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ADRIELLE MATIAS FERRINHO

**Expressão de genes envolvidos no perfil de ácidos graxos e proteólise
post mortem de bovinos Nelore e Rubia Gallega x Nelore**

Pirassununga

2020

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Zootecnia e Engenharia de Alimentos
da Universidade de São Paulo, como
parte dos requisitos para a obtenção do
título de Doutor em Ciências

Área de concentração: Qualidade e
Produtividade Animal

Orientadora: Angélica Simone Cravo
Pereira

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CERTIFICADO

Certificamos que a proposta intitulada "Expressão de genes envolvidos no perfil de ácidos graxos e proteólise post mortem de bovinos Nelore e Rubia Gallega x Nelore", protocolada sob o CEUA nº 2502020518 (ID 000950), sob a responsabilidade de **Adrielle Matias Ferrinho e equipe; Angélica Simone Cravo Pereira** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 22/06/2018.

We certify that the proposal "Expression of genes involved in fatty acid profile and post mortem proteolysis of Nellore and Blond Galician x Nellore cattle", utilizing 32 Bovines (32 males), protocol number CEUA 2502020518 (ID 000950), under the responsibility of **Adrielle Matias Ferrinho and team; Angélica Simone Cravo Pereira** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 06/22/2018.

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Espécie: [Bovinos](#)

sexo: [Machos](#)

idade: [10 a 12 meses](#)

N: [32](#)

Linhagem: [Nelore e Rubia Gallega x Nelore](#)

Peso: [220 a 300 kg](#)

Local do experimento: Os animais serão alojados no confinamento do Laboratório de Pesquisa em Gado de Corte, após o período experimental eles serão abatidos no abatedouro escola, ambos localizados no Campus Fernando Costa.

Pirassununga, 05 de abril de 2019

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Universidade de São Paulo - FZEA/USP

ADRIELLE MATIAS FERRINHO

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Produtividade Animal

Orientadora: Angélica Simone Cravo
Pereira

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Prof. Dr.: _____

Instituição: _____ Julgamento: _____

DEDICATÓRIA

Este trabalho é dedicado a Deus que me forneceu toda força e coragem necessárias; a minha família pela compreensão, apoio e amor dedicados a mim; e aos meus verdadeiros amigos pelo incentivo no decorrer deste processo.

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Muito obrigada!

“Você nunca sabe que resultados virão da sua ação.
Mas se você não fizer nada, não existirão resultados.”

Mahatma Gandhi

RESUMO

FERRINHO, A. M. **Expressão de genes envolvidos no perfil de ácidos graxos e proteólise *post mortem* de bovinos Nelore e Rubia Gallega x Nelore.** 2020. 124 f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2020.

Objetivou-se avaliar o perfil de ácidos graxos e a maciez da carne de bovinos Nelore e Rubia Gallega x Nelore, e entender as diferenças na qualidade da carne por meio da avaliação de parâmetros bioquímicos e metabólicos sanguíneos, da expressão de genes envolvidos no metabolismo lipídico e na proteólise *post mortem*, e da avaliação da abundância de proteínas envolvidas no processo proteolítico e crescimento muscular. Foram utilizados 16 bovinos Nelore (N) e 16 bovinos Rubia Gallega x Nelore (RGN), machos não castrados, com idade média de 11 meses e $280 \text{ kg} \pm 15 \text{ kg}$ de peso vivo inicial. Os animais foram mantidos nas mesmas condições de manejo e receberam a mesma dieta *ad libitum*. Após 120 dias de confinamento, os animais foram abatidos. Durante o abate, foram coletadas amostras de sangue para as análises de parâmetros bioquímicos e metabólicos e amostras do músculo *longissimus* para a extração de RNA e abundância proteica (zero dia). As amostras de músculo *longissimus* utilizadas nas análises de força de cisalhamento (FC), cor (COR), perda por cocção (PPC), degradação proteica, perfil de ácidos graxos e lipídeos totais (LT) foram coletadas durante a desossa. As amostras destinadas à FC, PPC, COR e degradação proteica foram maturadas por zero, 7, 14 e 21 dias. A expressão relativa de mRNA foi avaliada para os seguintes genes: *C/EBP α* , *ZFP423*, *FABP4*, *ACC*, *LPL*, *ACOX*, *LEP*, *SCD*, *TGF β* , *MSTN*, *CAPN1*, *CAPN2* e *CAST*. A abundância proteica foi realizada para as proteínas: troponina, calpaína, HSP 27, HSP 70 e miostatina. O delineamento foi inteiramente casualizado, considerando cada animal uma unidade experimental. Para as análises avaliadas ao longo do tempo de maturação, os dados foram analisados em parcelas subdivididas, considerando um arranjo fatorial 2x4, sendo os efeitos fixos: os grupos genéticos (N e RGN), os tempos de maturação (zero, 7, 14 e 21 dias) e suas interações. A significância foi declarada quando $P \leq 0,05$. Bovinos RGN apresentaram carnes mais macias, com menor PPC e menor teor de LT ($P \leq 0,05$) quando comparados aos N. Bovinos N apresentaram maiores concentrações de ácidos graxos hipercolesterolêmicos, 14:0 e 16:0 e maior atividade da enzima $\Delta 9$ dessaturase ($P \leq 0,05$). Por outro lado, o grupo RGN apresentou melhor índice

de saúde ($P \leq 0,05$) na carne, além de menor concentração sérica de colesterol total, HDL e LDL nos animais, quando comparados ao grupo N ($P \leq 0,05$). Em relação aos hormônios, as concentrações de hormônio do crescimento (GH) e leptina não foram influenciadas pelos grupos genéticos ($P > 0,05$), diferentemente do IGF-1 e da insulina que apresentaram maiores e menores concentrações em RGN, respectivamente, ($P \leq 0,05$). Ademais, os genes *TGF β* , *MSTN*, *C/EBP α* , *LPL* e *ACOX* foram mais expressos em animais cruzados RGN, indicando diferenças na formação de fibras musculares e no metabolismo lipídico entre os grupos genéticos. Não foram encontradas alterações na expressão gênica de *CAPN1*, *CAPN2* e *CAST* entre os grupos genéticos estudados ($P > 0,05$) apesar de terem apresentado valores de FC diferentes. Por outro lado, bovinos RGN apresentaram maior degradação de troponina-T ($P = 0,0244$). No entanto, não houve diferença entre os grupos genéticos para autólise de μ -calpaína ($P = 0,9854$), havendo apenas para o tempo de maturação ($P < 0,0001$). Observou-se interação para degradação de HSP 27 ($P = 0,0006$), indicando que a degradação desta proteína é maior e mais rápida em bovinos RGN. A abundância de HSP 70 foi alterada pelo tempo de maturação ($P < 0,0001$). A abundância proteica da miostatina não foi alterada pelo grupo genético ($P = 0,3489$). De modo geral, bovinos Rubia Gallega x Nelore apresentam carnes mais macias e mais desejável à saúde humana, quando comparados à raça Nelore. Estas diferenças podem ser explicadas pelos resultados hormonais de IGF-1 e insulina, pelos genes *TGF β* , *MSTN*, *LPL*, *ACOX* e pela degradação de troponina-T e abundância de HSPs.

Palavras-chave: *Bos taurus*, *Bos indicus*, lipídeos totais, dupla musculatura, miostatina e degradação proteica.

ABSTRACT

FERRINHO, A. M. **Expression of genes involved in fatty acid profile and proteolysis *post mortem* from Nelore and Rubia Gallega x Nelore cattle.** 2020. 124 f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2020.

The objective of this study was to evaluate the fatty acid profile and meat tenderness of Nelore and Rubia Gallega x Nelore cattle, and to understand the differences in meat quality through the evaluation of blood biochemical and metabolic parameters, expression of genes involved in lipid metabolism and *post mortem* proteolysis, and evaluation of the abundance of proteins involved in proteolytic process and muscle growth. Sixteen Nelore (N) and 16 crossbred Rubia Gallega x Nelore (RGN), non-castrated males, with an average age of 11 months and $280 \text{ kg} \pm 15 \text{ kg}$ of initial live weight were used. The animals were kept under the same management conditions and received the same diet *ad libitum*. After 120 days of feedlot finishing, the animals were harvested. During harvest, blood samples were collected for analysis of biochemical and metabolic parameters and *longissimus* muscle samples for RNA extraction and protein abundance (zero day). The *longissimus* muscle samples used in the shear force (SF), color, cooking loss (CL), protein degradation, fatty acid profile and total lipids analysis were collected during deboning. Samples from SF, color, CL, and protein degradation were aged for zero, 7, 14 and 21 days. Relative mRNA expression was evaluated for the following genes: *C/EBP α* , *ZFP423*, *FABP4*, *ACC*, *LPL*, *ACOX*, *LEP*, *SCD*, *TGF β* , *MSTN*, *CAPN1*, *CAPN2*, and *CAST*. Protein abundance was performed for the proteins: troponin, calpain, HSP 27, HSP 70 and myostatin. The design was completely randomized, considering each animal an experimental unit. For analysis evaluated over the aging time, the data were analyzed in subdivided plots, considering a 2x4 factorial arrangement. The model included the fixed effects: genetic groups (N and RGN), the maturation times (zero, 7, 14 and 21 days) and their interactions. Significance was declared when $P \leq 0.05$. RGN cattle had more tender meat, lower CL and lower total lipids content ($P \leq 0.05$) when compared to N. Beef from N Cattle presented higher concentrations of hypercholesterolemic fatty acids, 14: 0 and 16: 0 and higher enzyme activity $\Delta 9$ desaturase ($P \leq 0.05$). On the other hand, RGN group had better health index ($P \leq 0.05$) in meat, besides lower serum total cholesterol, HDL and LDL in animals when compared

to N group ($P \leq 0.05$). Regarding hormones, growth hormone (GH) and leptin concentrations were not influenced by genetic groups ($P > 0.05$), unlike IGF-1 and insulin, which presented higher and lower RGN concentrations, respectively, ($P \leq 0.05$). In addition, *TGF β* , *MSTN*, *C/EBP α* , *LPL*, and *ACOX* genes were more expressed in RGN animals, indicating differences in muscle fiber formation and lipid metabolism between genetic groups. No There were no differences in gene expression of *CAPN1*, *CAPN2* and *CAST* between the genetic groups studied ($P > 0.05$), although different SF values. On the other hand, RGN cattle showed greater degradation of troponin-T ($P = 0.0244$). However, there was no difference between the genetic groups for μ -calpain autolysis ($P = 0.9854$), except for aging time ($P < 0.0001$). There was interaction for degradation of HSP 27 ($P = 0.0006$), indicating that the degradation of this protein is greater and faster in RGN cattle than N. HSP 70 abundance was altered by aging time ($P < 0.0001$). Myostatin protein abundance was not influenced by genetic group ($P = 0.3489$). In general, Rubia Gallega x Nelore cattle provide more tender beef and more desirable to human health when compared to Nelore breed. These differences can be explained by the hormonal results of IGF-1 and insulin, besides *TGF β* , *MSTN*, *LPL*, *ACOX* genes and troponin-T degradation and abundance of HSPs.

Keywords: *Bos taurus*, *Bos indicus*, total lipids, double muscle, myostatin and protein degradation.

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CAPÍTULO

1. Considerações Iniciais

1.1 INTRODUÇÃO

A demanda por produtos de origem animal de boa qualidade e saudáveis para o consumo humano tem aumentado nos últimos anos. A maciez é um dos atributos mais importantes para definir qualidade da carne e garantir aceitação e satisfação pelos consumidores (KOOHMARAIE et al., 2002). Além dessa característica sensorial, há uma maior preocupação com a qualidade nutricional da carne, principalmente em relação à composição de ácidos graxos (AG), que pode ser associada ao aumento do colesterol plasmático e ao surgimento de doenças cardiovasculares (JENKINS et al., 2008).

A fim de atender às novas exigências dos consumidores, há necessidade de aplicar ferramentas mais eficientes para o sistema de produção de carne, tais como, planejamento nutricional, melhoramento genético, cruzamento industrial, dentre outros. Sabe-se que a raça com maior representatividade na bovinocultura de corte brasileira é a Nelore, a qual é conhecida por ser adaptada ao clima tropical, resistente a parasitas, eficiente na conversão de gramíneas tropicais, longevidade e fertilidade (ARBOITTE et al., 2004; KOURY FILHO, 2005). Por outro lado, a pecuária de corte nacional caracteriza-se pela baixa produtividade e ampla variação na qualidade da carne, principalmente em relação à maciez.

Nesse sentido, uma das estratégias aplicadas para melhorar a produtividade e a qualidade do produto final é a utilização de cruzamentos com raças de características complementares. Os cruzamentos entre raças europeias (*Bos taurus taurus*) com raças adaptadas ao clima tropical (*Bos taurus indicus*), possibilitam a geração de animais superiores em aspectos produtivos (ganho de peso, musculosidade, peso de carcaça, precocidade) e qualitativos da carcaça e da carne, como melhor acabamento, conformação e maciez, além de rendimento de cortes comerciais mais valorizados.

Em consonância, a fim de melhorar as características quantitativas e qualitativas da carcaça, a raça Rubia Gallega vem sendo introduzida no rebanho brasileiro para cruzamento com animais da raça Nelore, intensificando assim a produção e qualidade da carne (ALBERTÍ et al., 2005). A raça Rubia Gallega é conhecida pela alta taxa de crescimento e baixa deposição de gordura (MONSERRAT & SÁNCHEZ, 2000), com capacidade de produção de carcaças musculosas, que permite maior rendimento de cortes (OLIETE et al., 2006). Além de ser uma raça que se destaca por sua longevidade e rusticidade, adaptando-se em diversos ambientes (ALBERTÍ et al., 2005). Essa raça pode apresentar fenótipo de musculatura dupla, decorrente de mutações no gene da miostatina,

que é uma proteína reguladora da miogênese, podendo provocar alterações na multiplicação celular (MATSAKAS & PATEL, 2009, LANGLEY et al., 2002; LEE et al., 2009). Tal modificação no padrão de crescimento e desenvolvimento muscular do animal com fenótipo para dupla musculatura pode influenciar as características da carne, por obter maior produção de carne com menor deposição de gordura subcutânea e intramuscular (GERRARD et al., 1991; XAVIER, 2014).

