al., 2005).

Moreover, aged beef had higher abundance of proteins belonging to cell structures such as membranes or anchoring systems of the cytoskeleton (voltage-dependent anion-selective channel protein-1, vinculina and annexin fragments), suggesting a degradation of cell structures during meat aging and release of their protein constituent making them more extractable (LAVILLE et al., 2009b). In another study, the abundance of troponin T and myosin fragments increased, while the abundance of intact troponin T decreased during beef aging, indicating proteolysis and disruption of myofilaments (POLATI et al., 2012). More recently, it was reported that the protein spots number (fast troponin T, troponin I and myosin heavy chain-1 fragments) increased during beef aging, which could be associated with myofibril degradation (MARINO et al., 2013).

Furthermore, proteomics have been useful to provide a deeper understanding of postmortem proteolysis. Interestingly, a positive correlation was observed between abundance of heat shock protein-27 in fresh muscle and beef tenderness (MORZEL et al., 2008). In addition, the abundance of actin fragments increased in aged beef and the largest and smallest fragments of actin were positively correlated with the abundance of heat shock protein-27 in fresh

muscle. Taking these findings together and considering the pivotal role of heat shock protein-27 in the organization and maintenance of actin filament integrity, it was proposed that greater abundance of heat shock protein-27 in the muscle immediately after slaughter could prevent protein aggregation, which would facilitate the activity of proteolytic enzymes during beef aging. Additionally, it has also been reported that abundance of capping protein decreased in aged beef (BJARNADÓTTIR et al., 2010). Because capping protein plays a key role in the control of actin polymerization and uniformity of thin filament lengths interacting with actin and other Z-line-associated proteins, it was proposed that the capping protein degradation would be required for subsequent actin breakdown during beef aging.

The molecular mechanisms responsible for meat tenderization during aging have also gained new insight through phosphoproteomic approaches. A recent study has highlighted the role of apoptosis during beef aging (LONGO et al., 2015). It was observed that abundance of heat shock protein-B6 decreased in aged beef. As phosphorylated heat shock protein-B6 was also identified by western blotting and confirmed by mass spectrometry, it was suggested that the decrease in its abundance during aging could promote apoptosis, because heat shock protein-B6 phosphorylation enhances its anti-apoptotic role. In addition, it was also reported that abundance of two αB-crystallin spots with the lowest isoelectric point (probably more phosphorylated) increased, while the abundance of other two αβ-crystallin spots with the highest isoelectric point (probably less phosphorylated) decreased in aged beef. As phosphorylated αβ-crystallin was also identified by western blotting and confirmed by mass spectrometry, it was proposed that an increase in its phosphorylation level would promote apoptosis during beef aging.

Myofilament phosphorylation has also been reported to be important for meat tenderization. For example, it was observed that myosin light chain-2

doubled their phosphorylated sites from 0 to 24 hours after slaughter, without major changes after 14 days of aging, suggesting that double phosphorylation of myosin light chain-2 would play an important role in strong sustainable muscle shortening, because its phosphorylation could increase muscle contraction strength and keep it for a longer time (MUROYA et al., 2007a).

Moreover, another study showed that phosphorylated troponin T isoforms were detected on 2-DE gels of cattle muscle collected at 1 h after slaughter and beef aged for 1 day, but no troponina T-related polypeptide was detected on 2-DE gels of beef aged for 14 days (MUROYA et al., 2007b). So, it was suggested that this change in troponin T phosphorylation could be due to enzymatic dephosphorylation or cut-off of phosphorylated sites. According to authors, the troponin T phosphorylation sites were expected to be found in the N-terminal region. As the degraded troponin T fragments lacked N-terminal regions and phosphorylated sites, the cut-off of the N-terminal region by proteolysis would be more likely to explain the lack of phosphorylated troponin T fragments on 2-DE gels of beef aged for 14 days.

To the best of our knowledge, despite the pivotal role of phosphorylation on protein structure and function, large-scale phosphoproteomics studies have not been conducted to evaluate the meat aging. Nonetheless, proteomics and phosphoproteomics have provided new insights into the mechanisms of meat tenderization during aging. We can highlight that apoptosis and degradation of actin, myosin and troponin-T are important in this process. Furthermore, myofilament phosphorylation could be associated with the development of rigor mortis and postmortem proteolysis.

3. CONCLUSION

Meat quality can be affected by several factors. Proteomics and phosphosproteomics studies have provided a broader and deeper understanding of how these factors could contribute to these variations. They have confirmed and elucidated previous concepts and provided new insights into the mechanisms involved in meat quality. For example, differences in myofilaments expressed in fast or slow-twitch fibers can be related to variations between animals, genetic groups and nutrition and feeding management. Moreover, degradation of myofilaments and structural proteins has been confirmed as the main change taking place during meat aging and many studies have shown that proteolysis of actin and myosin can be as important for meat tenderization as degradation of troponin T and desmin. The identification of heat shock proteins related to meat tenderness has been one of the greatest contributions of proteomics to Meat Science, indicating the importance of apoptosis for meat tenderization. Phosphoproteomics has shown that the effects of individual variation and pre-slaughter stress on meat quality could be linked to differences in phosphorylation of myofilaments and glycolytic enzymes. Nonetheless, studies comparing the muscle proteome and phosphoproteome between taurine and zebu cattle have not been found.

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SECOND PART - ARTICLE

Understanding the difference in beef quality between Angus (*Bos taurus taurus*) and Nellore (*Bos taurus indicus*) cattle through a proteomic and phosphoproteomic approach

Article formatted according to the PloS one guidelines.

Abstract

Proteins are the major constituents of muscle and are key molecules regulating the metabolic changes during conversion of muscle to meat. Brazil is one of the largest exporters of beef and most of the Brazilian cattle are composed by zebu (Nellore) genotype. Zebu beef is generally leaner and tougher than taurine. The aim of this study was to compare the muscle proteomic and phosphoproteomic profile of Nellore and Angus cattle. Seven animals of each breed previously subjected to the same growth management were confined for 84 days. Proteins were extracted from *Logissimus dorsi* muscle samples collected immediately after slaughter and separated by two-dimensional electrophoresis. Pro-Q Diamond stain was used in phosphoproteomics. Proteins identification was performed using matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Tropomyosin alpha-1 chain, troponin-T, myosin light chain-1 fragment, cytoplasmic malate dehydrogenase, alpha-enolase and 78 kDa glucose-regulated protein were more abundant in Nellore, while myosin light chain 3, prohibitin, mitochondrial stress-70 protein and heat shock 70 kDa protein 6 were more abundant in Angus ($P<0.05$). Nellore had higher phosphorylation of myosin regulatory light chain-2, alpha actin-1, triosephosphate isomerase and 14-3-3 protein epsilon. However, Angus had greater phosphorylation of phosphoglucomutase-1 and troponin-T ($P<0.05$). Therefore, proteins involved in contraction and muscle organization, and heat shock proteins related to cell flux of calcium and apoptosis might be associated with differences in beef tenderness between Angus and Nellore. Furthermore, prohibitin might be linked to differences in intramuscular fat. Additionally, differences in phosphorylation of myofilaments and glycolytic enzymes could be involved with differences in muscle contraction force, susceptibility to calpain,

apoptosis and postmortem glycolysis, which might also be related to differences in beef tenderness among Angus and Nellore.

Introduction

Brazil is the second largest producer and one of the largest beef exporters in the world [1]. The majority of the Brazilian herd is composed of Zebu cattle (*Bos taurus indicus*), mainly Nellore. Zebu beef is usually less tender and has less marbling than that of taurine cattle (*Bos taurus taurus*), as Angus [2, 3]. This reduces attractiveness of zebu beef, because tenderness and marbling are considered the main sensory characteristics by consumers [4].