Apesar do cruzamento entre as raças Nelore e Rubia Gallega ser crescente no Brasil, ainda há escassez de dados na literatura que caracterizem o fenótipo obtido em progênes F1, para as características de maciez da carne e a qualidade da gordura. Diante disso, e, visto a importância crescente dada às características qualitativas da carne, faz-se necessário o maior entendimento no que se refere à composição lipídica e maciez da carne. As diferenças fenotípicas para qualidade de carne entre os animais Nelore e os Rubia Gallega x Nelore podem ser de origem intrínseca da raça, podendo ser explicadas por fatores genéticos e moleculares, como pela função de alguns genes relacionados a essas características.

Portanto, objetivou-se avaliar o perfil de ácidos graxos e a maciez da carne de bovinos Nelore e Rubia Gallega x Nelore, e entender as diferenças na qualidade da carne por meio da avaliação de parâmetros bioquímicos e metabólicos sanguíneos, da expressão de genes envolvidos no metabolismo lipídico e na proteólise *post mortem*, e da avaliação da abundância de proteínas envolvidas no processo proteolítico e crescimento muscular.

1.2 REVISÃO DE LITERATURA

1.2.1 Maciez e Proteólise

Dentre as características de qualidade de carne bovina, a maciez é considerada a mais importante, que influencia a satisfação do consumidor quanto à palatabilidade da carne (MILLER et al., 2001; OUALI et al., 2013). A variação desta característica é decisiva no retorno a compra, podendo ser o motivo da não aceitação pelos consumidores (SENTANDREU et al., 2002). Este fato preocupa as indústrias da carne que se importam por oferecer um produto com maciez garantida (WARNER et al., 2005; KOOHMARAIE; GEESINK, 2006).

A técnica de maturação na cadeia produtiva da carne é a alternativa mais utilizada para o amaciamento da carne. Além disso, esta técnica minimiza as diferenças de textura da carne entre as raças e até mesmo dentro da mesma raça, produzindo produto de melhor maciez e mais homogêneo ao consumidor (WHEELER et al., 2005). A maturação é um processo que envolve ação de sistemas proteolíticos intracelulares, os quais agem sobre as proteínas miofibrilares (OUALI et al., 2006). Dentre os diversos sistemas proteolíticos, o sistema calpaína-calpastatina é o principal sistema que afeta a degradação das fibras musculares durante o período *post mortem*, influenciando a maciez da carne (KOOHMARAIE, 1992; KOOHMARAIE & GEESINK, 2006; TAYLOR et al., 1995).

O sistema proteolítico calpaína-calpastatina pertence à família das proteases de cisteína não lisossomais e pode ser encontrado em diferentes tecidos. Este sistema é cálcio dependente, o qual caracteriza-se por três enzimas: μ -calpaína/calpaína 1 (requer baixa concentração de cálcio, entre 5 e 65 μM de Ca^{2+}), m -calpaína/calpaína 2 (exige alta concentração de cálcio, entre 300 e 1000 μM de Ca^{2+}) e o inibidor específico das calpaínas, a calpastatina (CROALL & DEMARTINO, 1991; SENTANDREU et al., 2002; GOLL et al., 2003; LI et al., 2009; VARRICCHIO et al., 2013). Essas proteínas são codificadas pelos genes *CAPN1*, *CAPN2* e *CAST*, respectivamente, e as principais proteínas miofibrilares alvos da ação desse sistema são: titina, desmina, nebulina, troponina T e tropomiosina (OUALI et al., 2013). As calpaínas degradam essas proteínas miofibrilares, rompendo a estrutura e a integridade do sarcômero, contribuindo para a maciez da carne (HUFF-LONERGAN et al., 1996, 1995; KOOHMARAIE, 1992; LOMIWES et al., 2014; WHIPPLE et al., 1990). Portanto, os produtos da transcrição

destes genes são considerados candidatos funcionais para características de qualidade de carne, devido sua ação proteolítica (CASAS et al., 2005). Segundo Wright et al. (2018), marcadores no gene CAPN1 estão associados à maciez da carne bovina, mas mecanismos específicos relacionados ao conteúdo ou atividade da calpaína ainda não foram estabelecidos.

A μ -calpaína destaca-se pela sua ação mais acentuada na proteólise *post mortem*, sendo mais importante que a m-calpaína no processo de amaciamento da carne (HOPKINS & THOMPSON, 2002; GEESINK, et al., 2006; KEMP et al, 2010). Isto ocorre devido a menor exigência de cálcio livre requerido pela μ -calpaína no período de *rigor mortis* (DRANSFIELD, 1999). Além da ativação pelos níveis de cálcio, as enzimas calpaínas agem em uma faixa de pH apropriado. Huff-Lonergan et. al. (2010) relataram que as μ -calpaínas são ativadas quando o pH declina de 6,8 para aproximadamente 5,7, enquanto que as m-calpaínas são ativadas em pH em torno de 5,7.

Dentre as proteínas miofibrilares atingidas pelo sistema proteolítico calpaína-calpastatina, a troponina-T é considerada um indicador confiável de proteólise *post mortem* (HUFF-LONERGAN et al., 2010). O desaparecimento da troponina T intacta e o surgimento de produtos de degradação de 28 a 32 kDa são as mudanças mais notáveis que ocorrem durante a maturação, como mensurado pela técnica de Western Blot (Koochmaraie, 1994). Segundo Harris et al., (2001) o fragmento de 30 kDa derivado da troponina T intacta é um ótimo indicador de maciez da carne.

Além do sistema proteolítico calpaína-calpastatina, a maciez é dependente de muitos fatores, como genética, idade ao abate, manejo nutricional, quantidade e solubilidade do colágeno, comprimento de sarcômero, declínio do pH *post mortem*, tipo de fibra e, conseqüentemente, tipo de músculo (SEIDEMAN & KOOCHMARAI 1987; KEMP & PARR, 2012; MARINO et al., 2013). Dentro do fator genética, muitos autores têm estudado a influência de bovinos de dupla musculatura nessa característica, porém os resultados ainda são controversos (WHEELER et al., 2001; CASAS et al., 1998; UYTTERHAEGEN et al., 1994; NGAPO et al., 2002).

Alguns autores clássicos relataram diferenças significativas na maciez da carne entre raças bovinas europeias e zebuínas (WHIPPLE et al., 1990; SHACKELFORD et al., 1991; O'CONNOR et al., 1997). Além disso, Crouse et al. (1993), Sherbeck et al. (1995) e Wright et al. (2018) observaram que a participação crescente de alelos específicos de *Bos indicus* em cruzamentos com *Bos taurus* diminui consideravelmente a maciez da carne.

Essa variabilidade da maciez entre subespécies tem sido atribuída à diferentes níveis de enzimas proteolíticas encontradas nos músculos dos animais (WHIPPLE et al., 1990), especialmente enzimas do sistema calpaína-calpastatina. Sabe-se que *Bos indicus* apresentam maior atividade de calpastatina quando comparados aos *Bos taurus*. Essa atividade elevada de calpastatina em bovinos *Bos indicus* diminui a proteólise *post mortem*, afetando negativamente a maciez (WHEELER et al, 1990; WHIPPLE et al., 1990; KOOHMARAIE, 1994; PRINGLE et al., 1997, 1999; MARTINS et al., 2017; WRIGHT et al., 2018). No entanto, a diferença na proteólise *post mortem* entre essas subespécies não tem sido associada apenas ao sistema calpaína-calpastatina, como também a outros sistemas proteolíticos e até mesmo a expressão gênica e abundância proteica de marcadores de maciez da carne (OUALI et al., 2006).

Martins et al. (2017), ao compararem animais zebuínos e taurinos britânicos, observaram que bovinos Nelore apresentam menor índice de fragmentação miofibrilar, comparados à raça britânica Angus ($47,6 \pm 4,28\%$ vs. $69,2 \pm 4,28\%$). Esse mesmo estudo avaliou a atividade da calpastatina, bem como a expressão gênica e abundância de proteínas envolvidas no amaciamento da carne. Os autores sugeriram que a maior proteólise *post mortem* no músculo esquelético de bovinos Angus, em comparação ao Nelore, não é causada por diferenças na expressão dos genes que codificam calpastatina ou calpaína, ou pela abundância dessas enzimas, mas sim devido à maior atividade da calpastatina no músculo esquelético de bovinos Nelore (MARTINS et al., 2017).

Assim, os mecanismos de amaciamento da carne têm despertado muito interesse dos pesquisadores da área de qualidade de carne. No entanto, apesar dos diversos estudos nem todos os mecanismos envolvidos no amaciamento são claramente entendidos (KEMP et al., 2010; OUALI et al., 2013), em especial em animais cruzados.

Além disso, outras proteínas, como as de choque térmico (HSPs) estão sendo avaliadas em estudos de maciez de carne (PICARD et al., 2017; GAGAOUA et al., 2018a). O papel das HSPs em animais vivos tem sido extensivamente descrito (MAYER, 2013). No entanto, a ação destas proteínas no músculo *post mortem* ainda não é clara, embora estudos tenham relatado associações entre HSPs, incluindo a HSP70, com a maciez da carne em bovinos (PICARD et al., 2017; CARVALHO et al., 2014), outras espécies (PHONGPA-NGAN et al., 2011) e em diferentes músculos (PICARD et al., 2017; PICARD et al., 2014, GAGAOUA et al., 2015, GAGAOUA et al., 2018b). Pesquisas de proteômica em carne bovina têm demonstrado que as HSPs mais pesadas diferem entre amostras de carne macia e dura (PICARD et al., 2017). No entanto, Bouley

et al. (2003) observaram que níveis mais baixos de HSP20 e HSP27 estão associados às carnes macias.

Contudo, ainda há pouca informação sobre o impacto do uso de animais cruzados *Bos taurus* e *Bos indicus* sobre a proteólise da carne. Portanto, é importante o conhecimento de quais sistemas poderiam estar inseridos no amaciamento da carne e consequentemente nas diferenças de *turnover* protéico. Além disso, a maioria dos estudos que contempla a maciez associa raças e não seus cruzamentos. Deste modo, o estudo da expressão gênica e a quantificação das proteínas é fundamental para o esclarecimento das influências destes fatores na maciez da carne dos animais entre subespécies.

1.2.2 Composição Lipídica da Carne

Nos últimos anos houve um aumento na conscientização da população em relação aos alimentos consumidos, considerando principalmente sua relação com a saúde, como por exemplo, a quantidade e qualidade da gordura, colesterol e sal, que fornecem riscos à saúde humana, como doenças cardiovasculares, alguns tipos de câncer, entre outras doenças (HOFFMAN et al., 2005; HOCQUETTE et al., 2012; TROY et al., 2016).

Nutricionistas recomendam uma moderada ingestão de gordura total e uma ingestão maior de ácidos graxos poli-insaturados (AGPI), principalmente AGPI ômega 3 (n3), necessários para o crescimento e desenvolvimento do embrião, além de apresentarem efeitos cardioprotetores e reduzirem o risco de certas doenças mentais (EUROPEAN FOOD SAFETY AUTHORITY, EFSA, 2010; RIEDIGER, OTHMAN, SUH, & MOGHADASIAN, 2009). O elevado consumo de ácidos graxos saturados (AGS) contribui para o desenvolvimento da síndrome metabólica, implicando em inflamação do tecido adiposo, aumento do estresse oxidativo e resistência à insulina (KENNEDY et al., 2009). Enquanto que os ácidos graxos monoinsaturados (AGMI) e poli-insaturados (AGPI) são benéficos, minimizando a resistência à insulina, influenciando no colesterol sanguíneo e reduzindo o risco de doenças coronárias (LOTTENBERG et al., 2012).

Diante disso, os consumidores estão cada vez mais interessados em alimentos de origem animal que apresentem menor teor de ácidos graxos saturados e calorias e maior teor de ácidos graxos insaturados, favorecendo uma vida saudável e prevenindo o desenvolvimento de doenças.

A composição de ácidos graxos (AG) da gordura intramuscular presente na carne é de suma importância para a saúde humana, uma vez que essa gordura não pode ser removida ou extraída antes do seu consumo (DE SMET, RAES & DEMEYER, 2004). Em geral, o conteúdo lipídico na carne bovina varia entre 4-15%, os AG da gordura intramuscular da carne bovina consistem proporcionalmente em média de 45-48% de AGS, 35-45% AGMI e até 5% de AGPI. A razão de ácidos graxos poli-insaturados:saturados (AGPI:AGS) da carne é geralmente baixa, em torno de 0,1; exceto para animais muito magros (<1% de gordura intramuscular), onde a razão AGPI:AGS é mais elevada, aproximadamente 0,5-0,7 (SCOLLAN et al., 2006).

Os AGS mais comuns na carne bovina são: mirístico (14:0), palmítico (16:0) e esteárico (18:0). Os principais AGMI incluem o palmitoleico (16:1) e oleico (18:1), e os principais AGPI são linoleico (18:2), linolênico (18:3), e araquidônico (20:4; SCOLLAN et al., 2006). Além desses, a carne bovina é uma das principais fontes do conjunto de isômeros do ácido graxo linolênico conjugado (CLA) na dieta humana, o qual tem sido identificado com diversas propriedades biológicas benéficas para a saúde como a redução do risco de câncer, aterosclerose e diabetes (BELURY, 2002).