Therefore, it is increasing the number of studies aiming to understand the molecular mechanisms related to the differences in beef quality between zebu and taurine cattle genotypes [5, 6]. These studies look for biomarkers that might be used in livestock breeding programs. Moreover, they may provide scientific support for the meat industry in the development of strategies to improve meat quality [7, 8, 9].

Proteomics has been widely used for identification of proteins related to meat quality features, because proteins are the major constituent of muscle tissue and also responsible for the regulation of metabolic routes involved in the conversion of muscle to meat [10, 11]. Furthermore, proteomics can be used to study post-translational modifications, which may modify structure and, consequently, protein activity. Phosphorylation stands out among the main post-translational modifications, and phosphoproteomics is a useful technique to study phosphorylated proteins. In muscle tissue, phosphorylation could modulate the interaction among myofilaments and the activity of metabolic enzymes [12, 13, 14].

Proteomic studies comparing fresh muscle or beef from cattle breeds with different beef quality merits were able to identify differentially abundant proteins related to beef sensorial attributes [15, 16, 17]. However, proteomics and phosphoproteomics studies comparing muscle or beef of zebu and taurine have not been conducted. Thus, the aim of this study was to compare the muscle proteomic and phosphoproteomic profile of Nellore and Angus cattle.

Materials and methods

Ethical approval

All animal procedures were approved by the Animal Care and Use Committee of the Universidade Federal de Lavras, Brazil, protocol number 048/12.

Animal handling, slaughter and muscle sampling

Seven Nellore (BW = 375 ± 13 kg) and seven Angus young bulls (BW = 383 ± 16 kg), with approximately 20 months of age and previously subjected to the same growth management under grazing were fed *ad libitum* for 84 days with a standard feedlot diet used in Brazil based on corn silage and a corn-soybean meal concentrate with a roughage to concentrate ratio of 30:70. The animals were housed in covered individual pens with concrete floor and equipped with drinking and feeding troughs. Detailed information about the diet and its chemical composition were previously published [6].

The slaughter was preceded by cerebral concussion followed by exsanguination. There was no electrical stimulation of carcasses. Immediately after exsanguinations, samples were collected from the *Longissimus dorsi* muscle between the 12th and 13th ribs, via incision through hide, and frozen in

liquid nitrogen. Samples were then powdered in liquid nitrogen and stored at -80 °C until protein extraction.

Protein extraction and quantification

Approximately 100 mg of frozen muscle was added to a microtube containing 1 mL of extraction solution [(7 M) urea, (2 M) thiourea, (4% w/v) CHAPS, (1% w/v) dithiothreitol, (2% v/v) immobilized pH gradient (IPG) buffer, pH 4 to 7, (0.5 mM) benzamidine hydrochloride hydrate and (0.5 mM) phenylmethanesulfonyl fluoride]. Muscle sample and extraction solution were homogenized using LabGEN 125 Homogenizer (Cole-Parmer, Bunker Hill, IL, USA) at 9,500 rpm, twice for 15 seconds, with an interval of 30 seconds on ice. Subsequently, the homogenate was centrifuged at 20,200 g at 4 °C for 30 minutes. The supernatant was collected and frozen at -80 °C. Protein quantification was performed using the Bradford Protein Assay (BioRad, Hercules, CA, USA).

Two-dimensional electrophoresis

The first dimension or isoelectric focusing (IEF) was performed in 24 cm pH 4-7 IPG strips (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Initially, the strips were rehydrated for 16 hours in 450 µL rehydration solution [extraction solution containing 1,200 µg of protein, DeStreak Rehydration Solution (GE Healthcare Bio-Sciences), and 2% pH 4-7 IPG-buffer]. The IEF was performed using Ettan IPGphor III System (GE Healthcare Bio-Sciences) at 20 °C through the following program: step and hold until 200 V (2 h), step and hold until 500 V (1 h), gradient mode at 1,000 V (800 V/h), gradient mode at 10,000 V (16,500 V/h), and step and hold until 10,000 V (27,500 V/h). The current limit was 50 µA per strip.

For the second dimension, the strips were initially equilibrated in two successive steps of 20 minutes each, first in 5 mL of equilibration solution (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 50 mM Tris-HCl pH 8.8) containing 1% DTT (reduction step), and then, in 5 mL of equilibration solution containing 2.5% iodoacetamide (alkylating step). Subsequently, the strips were placed on top of 1 mm thick 12.5% sodium dodecyl sulfate polyacrylamide gels and the proteins were separated using Ethan DALTsix (GE Healthcare Bio-Sciences) at 8 °C. Electrophoresis was performed with 20 mA per gel for 40 minutes, followed by 40 mA per gel until the end of the run. The Low Molecular Weight Calibration Kit was used (GE Healthcare Bio-Sciences). Seven gels for each breed were made, one for each animal.

Gel-staining and image analysis

After two-dimensional electrophoresis (2DE), the gels were immersed in fixing solution [10% acetic acid (v/v) and 50% methanol (v/v)] for 12 hours under constant shaking. Subsequently, the gels were stained with the specific fluorescent dye for phosphoproteins Pro-Q Diamond (Invitrogen Molecular Probes, Eugene, OR, USA). All procedures for phosphoproteins staining followed the optimized method described in previously suggested protocol [18]. Images of the gels with phosphoproteins were obtained using Fuji Film 5100 FLA Fluorescence Imaging System Scanner (Fuji Medical Systems, Hanover Park, IL, USA) in fluorescent scanning mode, resolution of 300 dpi, excitation filter of 532 nm and emission filter of 580 nm.

Immediately after obtaining the images of phosphorylated proteins, the gels were stained for total protein with a solution containing 8% ammonium sulfate (w/v), 0.8% phosphoric acid (v/v), 0.08% coomassie blue G-250 (v/v) and 20% methanol (v/v) [19] for 72 h and then washed with 1% acetic acid (v/v) until complete removal of excess dye. Gels were scanned using ImageScanner

III (GE Healthcare Bio-Sciences) at 300 dpi and subsequently stored in 2% acetic acid (v/v) at 20 °C until extraction and spots digestion.

Spots detection and quantification were performed with Image Master 2D Platinum version 7.0 software (GE Healthcare Bio-Sciences). The volume of each spot (optical density x area) was normalized to the total volume of spots detected on each gel for comparison between breeds. Differences were considered significant when *P*-value was lower than 5% by ANOVA. The comparison between breeds for each spot made by Image Master was confirmed manually. Due to the high background, the phosphoprotein image of one Nellore was not used for comparisons.

Spots digestion and protein identification

Images of the gels stained with Pro-Q Diamond and coomassie blue G-250 were overlaid using Adobe Photoshop CC 2015.0 (Adobe Systems, San José, CA, USA) to facilitate excision of differentially abundant spots in the phosphoproteomics analysis (S1 Figure). The spots were excised manually and subjected to trypsinization [20].

Peptide mass spectra (MS and MS/MS) was obtained using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF). A matrix α-cyano-4-hydroxycinnamic was used. MALDI analysis was performed using Ultraflex III MALDI-TOF/TOF system (Bruker Daltonics, Bremen, Germany). The MS analyses were performed with reflective positive peptide method, while the MS/MS analyses were performed using the LIFT positive method.

Protein identification was made using the MASCOT version 2.2 software (Matrix Science, Boston, MA, USA) at the MS/MS ion search mode, with the following parameters: tryptic specificity, one missed cleavage and a mass measurement tolerance of 0.2 Da for MS and 0.5 Da for MS/MS mode.

Cysteine carbamidomethylation was used as fixed modification, while methionine oxidation was used as variable modification. The database used was the Bovidae deposited in UniProt. The proteins identified in MASCOT were validated by SCAFFOLD version 3.6.4 software (Proteome Software, Portland, OR, USA). The criteria used for the validation was of at least one peptide, with a probability score greater than or equal to 90% for both peptides and proteins.

Results

A total of 423 matches ID were detected in the analysis of total protein and 1,093 in the analysis of phosphorylated proteins, of which 38 and 55 differed ($P<0.05$) between breeds, respectively. Excision of differentially abundant/phosphorylated spots was only performed for clearly visible and separable spots on gels (36 in the proteomic analysis and 23 in the phosphoproteomic study). Due to the 2DE/MS limitations for identification of low abundance proteins, it was not possible to identify all spots that were excised.

Differentially abundant proteins

The proteomic analysis identified sixteen differentially abundant spots ($P<0.05$). Seven spots were more abundant in Angus and nine were more abundant in Nellore (Fig 1 and Table 1). Nellore had greater abundance of tropomyosin alpha-1 chain (TPM1, two spots), troponin T (TNNT3), myosin light chain 1 fragment (MYL1), cytoplasmic malate dehydrogenase (MDH1) and alpha-enolase (ENO1). However, Angus had greater abundance of prohibitin (PHB). Furthermore, a spot identified in Angus as myosin light chain 3 (MYL3) was not detected in Nellore (S2 Figure). Four spots were identified as phosphoglucomutase 1 (PGM1), two of them more abundant in Nellore and two

in Angus. In addition, three proteins belonging to the heat shock protein (HSPs) family were identified, two with greater abundance in Angus, mitochondrial stress-70 protein (HSPA9, two spots) and heat shock 70 kDa protein 6 (HSPA6), and one with greater abundance in Nellore, 78 kDa glucose-regulated protein (HSPA5). HSPA6 had confirmation with the realization of a blast in UniProt. Interestingly, HSPA6 was detected in only one of Nellore bulls (S3 Figure).

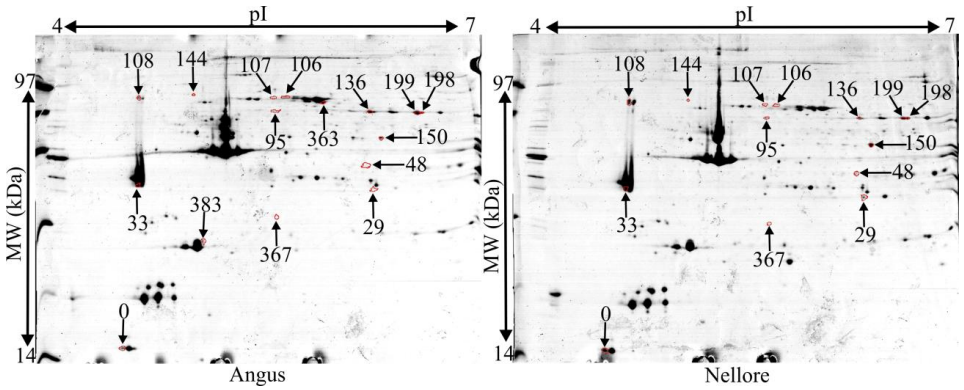

Fig 1. Representative 2-DE gel images showing differentially abundant proteins in the *Longissimus dorsi* muscle of Angus and Nellore bulls. Numbers found in the figure correspond to the Match ID shown in Table 1.

Table 1. Differentially abundant proteins in the *Longissimus dorsi* muscle of Angus and Nellore bulls.

Match ID[a]	Protein ID	ABV[b]	UniProt accession number	Mascot score[c]	Fold change	*P*-value[d]	Identified peptides[e]	pI observed/ expected	MW kDa observed/ expected
More abundant in Angus									
383	Myosin light chain 3	MYL3	P85100	188	ND[f] in Nellore	0.0007	2	5.12/5.00	25.7/22.1
136	Phosphoglucomutase-1	PGM1	Q08DP0	233	2.83	0.0031	3	6.30/6.36	63.1/61.8
199	Phosphoglucomutase-1	PGM1	Q08DP0	933	2	0.0079	11	6.64/6.36	62.6/61.8
106	Stress-70 protein, mitochondrial	HSPA9	Q3ZCH0	86	1.64	0.0111	1	5.72/5.97	75.8/74.0
107	Stress-70 protein, mitochondrial	HSPA9	Q3ZCH0	264	2.03	0.0183	3	5.63/5.97	75.9/74.0
363	Uncharacterized protein	HSPA6	F1MWU9	90	4.6	0.0349	1	5.98/5.74	68.3/71.4
367	Prohibitin	PHB	Q3T165	255	2.15	0.0238	3	5.65/5.57	29.7/29.8
More abundant in Nellore									
0	Myosin light chain 1 (fragment)	MYL1	Q08E10	209	1.89	0.0140	3	4.50/4.73	14.4/19.7
108	Tropomyosin alpha-1 chain	TPM1	Q5KR49	635	2.93	0.0197	7	4.66/4.69	75.3/32.7
33	Tropomyosin alpha-1 chain	TPM1	Q5KR49	337	1.72	0.0422	6	4.63/4.69	35.9/32.7
48	Troponin T	TNNT3	Q8MKI3	91	1.96	0.0084	1	6.30/5.99	40.3/32.1
29	Malate	MDH1	Q3T145	57	2.06	0.0009	1	6.34/6.16	35.2/36.7

	dehydrogenase, cytoplasmic								
150	Alpha-enolase	ENO1	Q9XSJ4	861	2.04	0.0152	11	6.39/6.37	49.0/47.6
198	Phosphoglucomutase-1	PGM1	Q08DP0	711	3.28	0.0079	8	6.67/6.36	63.4/61.8
95	Phosphoglucomutase-1	PGM1	A6QPB5	157	1.92	0.0160	1	5.64/5.48	65.2/62.5
144	78 kDa glucose-regulated protein	HSPA5	Q0VCX2	303	2	0.0131	4	5.07/5.07	78.7/72.4

[a] Match ID correspond to the numbers shown in Figure 1
[b] Protein abbreviation is in accordance with gene abbreviation in UniProt
[c] The Mascot baseline significant score is 31
[d] *P*-value obtained by ANOVA comparing spots abundance between Angus and Nellore muscle
[e] Number of peptides identified in Mascot and validated by Scaffold. The sequences of the peptides are found in S1 Table
[f] ND spot not detected

Differentially abundant phosphoproteins

The phosphoproteomic approach identified eleven differentially phosphorylated spots ($P<0.05$), three more abundant in Angus and eight more abundant in Nellore (Fig 2 and Table 2). There were two spots of myosin light chain 1/3 (MYL1) that were detected only in Nellore (S4 Figure). Additionally, Nellore had higher phosphorylation of myosin regulatory light chain 2 (MYLPF, two spots), alpha actin 1 (ACTA1, two spots), triosephosphate isomerase (TPI1) and 14-3-3 protein epsilon (YWHAE). However, Angus had greater phosphorylation of PGM 1 (two spots) and TNNT3.

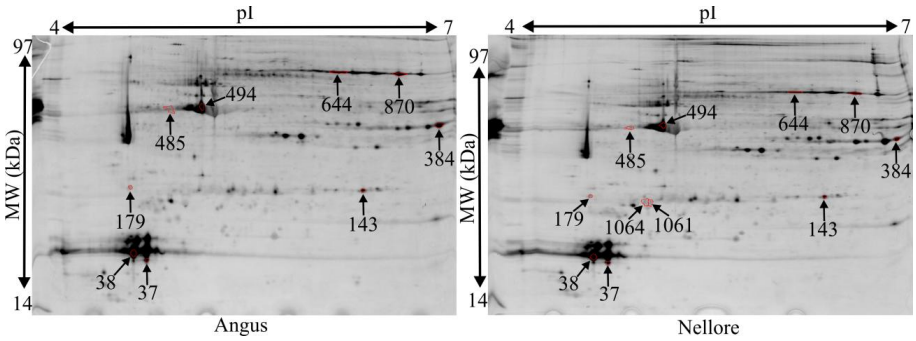

Fig 2. Representative 2-DE gel images showing differentially abundant phosphoproteins in the *Longissimus dorsi* muscle of Angus and Nellore bulls. Numbers found in the figure correspond to the Match ID shown in Table 2.