No entanto, a quantidade e a qualidade da composição de AG da carne bovina são dependentes do metabolismo lipídico que acontece ao longo da vida do animal. A deposição do tecido adiposo pode ocorrer devido à hipertrofia de adipócitos (lipogênese) e/ou hiperplasia de células adipogênicas (adipogênese) (HAUSMAN et al., 2009). Assim, uma vez que a gordura intramuscular é aquela que apresenta deposição tardia em relação aos demais tipos de tecido adiposo da carcaça, acredita-se que o aumento da deposição desse tecido se dê prioritariamente em virtude da lipogênese e não adipogênese. Contudo, a adipogênese intramuscular foi associada ao elevado teor de gordura intramuscular na carne de bovinos em fase de terminação (DUARTE et al., 2013).

A genética, a dieta, assim como a condição sexual, idade ao abate e taxa de crescimento são fatores que influenciam a composição de AG da carne (LADEIRA et al., 2016; WOODS & FEARON, 2009). Dentre esses fatores, a genética tem uma importante função no perfil de AG da carne de ruminantes (PITCHFORD et al., 2002; DE SMET, RAES & DEMEYER, 2004). Além disso, o conteúdo da gordura intramuscular relaciona-se intrinsecamente à raça (INOUE et al., 2011; KELLY et al., 2013; FEITOSA et al., 2017).

Nesse sentido, Martins et al. (2015), comparando dois grupos genéticos, demonstraram que a carne de bovinos Nelore apresenta menor conteúdo de gordura intramuscular ($2,7 \pm 0,08\%$ vs. $3,4 \pm 0,08\%$), do que a carne de bovinos Angus.

Posteriormente, Bressan et al. (2016) observaram que animais F1 cruzados taurinos x zebuínos, terminados com dieta de alto grão, apresentaram maior deposição de AGPI (28% a mais) e menor deposição de AGS (4% a menos), principalmente do ácido mirístico (C14:0), palmítico (C16:0) e esteárico (C18:0), quando comparados aos animais zebuínos puros. Esses resultados foram explicados pelos autores devido à maior porcentagem de gordura intramuscular (20%), maior atividade da enzima elongase C16-C18 (5%) presente nos animais cruzados e menor atividade da enzima delta-9-dessaturase no carbono 18 (3%) nos animais puros.

De acordo com De Smet, Raes e Demeyer (2004), em raças taurinas, a maior parte dos lipídeos intramusculares é basicamente formada por triacilgliceróis, os quais são armazenados na gota lipídica e, portanto, podem ser modificados mais facilmente, por meio do fornecimento de fontes de gorduras insaturadas na dieta. Por outro lado, em animais com baixa predisposição genética para a deposição de gordura intramuscular, como é o caso dos animais zebuínos e de dupla musculatura, a gordura presente na carne é composta basicamente pelos lipídeos de membrana, principalmente fosfolipídeos, os quais apresentam maiores proporções de AGPI, que são mais difíceis de serem alterados por meio do fornecimento de fontes dietéticas de gorduras insaturadas.

Portanto, diante dos estudos observados, grupos genéticos com menores taxas de deposição de gordura intramuscular produzem carne com maior quantidade de AGPI, dada a alta correlação negativa entre ambas as variáveis e a maior quantidade de fosfolipídeos (DE SMET, RAES & DEMEYER, 2004).

Além disso, segundo Oosterveer et al. (2009), há diferenças na atividade de algumas enzimas envolvidas no metabolismo lipídico, tais como as elongases, delta 4, delta 5, delta 6 e delta 9 dessaturase, entre as raças bovinas, que podem influenciar a regulação do teor de alguns ácidos graxos e, portanto, a qualidade do tecido adiposo.

1.2.3 Metabolismo Lipídico

1.2.3.1 Hormônios envolvidos no metabolismo lipídico

Em ruminantes, os adipócitos apresentam funções de reserva de energia, além de atuar como células endócrinas, secretoras de importantes hormônios, cujos sinais endócrinos estão relacionados com a produção animal. Dentre os hormônios, destaca-se a leptina, IGF-1, interleucinas, resistina, entre outros (MINER, 2004).

A leptina é produzida especificamente pelos adipócitos e quando secretada é transportada pelo sangue até atingir seus receptores localizados em diversos tecidos, inclusive o hipotálamo (MINER, 2004). A função da leptina é mediada através do neuropeptídeo Y, sendo responsável pela homeostase energética do organismo, capaz de alterar o consumo alimentar e o gasto energético corporal. Segundo Ji et al. (1998), Chillard et al. (1998) e Minton et al. (1998), a leptina está associada com a porcentagem de gordura corporal, sendo que animais com maior deposição de gordura apresentam maiores quantidades de leptina plasmática. A quantidade de gordura presente no citoplasma e o consequente tamanho do adipócito são refletidos pela expressão da leptina. Além disso, a leptina relaciona-se a aspectos reprodutivos, visto que sua presença facilita a secreção de gonadotrofinas (MINER, 2004).

Outra importante função da leptina está relacionada com o crescimento animal (GILIS et al., 2004). A leptina é um dos peptídeos que participa da liberação do hormônio do crescimento (GH), via neuropeptídeo Y (NPY), que estimula a secreção do hormônio liberador do hormônio do crescimento (GHRH) (MCMAHON et al., 2001). Portanto, a leptina estimula a secreção de GH via GHRH, através do NPY, uma vez que o efeito de estimulação da leptina é praticamente anulado na presença de anticorpos para GHRH (MCMAHON et al., 2001).

O GH é um hormônio importante na regulação do peso e metabolismo dos lipídeos, uma vez que o aumento em sua concentração estimula a síntese proteica, enquanto seu decréscimo irá sinalizar para lipogênese. Existe, assim, um *feedback* entre leptina, NPY e GH para regulação do metabolismo lipídico. Com o GH elevado, há mobilização de lipídeos e inibição de sua síntese, reduzindo os níveis de leptina, que, por sua vez, deixa de estimular a liberação de NPY (MCMAHON et al., 2001).

Outro hormônio envolvido na síntese de gordura é a insulina, que apresenta função de capturar glicose circulante para os tecidos periféricos, aumentando assim a lipogênese ou reduzindo a lipólise. Portanto, a concentração plasmática de insulina está positivamente correlacionada com a adiposidade da carcaça (RHOADES et al., 2007). Entretanto, existe variação em relação à sensibilidade à insulina entre os diferentes tecidos

corporais, sendo um dos principais fatores determinantes da partição de energia entre esses tecidos e o seu crescimento e desenvolvimento (HOSSNER, 2005).

1.2.3.2 Genes envolvidos no metabolismo lipídico

A adipogênese é regulada em grande parte pelos fatores de transcrição pertencentes à família *C/EBP* (Enhancer binding protein) e o *PPAR* (Peroxisome proliferator-activated receptor; DODSON et al., 2010; DU et al., 2010; YAMADA et al., 2009). Estes fatores transcricionais (*C/EBP* e *PPAR*) são autorreguladores e agem em conjunto a fim de estimular a diferenciação das células mesenquimais em pré-adipócitos, adipócitos maduros e, finalmente, em adipócitos (ROSEN et al., 2002).

O termo *PPAR* corresponde a uma família de receptores nucleares influenciados por AG que desempenham funções importantes na regulação do metabolismo de nutrientes e homeostase energética (LEMAY & HWANG, 2006). Existem três isoformas de *PPAR*, α , β e γ , que se diferenciam pelo tecido alvo, propriedades fisiológicas e estágios de desenvolvimento do tecido (DESVERGNE & WAHLI, 1999). O *PPAR γ* é o principal fator regulatório da adipogênese e do armazenamento de ácidos graxos, uma vez que se liga a genes associados ao metabolismo de gordura, tais como os que codificam a proteína de ligação ao ácido graxo (*FABP*), a qual desempenha o papel de transportar AG no tecido adiposo e contribuir para o metabolismo energético do músculo, direcionando AG de cadeia longa para a oxidação ou esterificação nas mitocôndrias e nos peroxissomos (BIONAZ, THERING & LOOR, 2012; GERBENS, 2004; VURAL et al., 2008). O *PPAR γ* tem maior expressão nos adipócitos e menor expressão no músculo (KERSTEN, 2014), sendo importante no controle da adipogênese (TAKADA, KOUZMENKO & KATO, 2009) e apresenta sensibilidade à insulina (OLEFSKY & SALTIEL, 2000). Além disso, o *PPAR γ* pode atuar na oxidação dos ácidos graxos de cadeia longa (SHARMA et al., 2012).

Outro fator envolvido no metabolismo lipídico é o elemento regulador de esterol ligado a proteína (*SREBP-1c*; XU et al., 2001). O *SREBP-1c* é associado à regulação dos genes envolvidos na lipogênese (HARVATINE & BAUMAN, 2006), atuando mais especificamente nos genes envolvidos na síntese de AG, principalmente os que codificam as enzimas acetil-CoA carboxilase (*ACC*) e ácido graxo sintase (*FAS*). No entanto, também foi relatado que o *SREBP* controla a expressão da enzima estearoil CoA dessaturase (*SCD*). Quando há alteração nos níveis de *SREBP*, a síntese da *SCD* é

influenciada, causando diferenças na composição de AG no tecido adiposo dos animais (MANNEN, 2011).

A *SCD* é responsável pela conversão de AGS em AGMI (MATSUSHI, FUJIMORI & OKITANI, 2001) e também está associada à conversão do ácido vacênico (C18:1 trans-11) a CLA (C18:2 cis-9 trans-11; SMITH et al., 2006). Existem algumas isoformas dessa enzima, a *SCD1* é a principal delas e está presente no fígado e no tecido adiposo (KIM, MIYAZAKI & NTAMBI, 2002). Uma transferência de elétrons permite a adição de uma dupla ligação entre os carbonos 9 e 10 do ácido esteárico (C18:0) e palmítico (C16:0), convertendo-os em ácido oleico (C18:1) e ácido palmitoleico (C16:1), respectivamente. Lee et al. (2011) observaram menor expressão dos genes *SCD* e *SREBP-1c* na gordura perirrenal de bovinos da raça Wagyu, o que acarretou maior concentração de AGS na carne desses animais.

As enzimas lipogênicas *ACC* e *FAS* estão associadas à síntese *de novo* de lipídeos, realizada no próprio tecido adiposo dos ruminantes (SMITH, WITKOWSKI & JOSHI, 2003). No entanto, qualquer alteração na ação dessas enzimas refletirá em mudanças nas taxas de síntese dos ácidos graxos. Ward et al. (2010) observaram que o conteúdo de gordura intramuscular está positivamente relacionada com a expressão dessas enzimas responsáveis pela síntese de ácidos graxos.

Nesse sentido, Underwood et al. (2007) relataram que bovinos com maior gordura intramuscular apresentaram maior taxa de ativação de *ACC*, enquanto que os animais com menor gordura intramuscular tiveram maior taxa de fosforilação e inativação de *ACC*, resultando em menor nível da enzima ativa. Zhang et al. (2008) relataram a ocorrência de polimorfismos de DNA no gene *FAS* em bovinos, sugerindo a importância desse gene para a regulação da formação de AG na composição da carne bovina. Posteriormente, Ward et al. (2010) avaliaram a atividade da *FAS* em diversos músculos de bovinos Limousin, Angus e seus mestiços e relataram maior atividade da *FAS* em músculos com maior escore de marmorização.

1.2.4 Bovinos Rubia Gallega

A raça Rubia Gallega (*Bos taurus*) é originária da Espanha, nativa da região da Galícia, onde é comercializada de duas maneiras, “Ternera Gallega Normal”, na qual os bezerros são desmamados antes de serem abatidos, e o “Ternera Gallega Suprema”, na qual os bezerros mamam até o momento do abate (TERNERA GALLEGA, 2008).

Segundo Domingo et al. (2014) a "Ternera Gallega" é referência espanhola no que diz respeito a comercialização de carne de alta qualidade, sendo 48% desse produto comercializado na Galícia, 50% em outras regiões da Espanha e 2% destinados à exportação.

A introdução dessa raça no Brasil foi em 2000 quando ocorreu a primeira experiência com inseminação artificial de matrizes Nelore com sêmen de Rubia Gallega. A cidade de Buri (SP) é considerada o centro de cruzamento da raça no Brasil. Esse cruzamento entre as raças foi iniciado para que houvesse uma combinação entre a rusticidade e adaptação com as características de produção de carne do Rubia Gallega (SANCHEZ et al., 2005), o cruzamento tem sido produzido em cerca de 40 propriedades brasileiras. Toda sua produção é destinada a uma grande rede varejista.

A raça Rubia Gallega é classificada como alta produtora de carne, porém, com maturidade tardia, apresenta elevada taxa de crescimento e baixa predisposição genética para a deposição de gordura (ALBERTÍ et al., 2005; MONSERRAT & SÁNCHEZ, 2000). Contudo, os animais desta raça apresentam um perfil lipídico de carne mais favorável à saúde humana, devido à gordura presente na carne ser composta basicamente pelos lipídeos de membrana, os fosfolipídios, que apresentam maiores proporções de ácidos graxos insaturados (DE SMET, RAES & DEMEYER, 2004). Segundo Oliete et al. (2006), bovinos Rubia Gallega produzem carcaças com elevado rendimento de peças comerciais, resultando em cortes cárneos mais pesados.

Albertí et al. (2005), ao analisarem sete raças de corte da Espanha, quanto às características de carcaça, observaram que a raça Rubia Gallega tendeu a produzir carcaças maiores, com pesos de abate mais elevados do que as demais raças. Além disso, o banco de dados analisado reflete a preferência de alguns mercados locais para carne magra, como produzido por Rubia Gallega e Asturiana de los Valles. Já Varela et al. (2004) mencionaram o potencial desses animais para serem abatidos em idade precoce, entre oito a dez meses, e com carcaça com deposição de gordura subcutânea favorável, a fim de impedir problemas, por exemplo, relacionados ao encurtamento das fibras pelo frio.