Table 2. Differentially abundant phosphoproteins in the *Longissimus dorsi* muscle of Angus and Nellore bulls.

Match ID[a]	Protein ID	ABV[b]	Uniprot accession number	Mascot score[c]	Fold change	*P*-value[d]	Identified peptides[e]	pI observed/ expected	MW kDa observed/ expected
More abundant in Angus									
384	Troponin T	TNNT3	Q8MKI4	103	3.56	0.0411	2	6.91/5.93	39.8/32.1
644	Phosphoglucomutase-1	PGM1	Q08DP0	199	1.65	0.0065	3	6.19/6.36	64.9/61.6
870	Phosphoglucomutase-1	PGM1	Q08DP0	377	1.95	0.0481	6	6.63/6.36	63.6/61.8
More abundant in Nellore									
1061	Myosin light chain 1/3	MYL1	A0JNJ5	412	ND[f] in Angus	0.0001	5	5.12/4.96	24.8/21.0
1064	Myosin light chain 1/3	MYL1	A0JNJ5	80	ND[f] in Angus	0.0001	1	5.07/4.96	25.0/21.0
38	Myosin regulatory light chain 2	MYLPF	Q0P571	212	1.45	0.0063	3	4.69/4.91	18.1/19.0
37	Myosin regulatory light chain 2	MYLPF	Q0P571	113	1.26	0.0213	1	4.79/4.91	17.6/19.0
485	Alpha actin 1	ACTA1	A4IFM8	465	1.82	0.0290	6	4.96/5.23	43.2/42.3
494	Alpha actin 1	ACTA1	A4IFM8	213	1.29	0.0470	2	5.22/5.23	43.9/42.3
143	Triosephosphate isomerase	TPI1	Q5E956	550	1.31	0.0113	6	6.40/6.45	26.4/26.9
179	14-3-3 protein épsilon	YWHAE	P62261	219	1.25	0.0228	3	4.67/4.63	26.8/29.3

[a] Match ID correspond to the numbers shown in Figure 2
[b] Protein abbreviation is in accordance with gene abbreviation in UniProt
[c] The Mascot baseline significant score is 31

[d] *P*-value obtained by ANOVA comparing spots abundance between Angus and Nellore muscle
[e] Number of peptides identified by Mascot and validated by Scaffold. The sequences of the peptides are found in S2 Table
[f] ND spot not detected

Discussion

The Nellore and Angus cattle used in our study showed significant differences in the main characteristics related to meat quality, which were evaluated in parallel studies of our research group. Nellore had tougher beef and lower myofibrillar fragmentation indices (MFI) [21]. Furthermore, Nellore had lower content of intramuscular fat (IMF). However, the content of total intramuscular collagen did not differ between Angus and Nellore [6].

Differentially abundant proteins

Nellore had greater abundance of proteins involved in the regulation of muscle contraction, which are important substrates of proteolytic enzymes during the meat aging

TNNT3 [15] and TPM1 [22] have been more abundant in tough beef. These findings are in agreement with our results, because Nellore muscle showed greater abundance of TPM1 and TNNT3 compared to Angus. Tropomyosin (Tpm) and troponin T (TnT) are among the main substrates of the proteolytic enzymes related to meat tenderization [23], and TnT degradation during aging has been positively associated with beef tenderization [17, 24].

In addition, due to the role of TnT in the regulation of the complex that controls the interaction among actin and myosin filaments, it has been suggested that changes in the relation between them could occur in response to TnT degradation [25]. The degradation of TnT could also be related to the disruption of its interaction with other thin filaments and, consequently, with the breaking of thin filaments in the sarcomeric I-band, which might lead to fragmentation of myofibrils.

Interestingly, the spot 108 that was identified as TPM1 had a molecular weight that was approximately twice the theoretical value (75.3 versus 32.7 kDa). Because TPM1 is a dimer of α-helices forming a coiled-coil [26], this result might indicate the presence of TPM1 dimer despite the denaturing conditions of electrophoresis. The presence of dimers in 2DE analysis has already been suggested [12].

Myofilaments expressed in fast or slow twitch fibers had different abundance between Angus and Nellore

MYL1 and MYL3 are the regulatory light chain of myosin. Nellore had increased abundance of MYL1, which is found in fast twitch fibers, while MYL3, which is found in slow twitch fibers, was only detected in Angus. Although we have not evaluated the proportion of muscle fiber types between breeds, the differences in the abundance of myosins expressed in fast or slow twitch fibers could suggest that muscle fiber type might have differed between Angus and Nellore. Likewise, TNNT3, an isoform also expressed in fast twitch fibers, was most abundant in Nellore. This is in line with a prior study, that observed lower proportion of fast twitch oxidative glycolytic fibers and higher of slow twitch oxidative fibers in the *Longissimus dorsi* muscle of crossbred Angus×Nellore compared to Nellore cattle [27]. In addition, Angus is considered to have a higher proportion of oxidative muscle fibers [28]. Muscle fiber type has been associated with meat quality [29]. However, more studies are needed to assess whether muscle fiber type is related to differences in beef quality between Angus and Nellore.

Similar to what was observed in our study, crossbred Angus×Holstein Friesian had lower abundance of MYL1 in *Longissimus lumborum* muscle than crossbred Belgian Blue×Holstein Friesian cattle, which were characterized by

later body maturity and leaner carcass [16]. In addition, Large White pigs (leaner carcass) had greater MYL1 abundance than Casertana pigs (fatter carcass) [30], and pigs with higher IMF content had lower abundance of fast twitch myofilaments and greater abundance of slow twitch myofilaments [31]. Furthermore, our results were also consistent with those obtained in a similar study, in which Podolian cattle had higher abundance of TnT and MYL1, tougher beef and lower MFI and IMF content than crossbreed Romagnola×Podolian and Friesian cattle [17]. Likewise, MYL1 was more abundant in Chianina cattle classified as tough beef [13].

Differences in abundance of enzymes involved in muscle energy metabolism between Angus and Nellore

Nellore had greater ENO1 abundance, a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. This result is in agreement with the higher abundance in Nellore of myofilaments expressed in fast twitch glycolytic fibers. Similarly, ENO1 was more abundant in steers compared to bulls and in *Semitendinosus* compared to *Longissimus thoracis* muscle, and this was consistent with the greater proportion of fast twitch glycolytic fibers reported for steers and *Semitendinosus* muscle [32].

Two spots identified as PGM1 were more abundant in Nellore, while two other spots, also identified as PGM1 were more abundant in Angus. PGM1 is involved in glycolysis and glycogenesis reversibly catalyzing the conversion of glucose 1-phosphate to glucose 6-phosphate. This could be related to metabolic differences in muscle tissue between breeds before slaughter. Glycogen synthesis and glucose degradation are both intense metabolic activities expected in muscles with a greater amount of fast twitch myofilaments [33], as it was observed in Nellore. Likewise, as the catalysis of glucose is the major

metabolic pathway for the supply of carbon and reduced cofactor to the synthesis of intramuscular fat [16, 30, 34], it was also expected great abundance of PGM1 in Angus, because they had higher IMF content. Similarly, cattle with greater genomic estimated breeding value for IMF had higher expression of PGM1 [35].

Furthermore, Nellore had higher abundance of MDH1, an important enzyme in gluconeogenesis that catalyzes the oxidation of malate to oxaloacetate, which may then be used as precursor for glucose synthesis. This result is in line with what has already been discussed for ENO1 and PGM1.

The activity of enzymes involved in energy metabolism is of great importance for the meat quality characteristics, because the formation of actin-myosin complex and pH drop are the main changes responsible for the conversion of muscle to meat, and they are strongly influenced by postmortem energy metabolism [10, 25]. However, the relation between abundance of energy metabolism enzymes and meat tenderness has been a controversial topic [10, 15, 32]. This discrepancy could be related to differences in enzyme activity, that may be altered by post-translational modifications such as phosphorylation [7, 12, 13].

Angus and Nellore differed in the abundance of heat shock proteins located in the mitochondria or sarcoplasmic reticulum that are involved in Ca^{2+}cellular traffic and apoptosis

Several studies have found a relationship between meat tenderness and HSPs abundance [22, 36, 37]. Due to the highly conserved chaperone capacity of proteins belonging to the HSPs family, many research groups have discussed the involvement of HSPs in the meat tenderizing process without considering their singularities. In our study, four spots belonging to the HSPs family were

identified, three more abundant in Angus and one in Nellore. Because HSPs have different cell locations, respond to different stimuli, and have different activities, we will discuss them separately to give greater biological significance to our results.

HSPA9 also known as mortalin and 75 kDa glucose-regulated protein (GRP-75) is the main mitochondrial HSP. It plays a key role in the translocation system that imports and exports protein across the mitochondrial membrane [38]. Despite belonging to the HSPs family, the expression of HSPA9 does not increase in response to heat stress. However, it is stimulated by glucose deprivation, Ca^{2+} influx and some cytotoxins [39]. Although HSPA9 is associated with anti-apoptotic processes due to its chaperone activity and inhibition of pro-apoptotic factors, it has also been proposed that under conditions of excessive stress, HSPA9 could not be able to prevent cell death and would change the mitochondrial functions leading to apoptosis [39].

HSPA9 abundance in *Longissimus thoraci* [40] and *Semitendinosus* [41] muscle immediately after slaughter had positive correlation with beef tenderness. These findings were in agreement with our result, because Angus had higher beef tenderness and greater HSPA9 abundance. This effect of HSPA9 on meat tenderness might be related to its anti-apoptotic effect, preventing the formation of protein aggregates, and might also be related to its role in triggering apoptosis. After slaughter and exsanguinations, muscle cells are subjected to various conditions of acute stress, such as interruption of the blood supply of glucose and oxygen, pH drop and increased Ca^{2+}concentration in sarcoplasm and mitochondria [10]. All these conditions may induce apoptosis, which has been described as one of the main mechanism responsible for the meat tenderization through caspase proteolytic system [42].

Several mechanisms may involve HSPA9 in the triggering of apoptosis. For example, HSPA9 connects the inositol 1,4,5-trisphosphate receptor of the

sarcoplasmic reticulum to the voltage-dependent anion channels of the mitochondria, facilitating the transfer of Ca^{2+} from the sarcoplasmic reticulum into the mitochondria. Overloading of Ca^{2+} in the mitochondria could lead to depolarization of the inner mitochondrial permeability transition pore and trigger apoptosis [43]. Additionally, the release of Ca^{2+} into the sarcoplasm leads to mitochondrial uptake of Ca^{2+} that cause conformational changes in the outer membrane-bound mitochondrial m-calpain large subunit, which leads to its binding to calpain small subunit and HSPA9. The formation of this complex allows the translocation of mitochondrial m-calpain from the outer membrane into the intermembrane space, where it is activated by a further increase of mitochondrial Ca^{2+} level triggering apoptosis [44].

Unlike what was observed for HSPA9, Nellore had greater abundance of HSPA5. This is the main HSP located in the sarcoplasmic reticulum, where is essential for the transport of newly synthesized polypeptides, for the folding and assembly of proteins, and for Ca^{2+} homeostasis [45]. HSPA5 has chaperone activity stimulated by its binding to Ca^{2+} and participates in the Ca^{2+} buffering in the sarcoplasmic reticulum. Ca^{2+} connected to HSPA5 corresponds to 25% of the Ca^{2+} reserves in the sarcoplasmic reticulum [46]. When Ca^{2+} reserves decrease or are depleted, there is a greater amount of unfolded proteins in the sarcoplasmic reticulum, which leads to increased expression of HSPA5 [47]. HSPA5 has been mainly related to inhibition of sarcoplasmic reticulum stress-related apoptosis [45]. To the best of our knowledge, HSPA5 has not been previously associated with meat tenderness differences.

The divergence in the abundance of HSPA9 and HSPA5 between Angus and Nellore was intriguing, as both proteins are related to cell flow of Ca^{2+}. After slaughter, Ca^{2+} retained in the sarcoplasmic reticulum is released into the sarcoplasm stimulating the rigor mortis and the calpain activity, which is considered one of the main responsible for myofibrillar degradation and meat

tenderization during aging [23, 25]. In addition, the output of Ca^{2+} from the sarcoplasmic reticulum to other cell compartments such as mitochondria triggers apoptosis [48].

A model has been proposed to explain how the Ca^{2+} flow could integrate the sarcoplasmic reticulum with the mitochondrial function [43]. In this model, HSPA5 is involved keeping Ca^{2+} within the sarcoplasmic reticulum, while HSPA9 is involved in the communication of sarcoplasmic reticulum and mitochondria that directs the Ca^{2+} flow from the former to the latter. Furthermore, it was suggested that the balance between the Ca^{2+} amount in the mitochondria and sarcoplasmic reticulum would be determinant to the decision between cell survival or death, wherein the Ca^{2+} overload within the mitochondria would direct to apoptosis. The massive Ca^{2+} influx into the matrix leads to mitochondria fission and accelerates the release of cytochrome c amplifying apoptosis via activation of caspases [8].

In this way, we could hypothesize that the greater abundance of HSPA5 in Nellore would delay, while the higher abundance of HSPA9 in Angus would accelerate apoptosis, rigor mortis, and beef tenderization. In agreement with this, HSPA9 level in muscle after slaughter had negative correlation with both pH at 3 hours and ultimate pH in the cattle carcass, and it was proposed that this result would be associated with an increased release of Ca^{2+} from the sarcoplasmic reticulum and, consequently, with higher enzyme activity and rigor mortis [49]. A positive relation found between μ-calpain and HSPA9 through correlation networks among protein biomarkers of beef tenderness also support our hypothesis [50]. Furthermore, it has been suggested that calcium-binding proteins, such as HSPA5, could contribute to the lower amount of free calcium after slaughter and, consequently, in reduced calpain activity [10].

Intriguingly, the calpastatin gene knockdown in bovine muscle satellite cells significantly increased the expression of μ-calpain, caspases and heat shock

proteins, suggesting that they are involved in apoptosis during the calpastatin gene silencing [51]. Additionally, it has been reported that caspase-3, an effector enzyme of apoptosis, could inhibit the calpastatin activity, which is the calpain inhibitor [11]. The high calpastatin activity has been considered one of the main factors related to lower zebu beef tenderness compared to taurine [52]. Differences in the abundance of calpastatin, calpain and caspases were not detected in our study. This result may be attributed to limitations of 2DE to detect differences in low-abundance proteins [12]. However, in a parallel study with the same animals used here, there was a higher calpastatin activity in Nellore beef [21]. In view of this, we could suggest that the calpain/calpastatin proteolytic system and caspase-dependent apoptosis together would be related to differences in beef tenderness between Nellore and Angus. Furthermore, a greater susceptibility to caspase-dependent apoptosis would be related to lower calpastatin activity and greater MFI in Angus. However, more investigations are needed to evaluate this hypothesis.

As HSPA5 and HSPA9 are mainly located within the sarcoplasmic reticulum and mitochondria, respectively, the higher abundance of HSPA5 in Nellore and the greater abundance of HSPA9 in Angus would also be related to differences in muscle fiber types among them, because fast twitch glycolytic fibers have higher volume of sarcoplasmic reticulum, while slow twitch oxidative fibers have greater mitochondrial volume and abundance [53].

Another HSP that differed between Angus and Nellore was HSPA6, which was more abundant in Angus and was detected only in one of Nellore bulls. HSPA6 expression has been reported to be strictly stimulated by heat in fibroblasts [54]. In another study, HSPA6 expression was strongly induced by heat, but it had no significant effect on protection of HEK-293 cells against heat-induced cell death [55]. These findings are interesting because in a parallel study

evaluating the same animals used in the current study, there was higher metabolic heat production and higher body temperatures in Angus [56].