De acordo com Taveira et al. (2014), a adoção do cruzamento Nelore x Rubia Gallega proporciona animais de desempenho superior, além de atender às preferências dos consumidores por carne de melhor qualidade. Em estudos realizados com animais da raça Rubia Gallega, comparando a idade ao desmame antes do abate, observou-se uma média de 0,70% de gordura na composição química do músculo *longissimus*, 54mg/100g

de ácido mirístico (C14:0), 910mg/100g do total de AGS e uma proporção AGPI:AGS de 0,20 no músculo *longissimus* (MORENO et al., 2006).

Outra particularidade que a raça Rubia Gallega pode apresentar é o fenômeno da dupla musculatura (DM). As raças bovinas de DM mais estudadas atualmente são: Belgian Blue e Piemontês, além de outras raças como, Asturiana de los Valles, Maine Anjou, Blond d'Aquitaine, Charolês, Limousin, Parthenaise, South Devon, Marchigiana e Rubia Gallega.

A DM é uma condição hereditária caracterizada pelo aumento dos músculos devido ao aumento do número de fibras musculares e crescimento individual dessas fibras, hiperplasia e hipertrofia, respectivamente (HANSET, 1982; ARTHUR, 1995). Essas características já são evidentes enquanto feto, pois durante os primeiros dois terços da gestação, fase de maior multiplicação celular, o número de fibras é maior em feto que possui característica DM (SWATLAND & KIEFFER, 1974; ARTHUR, 1995). Ao nascimento, bezerros DM têm cerca do dobro do número de fibras musculares brancas (SWATLAND, 1973;). Logo, bovinos DM têm maior proporção de fibras brancas, além de menor quantidade de mioglobina dos músculos (BOCCARD, 1982).

Os animais DM apresentam menor consumo e melhor conversão alimentar (ARTHUR, 1995), com carcaças que apresentam pouca gordura, tanto subcutânea quanto intramuscular (WIENER et al., 2009), maior porcentagem de cortes nobres, cerca de 30% a mais na relação músculo:osso (ARTHUR, 1995), menor porcentagem de ossos, maior rendimento de carcaça, maior área de lombo (WIENER et al., 2009).

A mutação genética de bovinos DM deve-se a um gene recessivo denominado *GDF8* (Growth Differentiation Factor 8), conhecido também como miostatina (*MSTN*; HAN, FORREST & HICKFORD, 2013) pertencente à família do *TGFβ* (McPHERRON, LAWLER & LEE, 1997). Tanto a *MSTN* como *TGFβ* estão envolvidos na regulação do crescimento do músculo esquelético e no catabolismo muscular (BUDASZ-RWIDERSKA et al., 2005), ambos agem como regulador negativo da síntese muscular em várias espécies (McPHERRON & LEE, 1997), restringindo o crescimento muscular. A *MSTN* pode contribuir também para a regulação da adipogênese (LEE & MCPHERRON, 2001), por meio da produção e secreção reduzida de leptina (McPHERRON & LEE, 2002).

O gene miostatina é expresso especificamente no músculo esquelético, durante as fases de desenvolvimento (miogênese) e adulto, variando de músculo para músculo. Nos primeiros estágios da embriogênese sua expressão está restrita ao miótomo e

continua a se expressar durante todo desenvolvimento muscular na embriogênese (BASS et al., 1999; THOMAS et al., 2000; KOCAMIS & KILLEFER, 2002; RIOS et al., 2002; SPILLER et al., 2002).

Em estudos envolvendo análises moleculares, McPherron e Lee (1997) testaram ratos e demonstraram que com o bloqueio da atividade do gene *MSTN* ocorre um aumento significativo na massa muscular dos animais, tendo esses a quantidade de gordura reduzida. Esses mesmos autores compararam as sequências gênicas da miostatina em dez espécies diferentes, confirmando que a hipertrofia muscular é causada pela perda da função da proteína miostatina.

Em relação ao conteúdo lipídico, bovinos DM são caracterizados pelo baixo teor de gordura intramuscular, apresentando um teor de gordura intramuscular inferior a 1% (WIENER et al., 2009; ALDAI et al., 2007). Além do menor teor de gordura, a composição de AG também é diferente em relação aos animais sem a condição de DM. A concentração de AGPI aumenta em animais DM, enquanto a concentração de AGS reduz, resultando em uma maior proporção AGPI / AGS (RAES, De SMET & DEMEYER, 2001; ALDAI et al., 2007; WIENER et al., 2009).

Raes et al. (2003) mostraram que na raça Belgian Blue o genótipo DM apresentou menor concentração de ácidos graxos monoinsaturados e maior concentração de ácidos poli-insaturados, em comparação ao genótipo não mutante. Esses autores atribuíram esse resultado a baixa concentração de lipídeos totais no músculo e a maior proporção de fosfolipídeos nos lipídeos totais.

Ademais, tem sido demonstrado que o metabolismo dos ácidos graxos n6 e n3 é afetado pelo gene *MSTN* e seu efeito é mais evidente em AGPI n3 da gordura intramuscular, em comparação com a gordura intermuscular e subcutânea (ALDAI et al., 2008), juntamente com uma proporção menor de AGPI de n6 / n3 (RAES, DE SMET, DEMEYER, 2001). Portanto, animais de DM produzem carne saudável.

Além dos estudos que avaliaram o conteúdo lipídico de bovinos DM, pesquisas têm relatado a influência deste fenótipo na qualidade de carne, principalmente maciez. Alguns polimorfismos do gene *MSTN* que suprimem a produção de miostatina funcional aumentam a maciez da carne (WHEELER et al., 2001; BENNET et al., 2019). De acordo com Hanset (1982), os músculos do bovino DM têm menor quantidade de colágeno (tecido conectivo), comprovada pela redução de cerca de 20 a 30% da quantidade de hidroxiprolina, o que implica diretamente na redução da resistência e, consequentemente,

em carne mais macia (UYTTERHAEGEN et al., 1994; ARTHUR, 1995; WIENER et al., 2009; FIEMS, 2012).

Por fim, particularmente no Brasil, onde a genética desempenha um papel importante no segmento de carne, espera-se um maior entendimento sobre o uso de animais cruzados para o aumento da eficiência da produção, a fim de elucidar as características descritas acima, resultando em melhor conhecimento dos fatores genéticos, biológicos e fisiológicos relacionados às características qualitativas e quantitativas de interesse de produtores e consumidores.

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CAPÍTULO

2. Artigo a ser submetido à revista científica

Rubia Gallega x Nelore crossbred can improve beef tenderness through changes in protein abundance and gene expression

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ABSTRACT

The objective was to evaluate meat quality of crossbred Rubia Gallega x Nelore compared to Nelore cattle, and understand it by mRNA of calpain 1 and 2, calpastatin, myostatin, and *TGFβ*; and protein abundance profile *post mortem* based on aged meat of the different genetic groups. Sixteen Nelore (N) and 16 crossbred Rubia Gallega x Nelore (RGN), non-castrated males, with 11 months of age and $280 \text{ kg} \pm 15 \text{ kg}$ of initial live weight were used. Animals were harvested after 120 days of feedlot finishing. RGN beef presented greater tenderness and lower coking loss ($P \leq 0.05$). Transcripts analysis demonstrated increased levels of *TGFβ* and *MSTN* in RGN, indicating differences in muscle fiber formation. However, we did not observe differences in mRNA abundance for *CAPN1*, *CAPN2* and *CAST* between genetic groups ($P > 0.05$). Protein analysis demonstrated increased troponin-T degradation in RGN cattle ($P = 0.0244$). No differences were observed between the genetic groups for calpain 1 autolysis ($P = 0.9854$), only for the aging time ($P < 0.0001$). There was interaction for degradation of HSP 27 ($P = 0.0006$), indicating that the degradation of this protein is faster and greater in RGN than N. HSP 70 abundance was altered by aging time ($P < 0.0001$). Myostatin protein abundance was not altered by the genetic group ($P = 0.3489$). In general, Rubia Gallega x Nelore cattle can improve beef traits and these differences may be explained by *TGFβ* and *MSTN* gene expression, troponin-T degradation and HSPs abundance.

Keywords: *Bos indicus*, *Bos taurus*, myostatin, proteolysis, HSP and troponin-T.

1. INTRODUCTION

Tenderness is one of the most important traits of meat quality, which will always be the first quality sought by the consumer (OUALI et al., 2013). Therefore, inconsistency in meat tenderness is a determining factor for the beef industry and affects consumer's purchase decisions (MILLER et al., 2001; OUALI et al., 2013; PICARD et al., 2014). However, different factors influence this attribute such as animal age at slaughter, collagen solubility, production system, diet, gender status, meat aging and is intrinsically related to genetic factors such as breeds and subspecies (STRYDOM et al. 2000; BONFATTI et al., 2013).

Bos indicus cattle, mainly Nelore breed, have been displayed to the production of low quality meat or with wide variability in meat tenderness (PEREIRA et al., 2015). This variability found within subspecies has been attributed to different levels of proteolytic enzymes found in animal muscles (WHIPPLE et al., 1990), especially calpain-calpastatin system enzymes. *Bos indicus* cattle have higher calpastatin activity when compared to *Bos taurus*, resulting in decreased *post mortem* proteolysis and lower meat tenderness (WHEELER et al, 1990; WHIPPLE et al., 1990; KOOHMARAIE, 1994; PRINGLE et al., 1997, MARTINS et al. 2017; WRIGHT et al., 2018).

Among the strategies to improve beef quality, mainly tenderness from *Bos indicus* beef, producers have been using European (*Bos taurus*) breeds for crossing. Thus, the introduction of breeds such as Rubia Gallega (*Bos taurus*) crossbred with Nelore animals can be an alternative to improve meat production and quality and allowing brand development by the meat industry. Rubia Gallega breed is known for its high growth rate and low-fat deposition (MONSERRAT & SÁNCHEZ, 2000), producing highly muscled carcasses and allowing higher cuts yield (OLIETE et al., 2006). In addition, this breed shown mutations in the myostatin gene (*MSTN*), resulting in inactivation of myostatin protein and consequently a higher muscle mass (DUNNER et al., 1997; GROBET et al., 1997; McPHERRON & LEE, 1997; KARIM et al., 2000; SMITH et al., 2000; PHOCAS, 2009), conferring the double muscled (DM) phenotype. Furthermore, according to Bocard (1982), Bailey et al. (1982), Gerrard et al. (1991), Wheeler et al. (2001), and Fiems (2012), cattle with DM phenotype result in tender meat when compared to normal cattle.

Besides genetic factor, aging process is other factor that alter beef tenderness, which involves the activation of intracellular proteolytic systems (WHEELER et al., 1990, DELGADO et al., 2001; OUALI et al., 2006). The calpain-calpastatin proteolytic

system is one of the major systems that affect the integrity of myofibrillar proteins during the *postmortem* period (KOOHMARAIE, 1992; KOOHMARAIE et al., 1987; KOOHMARAIE & GEESINK, 2006; TAYLOR et al., 1995). However, other proteolytic systems, gene expression, and protein abundance of meat tenderness markers have been studied, like proteasome, cathepsins, matrix metalloproteases, thrombin, and heat shock proteins (HSPs). Despite these extensive efforts by meat scientists, these mechanisms are still unclear (OUALI et al., 2013).

Based on that there is a lack of knowledge about the mechanisms influencing meat quality in crossbred cattle such as Rubia Gallega X Nelore. Thus, our results can contribute significantly to understand the genetic, biochemical and physiological mechanisms involved in meat tenderness of crossbred beef Rubia Gallega x Nelore and Nelore.

Therefore, the objective of this study was to evaluate the physical traits of meat quality and to quantify gene transcripts and proteins abundance involved in the *post mortem* proteolysis of Nelore and crossbred Rubia Gallega x Nelore cattle, to understand the molecular mechanisms involved in meat tenderness.

2. MATERIAL AND METHODS

The experiment was conducted at the feedlot of the University of Sao Paulo, located in Pirassununga, Sao Paulo, Brazil. All experimental procedures involving animals were approved by the Animal Ethics Committee of the College of Animal Science and Food Engineering, University of Sao Paulo, protocol number 2502020518.

2.1. Animal and Management

Sixteen Nelore bulls (N) and sixteen crossbred Rubia Gallega x Nelore bulls (RGN) with initial body weight 280 ± 15 kg and 11 months of age were used. Each genetic group, N and RGN, were from a single Nelore and Rubia Gallega bull, respectively. The Rubia Gallega bull used at the crossbreeding was homozygous for myostatin gene. These animals were housed in pens with two animals, according to genetic group. Animals were adapted to diet during twenty-one days. They were fed *ad libitum* with the same diet with a forage:concentrate ratio of 30:70 in finishing diet 1 during 50 days, then ratio of 85:15 in finishing diet 2 by 70 days (Table 1).

Table 1. Composition of experimental diets (% Dry Matter)

Ingredient	Finishing 1 ¹	Finishing 2 ²
Corn silage	21.00	4.400
Sugarcane bagasse	9.000	10.00
Ground corn	55.80	61.00
Soybean meal 45%	10.00	1.150
Whole cottonseed	-	20.00
Mineral mix ³	2.000	1.250
Urea	1.300	0.800
Potassium chloride	0.300	0.600
Calcareous	0.600	0.800
Composition		
Dry matter (% natural matter)	61.53	77.17
Crude protein (% DM)	15.53	13.25
Total digestible nutrient	73.40	78.88
Neutral detergent fiber (% DM)	17.87	16.14
Acid detergent fiber (% DM)	0.595	0.694

Diets formulated by software Ração de Lucro Máximo 3.2® (RLM) ESALQ-USP.

¹Offered between 1° to 50° day.

²Offered between 51° to 120° day.