Prohibitin could also be related to differences in apoptosis and intramuscular fat between Angus and Nellore

PHB are part of a group of proteins highly conserved and ubiquitously expressed in different cell tissues, being mainly located in the mitochondria, nucleus and plasma membrane [57]. Due to its location in several cellular compartments, translocation and interaction capacity with many transcription factors and proteins, PHB is involved in regulation of cell survival, apoptosis, metabolism and inflammation [58]. It may be upregulated under conditions of extreme stress and lead to apoptosis by modulating transcription factors and pro-apoptotic genes increasing caspases activity [59, 60].

In our study, PHB was more abundant in Angus. A greater abundance of PHB has already been described in bovine muscle classified as tender beef [61]. Furthermore, it was also observed by these authors higher abundance of other proteins of the inner and outer mitochondrial membranes, such as HSPA9, in the muscle of tender beef, which would be related to apoptosis. These findings support our suggestion that the difference in beef tenderness between Nellore and Angus would be partially explained by differences in apoptosis.

In addition to its relation with differences in beef tenderness, PHB could also be involved with differences in the IMF content between Nellore and Angus. It has been proposed that PHB would regulate adipocyte differentiation by modulating the insulin signaling pathway and mitochondrial biogenesis. Moreover, PHB also would regulate lipogenesis by modulating the pyruvate carboxylase and mitochondrial function [62]. PHB upregulation resulted in adipocyte hypertrophy associated with increase of white adipose tissue in mice

[63]. Treatment of fibroblasts with insulin or peroxisome proliferator-activated receptor gamma (PPAR-γ) caused PHB upregulation and induced adipogenesis with increased expression of PPAR-γ [62]. These findings are interesting, because in a parallel study, there was greater abundance of PPAR-γ in Angus muscle [6]. In addition, higher PHB abundance in IMF compared to subcutaneous, perirenal, and intermuscular fat has been observed in pigs [64]. These findings together with our results suggest that differences in PHB abundance might partially explain the lower deposition of IMF in Nellore compared to Angus.

Differentially abundant phosphoproteins

Only one differentially phosphorylated spot was also differentially abundant in proteomics analysis. It was the spot 870 (PGM1), which was more abundant in Angus and corresponded to spots 198 and 199 (PGM1) in proteomics analysis, which were more abundant in Nellore and Angus, respectively. The other differentially abundant spots in phosphoproteomics study showed no significant difference in proteomic analysis among Angus and Nellore. Thus, the differences observed in phosphoproteins could be attributed to differences in phosphorylation level instead of amount in total protein.

Angus and Nellore differed in phosphorylation of myofilaments, which is related to affect muscle contraction strength and susceptibility to calpain and apoptosis

Nellore presented higher phosphorylation of MYLPF, MYL1 and ACTA1, while Angus had only increased TNNT3 phosphorylation. MYLPF phosphorylation might alter the structure and motor function of the myosin to

increase the sensitivity of the contractile apparatus to Ca^{2+} [65]. Furthermore, MYLPF phosphorylation increased the contraction force in fast twitch skeletal muscle [66]. In addition, it has been suggested that phosphorylation of MYLPF might work as a kind of memory to enhance muscle contraction strength [67]. This hypothesis has been considered in an attempt to explain the relationship between tough meat and MYLPF phosphorylation [11]. These findings and hypotheses are consistent with our results, as Nellore had greater phosphorylation of MYLPF and tougher beef. A similar result was observed in a study with sheep, in which there was higher MYLPF phosphorylation in the group of animals classified as tough meat [14]. Moreover, greater phosphorylated MYLPF abundance has been reported in dark firm dry beef [68].

The phosphorylated MYLPF is expressed in fast twitch fibers. As there was no difference in abundance of this protein in proteomic analysis, we suggest that the difference found would be related to differences in phosphorylation and or MYLPF dephosphorylation rather than a possible difference in the fast and slow twitch fibers composition between Angus and Nellore. MYLPF is phosphorylated by Ca^{2+}/calmodulin-dependent myosin light chain kinase and is dephosphorylated by protein phosphatase 1 [69]. Despite the involvement of Ca^{2+} in MYLPF phosphorylation mechanism, this process does not require high Ca^{2+} concentrations to occur. Other factors such as myosin light chain kinase:protein phosphatase-1 ratio appear to be important to affect the MYLPF phosphorylation [67]. Additionally, it was demonstrated that myosin light chain kinase rather than calmodulin is limiting to the phosphorylation of MYLPF [66].

Although Angus had lower TNNT3 abundance in proteomic study, they showed greater phosphorylation of another TNNT3 isoform. Phosphorylation of skeletal troponins increased their susceptibility to degradation by calpain possibly due to dissociation from the native complex [70]. In addition, it has been suggested that TnT would undergo cut-off in phosphorylated sites during

post-rigor stage [71]. TnT is one of the main substrates for calpain and its degradation is related to the meat tenderization during aging [23]. Therefore, we could suggest that the greater phosphorylation of TNNT3 in Angus would partly explain its higher MFI and beef more tender compared to Nellore. Additionally, the lower TNNT3 phosphorylation in Nellore would be partially explained by greater TPM1 abundance observed in these animals, as was shown in proteomics analysis, because skeletal Tpm may inhibit the phosphorylation of skeletal TnT due to the strong interaction between them, reducing the exposure of TnT phosphorylation sites [72].

ACTA1 was other myofilament that had different phosphorylation level between Angus and Nellore. Likewise to what was observed in our study, higher level of ACTA1 phosphorylation was found in tough beef [13]. It has been suggested that phosphorylation of ACTA1 could prevent the onset of apoptosis and would be positively correlated with the meat toughness [11]. Our result and these findings give further support to our hypothesis that apoptosis would be involved in the difference of beef tenderness between Nellore and Angus.

Two enzymes involved in glucose metabolism had opposite levels of phosphorylation in Angus and Nellore

Angus and Nellore had greater abundance of PGM1 isoforms in the proteomics analysis, but the phosphoproteomics revealed higher PGM1 phosphorylation only in Angus. PGM1 is more active when phosphorylated due to a conformational change that exposes its active site in response to phosphorylation [73]. As previously discussed, this protein catalyzes reactions that drive glucose into glycolysis or glycogenesis. As muscle does not receive more nutrients after slaughter and as glycolysis becomes the major source of energy to the muscle cells, we could suggest that greater PGM1 phosphorylation

would contribute to a faster glycolysis in Angus. Supporting this hypothesis, it was proposed that phosphorylation of PGM1 is related to faster rates of glycolysis and pH drop in postmortem muscle [74]. Furthermore, an increase in the phosphorylated PGM1 abundance was observed in cattle muscle from 0 to 1 day after slaughter, which would be related to an increase in glycogenolysis and glycolysis due to increased anaerobic postmortem muscle metabolism [75].

On the other hand, TPI1 phosphorylation was higher in Nellore. Similarly to our study, TPI1 was more phosphorylated in tough beef [12]. TPI1 is a glycolytic enzyme that catalyzes the reversible conversion of D-glyceraldehyde 3-phosphate from glycerone phosphate. The phosphorylation of TPI1 decreased its activity in HeLa cells [76]. In addition, it was reported a higher abundance of phosphorylated TPI1 in the pigs muscle with slow pH decline compared to fast pH decline group [77]. A moderate rate of pH decline in cattle muscle could be beneficial to meat tenderness due to the lower risk of cold shortening and influence on the activity of proteolytic enzymes [25].

YWHAE phosphorylation might also be involved with differences in force of muscle contraction and apoptosis between Angus and Nellore

YWHAE belongs to 14-3-3 protein family working as adapters in the regulation of several signaling pathways due to their abilities to bind to a large number of proteins. It has been suggested that phosphorylation of 14-3-3 proteins might result in dimer formation or dissociation, and it might also cause changes in their binding sites, which would modulate their interaction with target proteins [78]. Specifically, we did not find studies about the effect of phosphorylation on the regulation of YWHAE. Anyway, it has been well documented that YWHAE negatively regulates apoptosis [79]. In addition, it

was proposed that 14-3-3 proteins might bind to phosphorylated myosin light chain kinase and this could influence its binding to myosin [80].

Because Nellore had greater phosphorylation of YWHAE and tougher beef, we could hypothesize that YWHAE phosphorylation would affect beef tenderness preventing apoptosis and enhancing the strength of muscle contraction. Additionally, this result might be involved with the difference in phosphorylation of MYLPF through its probable effect on the myosin light chain kinase activity. Other studies had already suggested the involvement of 14-3-3 proteins with the meat tenderness due to their likely involvement in apoptosis and muscle contraction force [15, 30].

Conclusions

We can conclude that differences in proteins involved in contraction and muscle organization, and heat shock proteins related to cell flux of calcium and apoptosis might be associated with differences in beef tenderness between Angus and Nellore. Furthermore, prohibitin might be linked to differences in intramuscular fat. Additionally, differences in phosphorylation of myofilaments and glycolytic enzymes could be involved with differences in muscle contraction force, susceptibility to calpain, apoptosis and postmortem glycolysis, which might also be related to differences in beef tenderness among Angus and Nellore.

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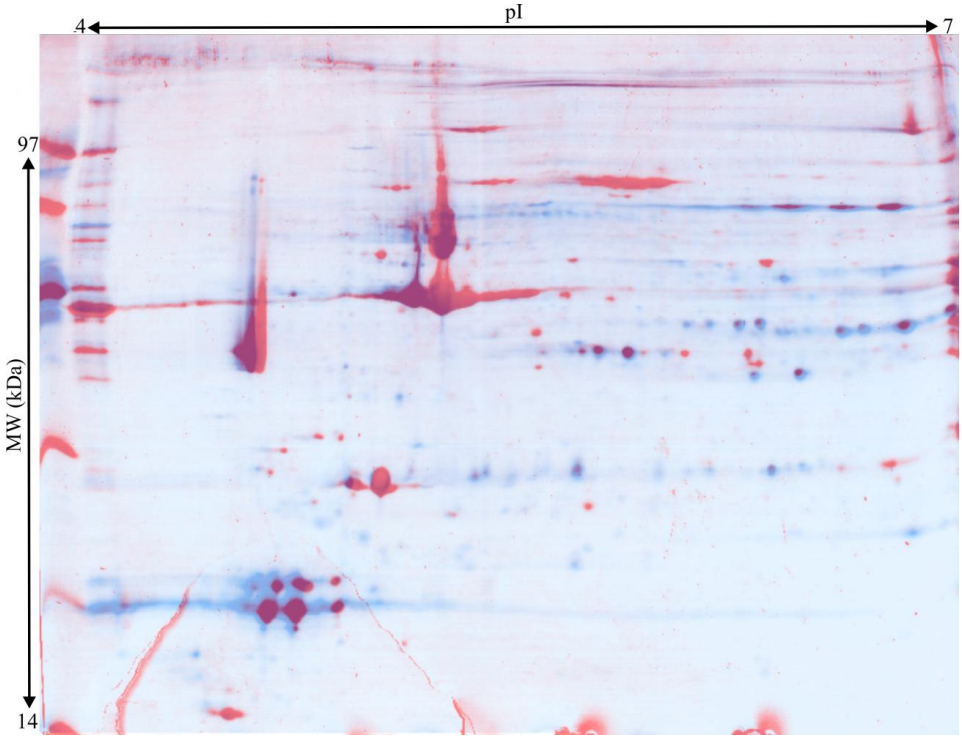
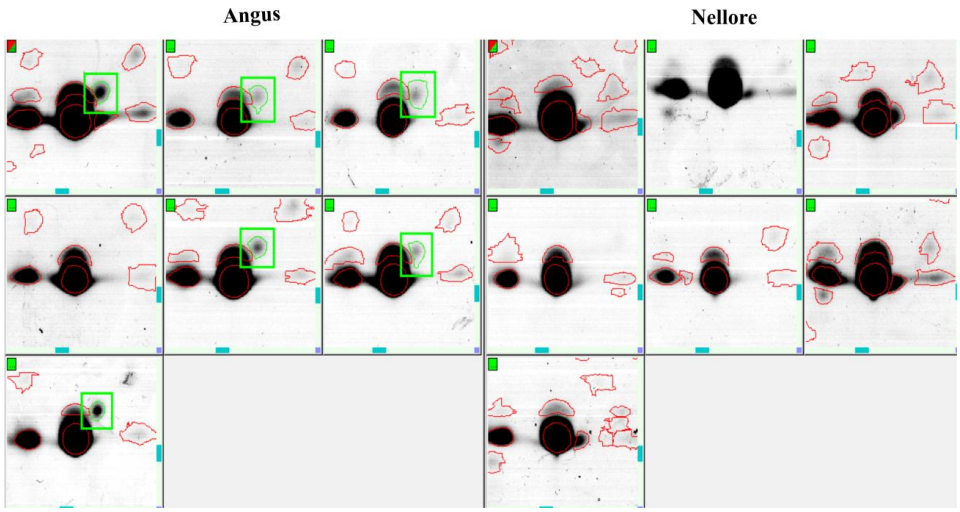
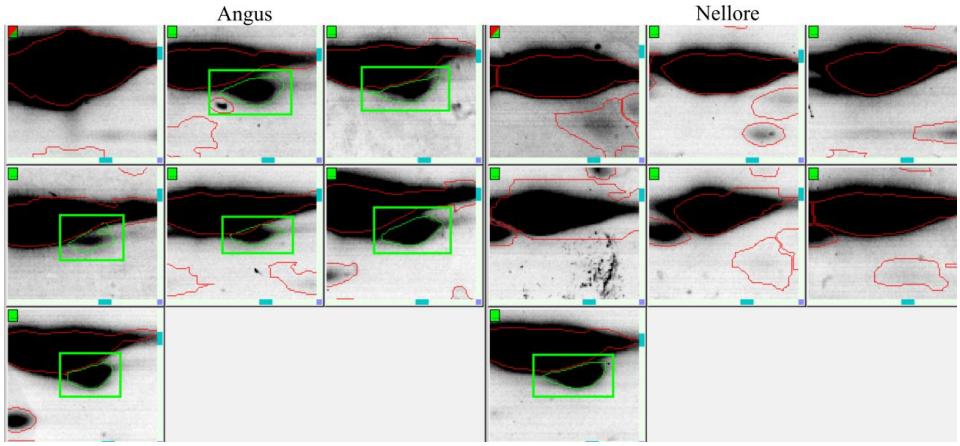
Supporting Information



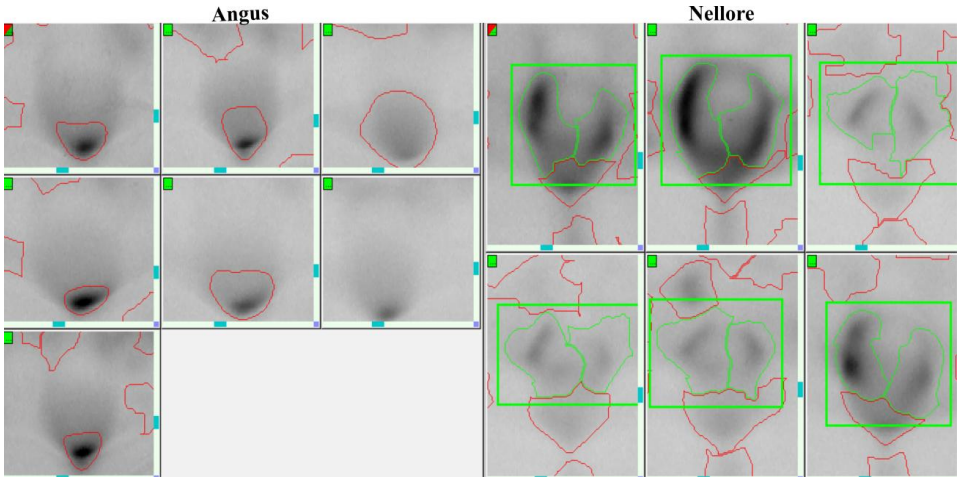
S1 Figure. Overlap of images of a gel stained with Pro-Q Diamond and Coomassie blue G-250. The image obtained with Pro-Q Diamond was colored in blue and the image obtained with Coomassie blue G-250 was colored in red. The overlap of spots in the two images produced brown color.