³Composition by quilogram: calcium 188 g; cobalt 24 mg; copper 720 mg; sulfur 74 g; fluorine 240 mg; phosphorus 24 g; iodine 40 mg; magnesium 30 g; manganese 1500 mg; selenium 8 mg; sodium 60 g; zinc 2080 mg; sodium monensin 1820 mg.

2.2. Sample collection

After 120 days of feeding, the animals were harvested (480.58 and 516.11 kg of final live weight for N and RGN respectively) at the slaughterhouse from the University of Sao Paulo in Pirassununga, SP, Brazil, supervised by the State Inspection Service. All samples were collected between 10th and 13th rib from *longissimus* muscle of each animal. Samples for RNA extraction and protein abundance (1 hour) were collected immediately after slaughter, and frozen in liquid nitrogen and subsequently kept at -80 °C in freezer.

Samples (2.5 cm thick) used for shear force (SF), cooking loss (CL), color analysis were collected 24 h after carcass cooling, individually vacuum-packed, and kept at 4 °C (aging period) by zero (24 hours *post mortem*), 7, 14 and 21 days, and then stored in -20 °C freezer. Samples for protein abundance were collected at 7, 14 and 21 days of aging and kept at -80 °C. Eight animals for each genetic group were randomly selected for gene expression and protein abundance analysis.

2.3. Shear Force and Cooking Loss Analysis

Aged samples by zero, 7, 14 and 21 days for shear force were thawed (4 °C) for 24 h and roasted in electric oven (Model F130 / L - Fornos Elétricos Flecha de Ouro Ind. E Com. Ltda, Sao Paulo, Brazil) at 180 °C until they reached an internal temperature of 71 °C, monitored by a digital thermometer inserted into the geometric center of each sample. The cooked samples were cooled at room temperature, and then individually wrapped in plastic film and stored over night at 4 °C. Six cylinders of each sample (1.27 cm in diameter) were removed in the muscle fibers direction using a bench drill (Ferrari FG13- 350W).

The cylinders were sheared transversely (across the predominant muscle fiber orientation) by Warner-Bratzler blade coupled to a Brookfield® CT-3 Texture Analyzer Texturometer (Brookfield, USA), according to methodology proposed by Wheeler, Shackelford and Koohmaraie (2001). Cooking loss (CL) was determined by the difference between the weight of sample before and after cooking, and the result was expressed in percentage.

2.4. Color

The samples were removed from the vacuum package and exposed to atmospheric air for 30 min at 4 °C to myoglobin oxygenation (blooming). After that, color reading was performed on the surface of steaks at each aging time using a portable colorimeter (mod. MiniScan EZ, Hunter Lab) with light source of D65, 10° standard observer and 30 mm of aperture, using the CIE L*, a*, b* system. Meat color parameters were lightness (L* from black to white), redness (a* from green to red) and yellowness (b* from blue to yellow) (HOUBEN et al., 2000).

2.5. Total RNA Extraction and cDNA Preparation

Samples from *longissimus* muscle were macerated and homogenized for total RNA extraction. Total RNA was extracted using Trizol® reagent according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Total RNA concentration was quantified using NanoDrop spectrophotometer (Thermo Scientific, Washington, DE). RNA samples were treated with DNase I (Invitrogen Life Technologies, Carlsbad, CA) prior to reverse transcription reaction. cDNA was produced using 30 ng of total RNA per analyzed gene using High Capacity kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The mRNA

levels were analyzed by quantitative real time polymerase chain reaction (qRT-PCR) using gene-specific primers (Table 2). Primers efficiency were obtained using different cDNA concentrations (5, 15, 30, 60 ng/μL).

Table 2. Genes and their respectively sequences of the *primers* used in this study

Gene	Abreviation		Sequence 5'-3'
Myostatin ¹	<i>MSTN</i>	Forward	TTGTGCACCAAGCAAACCCC
		Reverse	CCATGGCTGGAATCTTCCCGTA
Transforming growth β ²	<i>TGFβ</i>	Forward	AGCCAGGGGGATGTGCCA
		Reverse	TAGCACGCGGGTGACCTCCT
Calpastatin ³	<i>CAST</i>	Forward	GTCGGATCCAATGAGTTCTACC
		Reverse	CCTGCGATCCCTTCTTCTTTAT
Calpain 1 ¹	<i>CAPN1</i>	Forward	GACCATAGGCTTCGCTGTCT
		Reverse	AGGTTGATGAACTGCTCGGA
Calpain 2 ⁴	<i>CAPN2</i>	Forward	CGACTGGAGACACTGTTTCAGGA
		Reverse	CTTCAGGCAGATTGGTTATCACTT
Glyceraldehyde-3- phosphate	<i>GAPDH</i>	Forward	AGATAGCCGTAACCTTCTGTGC
		Reverse	ACGATGTCCACTTTGCCAG
β-Actin ⁶	<i>β-Actina</i>	Forward	CAGCAGATGTGGATCAGCAAGC
		Reverse	AACGCAGCTAACAGTCCGCC

¹ Designed by authors, ² Duarte et al 2013, ³ Martins et al. (2017), ⁴ Riggs et al. (2015), ⁵ Martins et al. (2015) and ⁶ Sangalli et al. (2014).

2.6. Gene Expression

Transcripts abundance were analyzed by QuantStudio 6 Real-Time PCR (Applied Biosystems) using SYBR Green RT-PCR Master Mix (GoTaq® Promega), following the cycle parameters: 95 ° C for 2 min, 45 cycles at 95 ° C for 15 s, and 60 ° C for 1 min. In each reaction were used 5 μL of SYBR Green RT-PCR Master Mix, 1.5 μL of each forward and reverse primer, 1 μL of cDNA (30ng of RNA) and MilliQ (ultrapure) water to complete the volume of 10 μL. After amplification, a melting curve (0.01 C / s) was used to confirm the purity of the product. The “thereshold cycle” (Ct) values were normalized (ΔCt) based on the geometric mean Cts of the values obtained for GAPDH and β actin. Relative levels of gene expression were calculated and represented as 2^{-ΔCt}.

2.7. Protein Abundance

For protein extraction, samples frozen at -80 °C were macerated and homogenized in 0.4 mL of Ripa buffer (150 mM sodium chloride, 1% Triton X-100, 0.5%

sodium deoxycholate, 0.1% SDS, 50 mM Tris - pH 8.0) with addition of 1% protease inhibitor MIX, 1 mM sodium orthovanadate, and 25 mM sodium fluoride in a tissue homogenizer (UltraStirrer, model BIOMT30K). Upon protein extraction samples were centrifuged at 13200 g for 30 min at 4 °C and then the supernatant was collected. Protein quantification was performed using Bradford Protein Assay reagent (# 500-0006, Bio Rad®, Hercules, CA), with 2mg/mL bovine albumin as solution standard.

For electrophoresis, aliquots with 50 µg of proteins were diluted 1:1 in Laemmli 2x buffer (#161-0737, Bio Rad®, Hercules, CA) plus 1% β mercaptoethanol. Samples were incubated at 95 °C for 5 min, and then applied to the 12% SDS-PAGE gel with a 4% stacking gel. A running buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS (# 161-0732, Bio Rad®, Hercules, CA) was used and the run was performed at 100 V for 2 hours.

Electrotransference was performed by wet transfer method using the transfer buffer (#161-0734, Bio Rad®, Hercules, CA) and PVDF membrane (#162-0177, Bio Rad®, Hercules, CA). The transfer occurred at 4 °C with magnetic stirring during 2 h at 100 V. To verify the complete protein transfer from gel to PVDF membrane was used the method of membrane staining with Ponceau S. Membranes were blocked in TBS-T (#170-6435, #170-6531, Bio Rad®, Hercules, CA) containing 5% of bovine albumin for 2 h at room temperature, and then washed with TBS-T for 5 min, 3 times. Membranes were incubated with specific primary antibody diluted in TBS-T with 2% of bovine albumin over-night at 4 °C. Conditions retained and suppliers for all primary antibodies dilutions are reported in Table 3.

Table 3. Primary antibodies dilutions used in this study

Protein	Product	Dilution
Calpain 1	#2556 Cell Sign	1:1,000
Troponin-T	T6277 Sigma	1:3,000
HSP 70	SC66048 Santa Cruz	1:500
HSP 27	SC13132 Santa Cruz	1:1,000
Myostatin	Ab201954 AbCam	1:2,000

After incubation with primary antibody, the membranes were washed 3 times with TBS-T (5 min per wash) and were then incubated with the appropriate secondary antibody for 1 h at room temperature with low stirring. Secondary antibodies were diluted in TBS-T with 2% of bovine albumin as follows: Anti-mouse IgG secondary antibodies

(A2554, Sigma®) were diluted 1:2,000 for HSP70, HSP27 and myostatin, while for troponin-T was diluted 1:3,000. The Anti-rabbit secondary antibody (A0545, Sigma®) was diluted in 1:5,000 for calpain 1. After incubation with secondary antibody the membranes were washed with TBS-T (3 times for 5 min per wash). Bands were visualized using Clarity Western ECL substrate (#170-5060, Bio-Rad®, Hercules, CA). The signals generated from the bands were captured and quantified by the Image Lab 6.0.1 software using ChemiDoc system (Bio-Rad®, Hercules, CA).

To account for potential variation in protein loading, intensities of protein bands (%) were expressed relative to the total intensities of all the protein bands within the lane for calpain, troponin-T and HSP27 proteins, while that for HSP70 and myostatin proteins, the total intensities were calculated as the ratio of sample band to reference pool present in each membrane.

2.8. Statistical analysis

The experimental design was completely randomized with 16 repetitions per treatment, each animal being considered an experimental unit. For the analysis of Warner Bratzler shear force, cooking losses, color and protein degradation, the data were submitted to ANOVA, and analyzed in subdivided plots, considering a 2x4 factorial arrangement. The model included genetic groups (Nelore and Rubia Gallega x Nelore) and aging time (1 hour/24 hours, 7, 14 and 21 days) and their interactions. Statistical analysis were performed using the Statistical Analysis System Version 9.3 program (SAS Institute Inc., Cary, NC). Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Means were compared using the Tukey test. For gene expression analysis, normalized data were submitted to Student's T test using the Excel OFFICCE tool.

3. RESULTS AND DISCUSSION

3.1. Meat quality traits

There was no interaction ($P > 0.05$) between genetic group and aging time for WBSF, CL, L*, a* and b*, therefore, each factor was studied separately (Table 4 and 5).

Genetic group influenced all these traits ($P \leq 0.05$; Table 4). Beef from RGN presented lower values for SF (53.05 ± 1.76 N), CL ($27.78 \pm 1.57\%$), L* (42.21 ± 0.46),

a* (17.16 ± 0.15) and b* (15.40 ± 0.16) when compared to N beef (58.84 ± 1.76 N, $29.61 \pm 1.57\%$, 43.22 ± 0.46 , 17.61 ± 0.15 , 16.09 ± 0.15 , respectively for the cited traits).

Table 4. Effect of genetic group on pH, shear force, cooking loss, and color of beef

Item	Genetic group		P value
	N	RGN	
pH	5.64 ± 0.039	5.69 ± 0.039	0.4360
SF (N)	58.84 ± 1.76^a	53.05 ± 1.76^b	0.0251
CL (%)	29.61 ± 1.57^a	27.78 ± 1.57^b	0.0043
L *	43.22 ± 0.46^a	42.21 ± 0.46^b	0.0157
a *	17.61 ± 0.15^a	17.16 ± 0.15^b	0.0365
b *	16.09 ± 0.16^a	15.40 ± 0.16^b	0.0028

Means in the same row followed by different letters are significantly different ($P \leq 0.05$).

N: Nelore; RGN: Rubia Gallega x Nelore.

SF, Warner Bratzler shear force; CL, cooking loss; L *, lightness; a *, redness; b *, yellowness.

The lower shear force value observed in RGN group can be due to the influence of double muscled present in these animals. According to Boccard (1982), Bailey et al. (1982) and Gerrard et al. (1991) meat from DM animals is relatively tender than normal cattle, due to lower collagen (connective tissue) content. Changes in connective tissue in meat from fast-growing animals may in part be derived from a small overall decrease in the percentage of total collagen, but it seems more likely that the large quantity of recently synthesized and poorly cross-linked collagen, which represents its soluble fraction, is diluting out the older fibres (ETHERINGTON, 1987). Studies by Bailey et al. (1982) further reported that not only was collagen content lower in meat from DM animals, but that it was made up of a lower proportion of stable non-reducible cross-links, making the meat more tender. Other factors such as the smaller muscle fibers in DM cattle (Bailey et al. 1982) may have associated with increased tenderness in meat.

For cooking loss, meat from RGN animals showed lower value of CL ($27.78 \pm 1.57\%$) compared to N ($29.61 \pm 1.57\%$). Chambaz et al. (2003) to evaluate different cattle breeds, observed lower CL in Limousin, Charolais and Simmental cattle when compared to Angus. Therefore, it is suggested that meat from double muscled cattle with low fat deposition retains more water between their muscle fibers. Silva (2015) and Sanchez et al. (2005) found CL values of 25.57% and 30.41%, respectively, for crossbred Rubia Gallega x Nelore, indicating that the value observed in the present study is characteristic

of this cross. It is noteworthy that CL values between 13.1% and 34.54% are considered normal for bovine species (MUCHENJE et al., 2009).

The average values of L* (lightness) and b* (yellowness) for genetic groups were above the variation considered normal for bovine species. According to Muchenje et al. (2009), the values of L* vary between 33.2 - 41 and for b* between 6.1-11.3. However, Pateiro et al. (2017), when evaluated crossbred animals Holstein x Rubia Gallega found values of 45.64 for L* and 14.76 for b*. The difference observed between genetic groups for b* may be associated with higher total lipid content in group N (2.03%) compared to RGN (1.43%; unpublished data). It is important to highlight that the mean pH value of the *longissimus* muscle after 24 hours *post mortem* was 5.64 ± 0.04 and 5.69 ± 0.04 for N and RGN group, respectively ($P > 0.05$). Values between 5.5 and 5.79 are considered normal for beef (PRIETO et al., 2018).