S2 Figure. Spot identified as myosin light chain 3 (MYL3). Spots highlighted in the green square (match ID 383) and only detected in Angus muscle.



S3 Figure. Spot identified as heat shock 70 kDa protein 6 (HSPA6). Spots highlighted in the green square (match ID 363) and only detected in the muscle of one of Nellore bulls.



S4 Figure. Two spots identified as myosin light chain 1/3 (MYL1). Spots highlighted in the green square (match IDs 1064 and 1061) and only detected in Nellore muscle.

S1 Table. Differentially abundant proteins between Angus and Nellore cattle muscle. Sequence of the peptides identified in Mascot and validated by the Scaffold.

Match ID	Protein ID	Peptide sequence
More abundant in Angus		
383	Myosin light chain 3	(R)ALGQNPTQAEVLR(V) (K)DTGTYEDFVEGLR(V)
136	Phosphoglucomutase-1	(K)AYQDQKPGTSGLR(K) (K)LSLcGEESFGTGSDHIR(E) (K)IDNFEYSDPVDGSISR(N)
199	Phosphoglucomutase-1	(K)AYQDQKPGTSGLR(K) (R)QEATLVVGGDGR(F) (K)EAIQLIVR(I) (K)TIEEYAIcPDLHVDLGVLGK(Q) (R)NIFDFNALK(E) (R)IDAMHGVVGPYVK(K) (R)IDAmHGVVGPYVK(K) (K)TGEHDFGAAFDGDGDR(N) (K)IALYETPTGWK(F) (K)LSLcGEESFGTGSDHIR(E) (K)IDNFEYSDPVDGSISR(N)
106	Stress-70 protein, mitochondrial	(R)AQFEGIVTDLIR(R)

107	Stress-70 protein, mitochondrial	(R)AQFEGIVTDLIR(R)
		(K)VQQTVQDLFGR(A)
		(K)LLGQFTLIGIPPAPR(G)
363	Uncharacterized protein	(R)IINEPTAAAIAYGLDR(L)
367	Prohibitin	(R)ILFRPVASQLPR(I)
		(R)FDAGELITQR(E)
		(R)KLEAAEDIAYQLSR(S)
More abundant in Nellore		
0	Myosin light chain 1 (fragment)	(K)EAFLLFDR(T)
		(K)ITLSQVGDVLR(A)
		(K)DQGTYEDFVEGLR(V)
108	Tropomyosin alpha-1 chain	(K)ATEDELDKYSEALK(D)
		(K)KATDAEADVASLNR(R)
		(R)RIQLVEEELDR(A)
		(R)IQLVEEELDR(A)
		(R)KYEEVAR(K)
		(R)KLVIIESDLER(A)
		(K)SIDDLEDELYAQK(L)
33	Tropomyosin alpha-1 chain	(R)KLVIIESDLER(A)
		(K)ATEDELDKYSEALK(D)
		(R)RIQLVEEELDR(A)
		(R)IQLVEEELDR(A)
		(R)KYEEVAR(K)
		(K)ATEDELDKYSEALK(D)
48	Troponin T	(R)KPLNIDHLSEDKLR(D)
29	Malate dehydrogenase, cytoplasmic	(K)VVEGLPINDFSR(E)
150	Alpha-enolase	(R)EIFDSR(G)
		(R)GNPTVEVDLFTAK(G)
		(R)AAVPSGASTGIYEALELR(D)
		(K)LAmQEFMILPVGAENFR(E)
		(K)LAmQEFmILPVGAENFR(E)
		(R)IGAEVYHNLK(N)
		(K)DATNVGDEGGFAPNILENK(E)
		(K)VVIGmDVAASEFYR(S)
		(K)VVIGMDVAASEFYR(S)
		(R)YITPDELANLYK(S)
		(K)VNQIGSVTESLQAcK(L)

198	Phosphoglucomutase-1	(K)AYQDQKPGTSGLR(K) (R)QEATLVVGGDGR(F) (K)EAIQLIVR(I) (R)IDAmHGVVGPYVK(K) (K)TGEHDFGAAFDGDGDR(N) (K)IALYETPTGWK(F) (K)LSLcGEESFGTGSDHIR(E) (K)IDNFEYSDPVDGSISR(N)
95	Phosphoglucomutase-1	(K)TGEHDFGAAFDGDGDR(N)
144	78 kDa glucose-regulated protein	(R)ITPSYVAFTPEGER(L) (K)KSDIDEIVLVGGSTR(I) (K)SDIDEIVLVGGSTR(I) (K)DNHLLGTFDLTGIPPAPR(G)

S2 Table. Differentially abundant phosphoproteins between Angus and Nellore cattle muscle. Sequence of the peptides identified in Mascot and validated by the Scaffold.

Match ID	Protein ID	Peptide sequence
More abundant in Angus		
384	Troponin T	(K)ALSSmGANYSSYLAK(A) (R)RKPLNIDHLSEDK(L)
644	Phosphoglucomutase-1	(K)AYQDQKPGTSGLR(K) (K)TGEHDFGAAFDGDGDR(N) (K)IDNFEYSDPVDGSISR(N)
870	Phosphoglucomutase-1	(K)AYQDQKPGTSGLR(K) (R)QEATLVVGGDGR(F) (K)EAIQLIVR(I) (R)IDAMHGVVGPYVK(K) (R)IDAmHGVVGPYVK(K) (R)YDYEEVEAEGANK(M)
More abundant in Nellore		
1061	Myosin light chain 1/3	(K)QQQDEFKEAFLLFDR(T) (K)ITLSQVGDVLR(A) (K)KIEFEQFLPmLQAISNNK(D) (K)DQGTYEDFVEGLR(V) (R)HVLATLGEK(M)
1064	Myosin light chain 1/3	(R)HVLATLGEK(M)
38	Myosin regulatory light chain 2	(K)EAFTVIDQNR(D) (K)GADPEDVITGAFK(V)

		(K)NMWAAFPPDVGGNVDYK(N)
37	Myosin regulatory light chain 2	(K)FLEELLTTQcDR(F)
485	Alpha actin 1	(R)AVFPSIVGRPR(H) (K)IWHHTFYNELR(V) (R)GYSFVTTAER(E) (K)SYELPDGQVITIGNER(F) (K)DLYANNVmSGGTTMYPGIADR(M) (K)QEYDEAGPSIVHR(K)
494	Alpha actin 1	(R)AVFPSIVGRPR(H) (K)IWHHTFYNELR(V)
143	Triosephosphate isomerase	(K)VPADTEVVcAPPTAYIDFAR(Q)
179	14-3-3 protein epsilon	(K)DLGATWVVLGHSER(R) (R)HVFGESDELIGQK(V) (K)TATPQQAQEVHEK(L) (K)SNVSDAVAQSAR(I) (R)IIYGGSVTGATcK(E) (K)VAGmDVELTVEER(N) (K)AASDIAMTELPPTHPIR(L) (K)AASDIAmTELPPTHPIR(L)