Shear force and color were also influenced by the aging time ($P \leq 0.05$). The results obtained according to aging days, regardless of genetic group, are shown in Table 5.

Table 5. Effect of aging time on shear force, cooking loss and color of beef from *longissimus* muscle

	Aging time				P valor
	24 hours	7 days	14 days	21 days	
SF (N)	76.79 ^a ± 3.43	53.84 ^b ± 2.35	46.87 ^c ± 2.25	46.29 ^c ± 1.76	<0.0001
CL (%)	29.90 ± 1.60	28.12 ± 1.67	28.23 ± 1.67	28.55 ± 1.58	0.1249
L*	40.60 ^b ± 0.55	43.33 ^a ± 0.55	43.66 ^a ± 0.55	43.27 ^a ± 0.55	<0.0001
a*	17.02 ^b ± 0.16	17.20 ^{ab} ± 0.26	17.80 ^a ± 0.23	17.54 ^a ± 0.18	0.0265
b*	14.76 ^b ± 0.20	15.68 ^a ± 0.27	16.39 ^a ± 0.26	16.15 ^a ± 0.15	<0.0001

Means in the same row followed by different letters are significantly different ($P \leq 0.05$).

SF, Warner Bratzler shear force; CL, cooking loss; L *, lightness; a *, redness; b *, yellowness.

The shear force values decreased during the aging from 24 hours (76.79 ± 3.43 N) to day 14 (46.87 ± 2.25 N). However, tenderness values obtained on day 14 and 21 (46.29 ± 1.76 N) were not different, suggesting that proteolytic activity in meat would be more intense until 14 days *post mortem*, regardless of the genetic group studied. Therefore, meat aged by 21 days would be unnecessary for these studied genotypes, thus reducing costs and space in the industry. On the other hand, Oliete et al. (2006) found that

beef from Rubia Gallega showed an intense decrease in shear force values from the first day to the 21st day of aging, with 7.72 kg (approximately 75.71 N) on the first day and 5.21 kg (approximately 51.10 N) on the 21st day. However, the authors report that aging intensity is reduced over time, and the last week of aging, from day 14 to day 21, it is not efficient to tenderize meat. Eilers et al. (1996), to evaluate some cattle breeds and crosses, including *Bos taurus* and *Bos indicus* breeds, concluded that the tenderness is improved in the first 12 days of aging, not necessary to aging the meat for longer time.

For color, day 14 and 21 presented higher values of L*, a* and b*, when compared to 24 hours ($P \leq 0.05$). Oliete et al. (2006) and Stanišić et al. (2012) also observed an increase in the values of a* and b* in beef over the aging time. Tullio et al. (2013) reported that L* of meat is altered by *post mortem* aging. These changes are expected due to the proteolysis processes that occur in the cellular structures, such as the Z disc disintegration, which allows the dissemination of light (HUFF-LONERGAN & LONERGAN, 2005), contributing to greater reflection in the meat. According to Muchenje et al. (2009), the values of a* may vary from 11.1 to 23.6 for bovine. It is important to emphasize that in the present study, the values of a* vary between 17.02 and 17.80; this difference between values certainly would not interfere in the consumer's decision during the purchase.

3.2. Gene expression

There was no genetic groups effect on transcript levels of calpain 1 (*CAPN1*, $P = 0.4821$), calpain 2 (*CAPN2*, $P = 0.1003$) and calpastatin (*CAST*, $P = 0.4309$; Figure 1).

Figure 1. Transcript levels of calpastatin (*CAST*; $P = 0.4309$), calpain 1 (*CAPN1*; $P = 0.4821$) and calpain 2 (*CAPN2*; $P = 0.1003$) gene in *longissimus* from Nelore and Rubia Gallega x Nelore cattle finished in feedlot

The calpain-calpastatin system is known to play a key role in meat tenderness, calpastatin (*CAST*) inhibits both calpain 1 (*CAPN1*) and calpain 2 (*CAPN2*) and requires calcium concentrations near or below those required for activate calpain (KEMP et al., 2010). In the present study, it was expected to find higher *CAST* gene expression in pure Nelore animals since higher *CAST* expression has been associated to greater inhibitory activity and greater shear force in aged beef from *Bos indicus* (GIUSTI et al., 2013; KOOHMARAIE, 1994; WHEELER et al, 1990; WRIGHT et al., 2018). However, the results reveal that the slightly lower tenderness of meat from Zebu animals is probably not a result of lower expression of genes encoding proteases (*CAPN1* and *CAPN2*), nor due to the increased expression of *CAST*, an inhibitor of calpains, which was also no different between genetic groups. This assumption is reinforced by the results obtained by Whipple et al. (1990), whom found higher enzymatic activity of calpastatin in Zebu animals when compared to Taurine, but there were no differences in activity of the enzyme calpain in the two genetic groups.

It is known that there are other proteolytic systems besides calpain-calpastatin that may be involved in meat tenderness (OUALI et al., 2006), such as caspase systems

and heat shock proteins (HSP; OUALI et al., 2013; SACCÀ et al., 2018). In addition, Moloto et al. (2017) emphasized that the complexity of the tenderness process and the different metabolic pathways involved in muscle-to-meat transformation lead to the difficulty of identifying markers for meat tenderness. Moreover, Martins et al. (2017) assessing the proteolysis of Angus and Nelore cattle, observed that the higher proteolysis in *longissimus* muscle of Angus compared to Nelore cattle was not caused by differences in the expression of genes that encoding calpastatin or calpain, nor due to the abundance of these enzymes, but due to the higher calpastatin activity in skeletal muscle of Nelore compared to Angus animals. Therefore, our study indicate that differences found in shear force values between genetic groups involve others complex systems and only the gene expression from calpain-calpastatin proteolytic system is not able to reply our results.

For the transforming growth factor β (*TGF β*), the expression of this gene tended ($P = 0.0847$) an increasing in levels of transcript in RGN when compared to N (Figure 2). According to Kollias and McDermott (2008), *TGF β* superfamily members have been shown to have potent effects on both muscle development and postnatal skeletal muscle mass. Moreover, there is integration between *TGF β* and myostatin with skeletal muscle-specific transcriptional regulators to control gene expression, which is important to understand how these growth factors modulate the muscle phenotype during development and regeneration.

Figure 2. Transcript levels of transforming growth factor beta (*TGF β*) gene in *longissimus* from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.0847$)

Regarding the myostatin gene (*MSTN*), RGN cattle showed an increase in *MSTN* transcript levels ($P = 0.0434$; Figure 3) when compared to N. Myostatin acts as a negative regulator of myogenesis, besides maintaining the tissue homeostasis in adults (McPHERRON, LAWLER & LEE, 1997; PENG et al., 2013). Bass et al. (1999), Oldham et al. (2001) and Berry et al. (2002) observed higher expression of myostatin mRNA in muscle of double muscle cattle carrying the myostatin mutation. The same authors associated increased transcription of the myostatin locus with nonfunctional myostatin protein in these animals. Subsequently, Forbes et al. (2006) reported that increasing levels of active myostatin protein decrease its own expression and promoter activity, representing a classic example of negative feedback. However, the same does not occur when there is non-functional myostatin, allowing greater expression of this gene. In addition, Wheeler et al. (2001) related the greater tenderness in DM animals with inactivated myostatin protein. Therefore, such as previously mentioned, genes related to calpain-calpastatin proteolytic system would not be the best to explain the differences observed in shear force between these genetic groups. Other factors, like genes involved in muscle growth, such as myostatin could account for this difference in meat tenderness.

Figure 3. Transcript levels of myostatin (*MSTN*) gene in *longissimus* from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.0434$)

The results of *TGFβ* and *MSTN* for RGN cattle suggest that *TGFβ* gene probably affected the expression of myostatin in skeletal muscle. Jank et al. (2006), to study the polymorphism of the *MSTN* gene, in double muscle cattle with different genotypes, also observed similar results for *TGFβ* and *MSTN* expression. In the present study, increase transcript levels were already expected in RGN, since there is a close interdependence between *TGFβ* and *MSTN* expression (both belonging to the TGFβ protein superfamily) showing strong structural and functional similarities in bovine skeletal muscle (JANK et al., 2006; BUDASZ-ŚWIDERSKA et al., 2005). Thus, both *TGFβ* and *MSTN* are involved in regulating skeletal muscle growth and muscle catabolism (BUDASZ-ŚWIDERSKA et al., 2005). Observing changes in these genes may provide useful insights into the common and divergent aspects of intracellular signaling of these pathways. Thus, the manipulation of *TGFβ* and *MSTN* signaling is considerable for muscle mass modulation in cattle (BUDASZ-ŚWIDERSKA et al., 2005). However, according to Kollias and McDermott (2008), studies still needs to be done to fully understand the intracellular mechanisms of the TGFβ and myostatin signaling pathways in skeletal muscle.

These results in gene expression of *TGFβ* and *MSTN* are explained due to RGN progenies, which were obtained from a bull with a double muscle phenotype. According to Widyas et al. (2018), crossing between a DM and a normal animal ensures a progeny with meat production potential, higher carcass yield and better meat quality, these traits influenced by DM genetics and the transfer of mutation of *MSTN* gene to progenies.

3.3. Protein abundance

In *post mortem* muscle, three bands referring to troponin-T were detected, the intact band and more two bands from degradation of troponin-T are illustrated in figure 4.A. There was no interaction between genetic group x aging time for the intact troponin-T band ($P = 0.2732$). However, the abundance of intact troponin-T was different between genetic groups ($P = 0.0244$; Figure 4.B) and between aging time ($P < 0.0001$; Figure 4.C). Nelore cattle showed higher abundance of intact troponin-T when compared to RGN, 40.34% and 29.75%, respectively. This result corroborates with shear force results for the different genetic groups, and the lower abundance of intact troponin-T is associated with greater tenderness (MARINO et al., 2013). According to Wright et al. (2018) this protein is a reliable indicator of *post mortem* proteolysis.

Figure 4. (A) Representative Western Blot image showing the protein abundance of troponin-T in *longissimus* muscle from Rubia Gallega x Nelore (RGN) and Nelore (N) cattle over the aging time (1 hour, 7, 14 and 21 days). (B) Percentage of intact troponin-T abundance of different genetic groups ($P = 0.0244$). (C) Percentage of intact troponin-T abundance over the aging time ($P < 0.0001$), regardless of genetic group. Troponin-T degradation was determined as the ratio of each band to total of bands.

Over the aging time, we observed a reduction in the abundance of intact troponin-T at each aging interval, reaching 0.25% on the 21st day. This was due to the degradation process, which results in loss of integrity of the thin filaments, causing rupture of myofibrils, which leads to improve meat tenderness (TAYLOR et al., 1995).

There was interaction between genetic group x aging time for degraded troponin-T 1 ($P = 0.05$; Figure 5). At first hour, RGN cattle presented 14% more product of troponin-T degradation when compared to N. This result can be explained, because according to Wheeler et al. (1990), the appearance of protein band with approximately 30 kDa molecular weight (degraded troponin-T 1) is more evident in the first 14 days *post mortem* for *Bos taurus* meat than *Bos indicus*. In addition, this 30 kDa fragment has been indicated as a tenderness marker during meat aging (HUANG et al., 2012; PRATES et al., 2001).

Figure 5. Percentage of degraded 1 troponin-T abundance, interaction between genetic group and aging time ($P = 0.05$). Troponin-T degradation was determined as the ratio of each band to total of bands.

There was no interaction between genetic group x aging time ($P = 0.8572$) for degraded troponin-T 2. Neither for the genetic group ($P = 0.2002$). However, the abundance of degraded troponin-T 2 increased over the aging, regardless of the genetic group ($P < 0.0001$; Figure 6), reaching an abundance average of 86.60% on the 21st day. Under *post mortem* conditions, troponin-T is a substrate for calpain 1 and its product of degradation is identified as a sequence of polypeptides of approximately 30 kDa and lower weight 28 kDa (degraded troponin-T 2) after the aging period (HO, STROMER & ROBSON, 1994). The appearance of these polypeptides was considered an excellent indicator of the tenderization process of beef during aging (PENNY & DRANSFIELD, 1979). These results of troponin degradation are consistent with shear force results, which presented lower values for RGN than N, and decreasing over the aging time until day 14. Thus, troponin-T may be more reliable at predicting shear force when proteolysis is significantly decreased.

Figure 6. Percentage of degraded 2 troponin-T abundance over the aging time ($P < 0.0001$), regardless of genetic group. Troponin-T degradation was determined as the ratio of each band to total of bands.

The calpain 1 autolysis did not present interaction between genetic group x aging time ($P = 0.5035$). The genetic group also did not influence this trait ($P = 0.9854$; Figures 7.B and 7.D), corroborating with result obtained in the *CAPNI* gene expression. It was expected to find difference in the calpain 1 autolysis between genetic group, since Wheeler et al. (1990) found a lower calpain 1 protein expression and higher calpastatin protein expression in Brahman (*Bos indicus*) when compared to Hereford (*Bos taurus*), which resulted in lower meat tenderness in *Bos indicus* animals, because calpastatin is a calpain inhibitor.

Many authors cited the calpain-calpastatin system as a major contributor to meat tenderness (BHAT et al., 2018; DELGADO et al., 2001; KOOHMARAIE, 1992; KOOHMARAIE & GEESINK, 2006). On the other hand, other studies have shown that there is a set of physicochemical factors such as pH and cooling, enzymatic systems such as caspases, serine peptidases, proteasome, cathepsins, matrix metalloproteases, among others, and heat shock proteins that influence meat tenderness (SENTANDREU et al., 2002 ; OUALI et al., 2006; 2013). Therefore, in this study, we indicate that the difference found in the shear force between genetic groups cannot be justified by this enzyme.

The abundance of intact calpain 1 and autolysed calpain 1 were different for aging time ($P < 0.0001$; Figures 7.C and 7.E). Our data demonstrated that calpain 1

autolysis occurred more efficiently in the first 7 days *post mortem*, after this period there were no differences in the abundance of intact calpain 1 and autolysed calpain 1 bands. According to our results, Bhat et al. (2018) also reported that the autolysis of calpain 1 and loss of most of the activity to occur within 7 days *post mortem* whereas calpain 2 activity did not decrease in lamb *Biceps femoris* up to 56 days *post mortem*.

Figure 7. (A) Representative Western Blot image showing the protein abundance of calpain 1 in *longissimus* muscle from Nelore (N) and Rubia Gallega x Nelore (RGN) cattle over the aging time (1 hour, 7, 14 and 21 days). (B) Percentage of calpain 1 abundance of different genetic groups ($P = 0.9854$). (C) Percentage of calpain 1 abundance over the aging time ($P < 0.0001$), regardless of genetic group. (D) Percentage of calpain 1 autolyzed abundance of different genetic groups ($P = 0.9854$). (E) Percentage of calpain 1 autolyzed abundance over the aging time ($P < 0.0001$), regardless of genetic group. Calpain 1 autolysis was determined as the ratio of each band to total of bands.

There was interaction between genetic group x aging time for degradation of HSP 27 ($P = 0.0006$; Figure 8). At first hour, the abundance of HSP 27 and degraded HSP 27 was equal for both genetic groups. However, between day zero and 7, RGN cattle presented intense degradation of HSP 27 when compared to N, however this degradation process does not extend over the days, and no difference in degradation was observed between day 7 and 21 for the RGN group. On the other hand, group N showed low degradation between periods 0 - 7 and 14 - 21. Based on that, HSP 27 degradation is more intense and efficient during the early days of aging in RGN than N cattle. Therefore, we can suggest that RGN cattle showed greater ability to degrade myofibrillar proteins, since there was less abundance of intact HSP 27 in RGN, favoring meat tenderness, which was observed with the lowest shear force value for this genetic group.

(C)

Figure 8. (A) Representative Western Blot image showing the protein abundance of HSP 27 in *longissimus* muscle from Nelore (N) and Rubia Gallega x Nelore (RGN) cattle over the aging time (1 hour, 7, 14 and 21 days). (B) Percentage of HSP 27 abundance interaction between genetic group and aging time ($P = 0.0006$). (C) Percentage of degraded HSP 27 abundance interaction between genetic group and aging time ($P = 0.0006$). HSP 27 degradation was determined as the ratio of each band to total of bands.

Heat shock proteins (HSPs) are anti-apoptotic chaperone proteins. The activity or expression of HSPs increases dramatically to preserve cellular proteins from denaturation or oxidative stress-related damage (KÜLTZ, 2003). Results from previous proteomic studies indicate that different levels of chaperone protein expression, specifically small HSPs (20 and 27) may have implications for variability in meat quality characteristics such as tenderness, color, water holding capacity, juiciness and flavor. (JOSEPH et al., 2012; KIM et al., 2008; PICARD & GAGAOUA, 2017; ZHAO et al., 2012). In addition, their possible involvement in proteolytic systems delays meat aging (LOMIWES et al., 2014; OUALI et al., 2006; PICARD & GAGAOUA, 2017).

Regarding the abundance of HSP 70, there was no interaction between genetic group x aging time ($P = 0.4508$). However, RGN genetic group tended to present lower HSP70 abundance ($P = 0.0781$; Figure 9.B). In relation to aging time, HSP70 abundance remained constant from first hour to day 14, but showed a 32% increase in abundance between day 14 and 21 *post mortem* ($P < 0.0001$; Figure 9.C).

Figure 9. (A) Representative Western Blot image showing the protein abundance of HSP 70 in *longissimus* muscle from Nelore (N) and Rubia Gallega x Nelore (RGN) cattle over the aging time (1 hour, 7, 14 and 21 days). (B) Percentage of HSP 70 abundance of different genetic groups ($P = 0.0781$). (C) Percentage of HSP 70 abundance over the aging time ($P = 0.0001$). HSP 70 degradation was determined as the ratio of sample band to reference pool presents in each membrane.

Although HSPs have anti-apoptotic functions, researchers have reported differences in expression between HSP27 and HSP70. Some authors indicate to increases in HSP27 abundance in less tender meat (KIM et al., 2008; PULFORD et al., 2008; 2009) and increases in HSP70 abundance in more tender meat (PICARD et al., 2010, 2014). Moncau et al. (2018) to evaluate F1 Simmental South African x Nelore cattle, observed the same behavior for HSPs 27 and 70, with reduction and increase in concentration over aging time, respectively. We suggest that the increase in HSP70 abundance over the aging time can be attributed to HSP90 degradation products, since the antibody used recognizes a sequence of common amino acids among the isoforms. Our results corroborate previous researches, suggesting that HSP 27 and HSP 70 could be markers to tenderness. However, further studies should be conducted to elucidate the mechanisms of HSP 27 and 70.

Myostatin protein was not differently abundant between genetic groups ($P = 0.3489$; Figure 10), unlike the observed in gene expression analysis. Because post-transcriptional modifications may occur, differences (or lack thereof) in gene expression may not lead to differences in protein abundance. According to Schwanhäusser et al. (2011), about 40% of the variance in protein level is explained by relative mRNA

expression. Therefore, a possible contribution of the myostatin gene and / or protein in the shear force difference between genetic groups is suggested. Due to gene that controls the proliferation of myoblasts and muscle fiber formation, its overexpression leads to muscular hyperplasia, which is closely related to calpastatin expression. Therefore, some research groups have reported the genetic polymorphisms that could be associated with meat tenderness.

According to Shackelford et al. 1994, the contribution of myofibrillar tenderization to the increased tenderness in meat from double-musced cattle is not fully understood. However, further investigations should be conducted in order to gain a better understanding of the possible mutation types and their implications on meat quality.

Figure 10. (A) Representative Western Blot image showing the protein abundance of myostatin in *longissimus* muscle from Nelore (N) and Rubia Gallega x Nelore (RGN) cattle at zero day of aging. (B) Percentage of myostatin abundance of different genetic groups ($P = 0.3489$). Myostatin expression was determined as the ratio of sample band to reference pool presents in each membrane

4. CONCLUSION

Despite crossing between Rubia Gallega and Nelore breeds provide a more tender meat with less cooking loss, the observed differences may not be perceptible to the consumer.

However, the variation found in tenderness values can be assigned to results of *TGFβ* and *MSTN* gene expression, indicating that these two genes may participate in the formation of muscle fibers, with a different profile in Rubia Gallega x Nelore cattle, may interfere in meat tenderness of this genetic group. In addition, it is evident that heat shock proteins (*HSP* 27 and 70) have an intense contribution to tenderness of Rubia Gallega x Nelore meat, and troponin-T degradation is a potential indicator of tenderness.

Further studies are needed to confirm the possible use of these biomarkers in the meat industry. Once, the expression of *CAPN1*, *CAPN2* and *CAST* genes are not good indicators to tenderness in these genetic groups evaluated.

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CAPÍTULO

3. Artigo a ser submetido à revista científica

**Fatty acid composition and gene expression involved in lipid metabolism from
Nelore and Rubia Gallega x Nelore cattle**

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ABSTRACT

The objective was to evaluate the fatty acid composition of crossbred Rubia Gallega x Nelore compared to Nelore cattle, and understand the differences on lipid composition by biochemical and metabolic parameters and expression of genes related to lipid metabolism. Sixteen Nelore (N) and 16 crossbred Rubia Gallega x Nelore (RGN), non-castrated males, with an average age of 11 months and $280 \text{ kg} \pm 15 \text{ kg}$ of initial live weight were used. Animals were harvested after 120 days of feedlot finishing. Nelore cattle showed higher total lipids content, higher concentrations of hypercholesterolemic fatty acids, 14:0 and 16:0, and higher activity of $\Delta 9$ desaturase enzyme ($P \leq 0.05$). While RGN group presented higher total polyunsaturated fatty acid (Σ PUFA) and better health index ($P \leq 0.05$). In addition, RGN animals had lower serum total cholesterol, HDL and LDL concentration, when compared to N ($P \leq 0.05$). Regarding to hormones, RGN group presented higher IGF-1 and lower insulin ($P \leq 0.05$) than N. Furthermore, RGN group presented higher *LPL* ($P \leq 0.05$) and subtle increasing *ACOX* genes expression levels ($P = 0.0998$). Our results indicate that Rubia Gallega x Nelore cattle provide healthier meat for consumption, besides indicating greater lipid *turnover*.

Keywords: *Bos taurus*, *Bos indicus*, total lipids, cholesterol, IGF-1, insulin.

1. INTRODUCTION

In recent years, the demand for animal products with great quality for human consumption has increased, especially in relation to nutritional quality. Nutritionists recommend moderate intake of total fat and higher intake of polyunsaturated fatty acids (PUFA), especially omega 3 (n3) PUFA, necessary for embryo growth and development, capable of presenting cardioprotective effects and reducing the risk of certain diseases (EUROPEAN FOOD SAFETY AUTHORITY, EFSA, 2010; RIEDIGER, OTHMAN, SUH, & MOGHADASIAN, 2009).

However, the content and quality of fatty acid (FA) composition of beef are dependent on the lipid metabolism that occurs throughout the animal's life (HAUSMAN et al., 2009). Thus, diet, as well as sexual condition, age at slaughter, growth rate and genetics are factors that influence the FA composition of beef (PITCHFORD et al., 2002; WOODS & FEARON, 2009; CESAR et al., 2014; 2015 and 2016; LADEIRA et al., 2016; ABOUJAOUDE et al., 2006).

Several authors have reported the genetic as a strategy in modifying the FA profile of ruminant meat (PITCHFORD et al., 2002; DE SMET, RAES & DEMEYER, 2004; ALDAI et al., 2006; EKINE-DZIVENU et al., 2014). Therefore, genetic may influence the activities involved in lipid metabolism, gene expression or enzymatic activity, including elongases and desaturases (SCOLLAN et al., 2006; BARTOŇ et al., 2007; OOSTERVEER et al., 2009; GAMA et al., 2013; GRUFFAT et al., 2013). Furthermore, growth factors present in the blood, such as insulin, insulin-like growth factor (IGF-I), growth hormone, thyroid hormones, as well as vitamins (PYATT et al., 2005), are needed to stimulate proliferation of pre adipocytes (HAUSMAN et al., 2009), collaborating with changes in beef composition.

According to De Smet, Raes & Demeyer (2004), genetic groups with a lower rate of intramuscular fat deposition display beef with higher PUFA content, due to fat be mainly composed of phospholipids. In addition, beef from double muscled cattle has a lower saturated fatty acids (SFA) content and a more favorable PUFA/SFA ratio than meat from other genotypes (FIEMS, 2012). Several beef breeds are characterized by the double muscling phenomenon, such as Belgian Blue, Piedmontese, Charolais, Asturiana de los Valles, Marchigiana, and Rubia Gallega (FIEMS et al., 2012; MIRANDA et al., 2002).

Rubia Gallega (*Bos taurus*) breed has been used to cross with Nelore (*Bos indicus*) breed in Brazil to improve the quantitative and qualitative carcass traits

(ALBERTÍ et al., 2005). These animals have a high growth rate and low genetic predisposition to fat deposition (ALBERTÍ et al., 2005; MONSERRAT & SÁNCHEZ, 2000), providing fatty acid composition more favorable for human consumption. Nevertheless, there are studies in the literature that demonstrated that Nelore beef is also considered desirable in relation to human health (FEITOSA et al., 2016; ROSSATO et al., 2010).

Hence, researches have been conducted to understand the influence of genes expression involved in adipogenesis and lipogenesis of ruminants between subspecies and with different beef fatty profile (GRUFFAT et al., 2013; TEIXEIRA et al., 2017), displaying differences on beef fatty acid profile. The level expression of adipogenic and lipogenic genes is known to be regulated by several transcription factors (MANNEN, 2011), whose differential expression is known to play a fundamental role in lipid metabolism of bovine adipocytes (TANIGUCHI et al., 2008).

In the present study, we evaluated the expression of genes that encode key proteins involved in biosynthesis, fat mobilization and fatty acid oxidation in *Bos indicus* and cross *Bos indicus* x *Bos taurus* animals, such as *C/EBPα*, *ZFP423*, *ACC*, *LPL*, *FABP4*, *LEP*, *SCD* and *ACOX*. Therefore, the objective of this study was to evaluate the fatty acid composition of crossbred Rubia Gallega x Nelore compared to Nelore cattle, and understand the differences on lipid composition by biochemical and metabolic parameters and expression of genes related to lipid metabolism.

2. MATERIAL AND METHODS

The experiment was conducted at the feedlot of the University of Sao Paulo, located in Pirassununga, Sao Paulo, Brazil. All experimental procedures involving animals were approved by the Animal Ethics Committee of the College of Animal Science and Food Engineering, University of Sao Paulo, protocol number 2502020518.

2.1. Animal and Management

Sixteen Nelore bulls (N) and sixteen crossbred Rubia Gallega x Nelore bulls (RGN) with initial body weight 280 ± 15 kg and 11 months of age were used. Each genetic group, N and RGN, were from a single bull Nelore and Rubia Gallega, respectively. The Rubia Gallega bull used at the crossbreeding was homozygous for myostatin gene. These animals were housed in pens with two animals, according to genetic group. Animals were adapted to diet during twenty-one days. They were fed *ad libitum* with the same diet with

a forage:concentrate ratio of 30:70 in finishing diet 1 during 50 days, then ratio of 85:15 in finishing diet 2 by 70 days (Table 1).

Table 1. Composition of experimental diets (% Dry Matter)

Ingredient	Finishing 1¹	Finishing 2²
Corn silage	21.00	4.400
Sugarcane bagasse	9.000	10.00
Ground corn	55.80	61.00
Soybean meal 45%	10.00	1.150
Whole cottonseed	-	20.00
Mineral mix ³	2.000	1.250
Urea	1.300	0.800
Potassium chloride	0.300	0.600
Calcareous	0.600	0.800
Composition		
Dry matter (% natural matter)	61.53	77.17
Crude protein (% DM)	15.53	13.25
Total digestible nutrient	73.40	78.88
Neutral detergent fiber (% DM)	17.87	16.14
Acid detergent fiber (% DM)	0.595	0.694

Diets formulated by software Ração de Lucro Máximo 3.2® (RLM) ESALQ-USP.

¹Offered between 1^o to 50^o day.

²Offered between 51^o to 120^o day.

³Composition by quilogram: calcium 188 g; cobalt 24 mg; copper 720 mg; sulfur 74 g; fluorine 240 mg; phosphorus 24 g; iodine 40 mg; magnesium 30 g; manganese 1500 mg; selenium 8 mg; sodium 60 g; zinc 2080 mg; sodium monensin 1820 mg.

2.2. Sample collection

After 120 days of feeding, the animals were harvested (480.58 and 516.11 kg of final live weight for N and RGN respectively) at the slaughterhouse from the University of Sao Paulo in Pirassununga, SP, Brazil, supervised by the State Inspection Service. During slaughter, blood samples were collected from animals to measure biochemical and metabolic parameters (glucose, triglycerides, total cholesterol, HDL, LDL, VLDL, insulin, leptin, GH, and IGF-1). Samples for RNA extraction were collected between 10th and 13th rib from *longissimus* muscle of each animal, immediately after slaughter, and frozen in liquid nitrogen and subsequently kept at -80 °C in freezer. Eight animals from each genetic group were randomly selected for gene expression analysis.

Samples used for fatty acid profile composition and total lipids analysis were obtained between 10th and 13th rib *longissimus* muscle at 24 h after carcass cooling, in a vacuum package, and kept at -80°C freezer.

2.3. Total lipids and fatty acid composition

The total lipids were determined in fresh samples following the methodology of Bligh & Dyer (1959) in duplicate. Meat fatty acids were extracted from the intramuscular fat of the *longissimus* muscle as described by Folch, Lees, & Sloane Stanley (1957). Aliquots of muscle lipids were methylated separately using base (0.5 N sodium methoxide) and acid (5% methanolic HCl) reagents, according to Kramer et al. (1997). The FAs were quantified by gas chromatography (GC-2010 Plus - Shimadzu AOC 20i auto-injector) with an SP-2560 capillary column (100 m $\times$ 0.25 mm diameter, 0.02 mm thick, Supelco, Bellefonte, PA). The initial temperature was 45°C with an increase ($13^{\circ}\text{C}/\text{min}$) to 175°C , which was held for 27 min before a further increase to 215°C ($4^{\circ}\text{C}/\text{min}$); the final temperature was maintained for 35 min. Hydrogen (H_2) was used as the carrier gas at $40\text{ cm}^3/\text{s}$. The temperature of the flame ionization detector (FID) was 250°C , the H_2 flow rate was $40\text{ mL}/\text{min}$, the air flow was $400\text{ mL}/\text{min}$ (synthetic air), the make-up gas flow was $30\text{ mL}/\text{min}$ kPa (N_2), and the sampling rate was 40 msec. The FAs were identified by comparing the retention times of methyl esters in the samples with those of the standards, FA C4-C24 (F.A.M.E. mix, Sigma®), GLC 463 Reference Mixture Nu Check, vaccenic acid (V038-1G, Sigma®), linoleic acid (UC-61 M 100 mg), conjugated linoleic acid (CLA) (UC-60M 100 mg, Sigma®), and tricosanoic acid (Sigma®). The FA were quantified by normalizing the area under the curve of methyl esters using GS Software Solutions (version 2.42). The FA contents were expressed as a percentage of total FA methyl ester quantified.

$\Delta 9$ desaturase activity was calculated according to Aldai et al. (2006):

$$\Delta 9 \text{ desaturase } 14 = (100 * (14:1 \text{ cis}9 / (14:1 \text{ cis}9 + 14:0)));$$

$$\Delta 9 \text{ desaturase } 16 = (100 * (16:1 \text{ cis}9 / (16:1 \text{ cis}9 + 16:0)));$$

$$\Delta 9 \text{ desaturase } 18 = (100 * (18:1 \text{ cis}9 / (18:1 \text{ cis}9 + 18:0)));$$

Index related to human health and nutritional quality were calculated according to Zhang et al. (2008), Ulbricht and Southgate (1991), and Santos-Silva, Bessa and Santos-Silva (2002).

$$\text{Health Index} = (\text{MUFA} + \text{PUFA}) / (4 * 14:0 + 16:0);$$

$$\text{Atherogenicity Index} = (12:0 + (4 * 14:0) + 16:0) / (\Sigma n6 + \Sigma n3 + \Sigma \text{MUFA});$$

Thrombogenicity index =

$$(14:0+16:0+18:0)/((0,5*\Sigma\text{MUFA})+(0,5*\Sigma n6)+(3*\Sigma n3)+n3/n6);$$

$$\text{Hypocholesterolemic/Hypercholesterolemic} = (18:1\ n9\text{cis}+18:2\ n6\text{cis}+20:4\ n6+18:3\ n3+20:5\ n3+22:5\ n3+22:6\ n3)/(14:0+16:0).$$

2.4. Biochemical and metabolic parameters

Serum concentrations of glucose, triglycerides, total cholesterol, and high density lipoprotein (HDL) were determined by immunoenzymatic procedure using Bioclin Diagnostics kits (Belo Horizonte, Brazil) in automated biochemical equipment (Mindray BS-200E), according to manufacturer's instructions. Serum values of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were calculated according to Friedewald et al. (1972), being, VLDL = triglycerides/5; and LDL = total cholesterol – (HDL + VLDL).

Insulin, leptin, GH (growth hormone), and IGF-1 (insulin-like growth factor-1) hormones were determined by Immunoenzymatic Assay method according to Biotisa Microplate Reader New Biotechnology and Sigma kits (Bioclin Quibasa, Belo Horizonte, Brazil), following manufacturer's standards.

2.5. Total RNA Extraction and cDNA Preparation

Samples of *longissimus* muscle were macerated and homogenized for total RNA extraction. Total RNA was extracted using Trizol[®] reagent according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Total RNA concentration was quantified using NanoDrop spectrophotometer (Thermo Scientific, Washington, DE). RNA samples were treated with DNase I (Invitrogen Life Technologies, Carlsbad, CA) prior to reverse transcription reaction. cDNA was produced using 30 ng of total RNA per analyzed gene using High Capacity kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The mRNA levels were analyzed by quantitative real time polymerase chain reaction (qRT-PCR) using gene-specific primers (Table 2). Primers efficiency were obtained using different cDNA concentrations (5, 15, 30, 60 ng/μL).

Table 2. Sequences of gene *primers*

Gene	Abbreviation		Sequence 5'-3'
CCAAT enhancer binding ¹	<i>C/EBPα</i>	Forward	TGCGCAAGAGCCGGGACAAG
		Reverse	ACCAGGGAGCTCTCGGGCAG
Fatty acid binding protein 4 ¹	<i>FABP4</i>	Forward	GGATGATAAGATGGTGCTGGA
		Reverse	ATCCCTTGGCTTATGCTCTCT
Acetyl-CoA carboxylase ¹	<i>ACC</i>	Forward	TGAAGAAGCAATGGATGAACC
		Reverse	TTCAGACACGGAGCCAATAA
Lipoprotein lipase ¹	<i>LPL</i>	Forward	CTCAGGACTCCCGAAGACAC
		Reverse	GTTTTGCTGCTGTGGTTGAA
Acyl-CoA oxidase ¹	<i>ACOX</i>	Forward	GCTGTCCTAAGGCGTTTGTG
		Reverse	ATGATGCTCCCCTGAAGAAA
Leptin ²	<i>LEP</i>	Forward	GGGCACGTCAGCATCTATTA
		Reverse	CCTGTCTGCTGTTATGGTCTTA
Zinc finger protein ¹	<i>Zfp423</i>	Forward	GGATTCTCCGTGACAGCA
		Reverse	TCGTCCTCATTCCTCTCTCT
Esteroil-CoA desaturase ³	<i>SCD</i>	Forward	TTATTCCGTTATGCCCTTGG
		Reverse	TTGTCATAAGGGCGGTATCC
Glyceraldehyde-3-phosphate Dehydrogenase ¹	<i>GAPDH</i>	Forward	AGATAGCCGTAACCTTCTGTGC
		Reverse	ACGATGTCCACTTTGCCAG
β -Actin ⁴	<i>β-Actin</i>	Forward	CAGCAGATGTGGATCAGCAAGC
		Reverse	AACGCAGCTAACAGTCCGCC

¹ Martins et al 2015, ² Mota et al 2017, ³ Teixeira et al 2017, ⁴ Sangalli et al. (2014).

2.6. Gene Expression

Transcripts abundance were analyzed by QuantStudio 6 Real-Time PCR (Applied Biosystems) using SYBR Green RT-PCR Master Mix (GoTaq® Promega), following the cycle parameters: 95 ° C for 2 min, 45 cycles at 95 ° C for 15 s, and 60 ° C for 1 min. In each reaction were used 5 μ L of SYBR Green RT-PCR Master Mix, 1.5 μ L of each forward and reverse primer, 1 μ L of cDNA (30ng of RNA) and MilliQ (ultrapure) water to complete the volume of 10 μ L. After amplification, a melting curve (0.01 C / s) was used to confirm the purity of the product. The “threshold cycle” (Ct) values were normalized (Δ Ct) based on the geometric mean Cts of the values obtained for GAPDH and β actin. Relative levels of gene expression were calculated and represented as $2^{-\Delta$ Ct}.

2.7. Statistical Analysis

The experimental design was completely randomized with 16 repetitions per treatment, each animal being considered an experimental unit. For the analysis of total

lipids, fatty acid composition and biochemical and metabolic parameters, the model included the fixed effects of genetic group evaluated by variance analysis, using SAS version 9.3 software (SAS Institute Inc., Cary, NC). For fatty acid composition, the model included total lipids as covariable. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. For gene expression analysis, normalized data were submitted to Student's T test using the Excel OFFICCE tool.

3. RESULTS AND DISCUSSION

3.1. Meat quality

The genetic group influenced the total lipids concentration of the beef ($P \leq 0.05$; Table 3). Nelore cattle showed higher lipid deposition (2.03 ± 0.65 %) when compared to RGN (1.43 ± 0.65 %). This result is due to low fat deposition that occurs in the Rubia Gallega breed (MONSERRAT & SÁNCHEZ, 2000). Besides that beef from double muscled animals present an amount of intramuscular fat about 1% (FIEMS, 2012). Pateiro et al. (2017) found similar values of total lipid when evaluated Holstein x Limousin and Holstein x Rubia Gallega crossbred cattle, 1.56% and 1.63%, respectively. Moreno et al. (2006) and Webb et al. (1998) reported that the increase of total lipids in beef is associated with the increase of MUFA, this information corroborates the results obtained in this study, as observed in table 3.

Tabela 3. Total lipids and fatty acid composition of *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot

Variable	Genetic Group		P value
	N	RGN	
Total lipids	2.03 ^a ±0.649	1.43 ^b ±0.647	0.0488
ΣSFA	43.33 ±0.860	44.20 ±0.860	0.4820
10:0	0.05 ±0.004	0.04 ±0.004	0.3445
12:0	0.06 ±0.003	0.06 ±0.003	0.6372
14:0	2.82 ^a ±0.096	2.27 ^b ±0.096	0.0003
16:0	23.56 ^a ±0.382	21.91 ^b ±0.382	0.0046
17:0	0.78 ±0.034	0.74 ±0.034	0.4114
18:0	15.24 ^b ±0.561	18.26 ^a ±0.561	0.0007
19:0	0.04 ±0.004	0.05 ±0.004	0.0864
20:0	0.07 ^b ±0.004	0.09 ^a ±0.004	0.0001
21:0	0.013 ±0.005	0.016 ±0.005	0.4074
22:0	0.27 ±0.036	0.29 ±0.035	0.6373

24:0	0.14 ±0.022	0.15 ±0.022	0.7794
ΣBCFA	0.42 ±0.038	0.50 ±0.038	0.0833
14:0 ISO	0.02 ±0.003	0.03 ±0.003	0.0830
15:0 ISO	0.09 ±0.008	0.10 ±0.008	0.2016
15:0 ANTEISO	0.11 ^b ±0.010	0.15 ^a ±0.010	0.0155
16:0 ISO	0.10 ±0.011	0.12 ±0.011	0.1868
17:0 ISO	0.01 ^b ±0.002	0.02 ^a ±0.002	0.0008
18:0 ISO	0.08 ±0.012	0.09 ±0.012	0.7712
ΣMUFA	46.04 ^a ±0.767	42.14 ^b ±0.767	0.0009
14:1 cis9	0.60 ^a ±0.026	0.35 ^b ±0.025	<.0001
16:1 cis9	2.73 ^a ±0.086	2.16 ^b ±0.086	<.0001
16:1 cis10	0.10 ±0.014	0.09 ±0.014	0.3017
16:1 cis11	0.15 ^a ±0.007	0.08 ^b ±0.007	<.0001
16:1 trans9	0.27 ^b ±0.011	0.32 ^a ±0.011	0.0036
15:1 cis10	0.08 ±0.018	0.08 ±0.018	0.8828
16:1 cis12 trans14	0.02 ±0.002	0.02 ±0.001	0.3456
16:1 trans10	0.03 ±0.003	0.03 ±0.003	0.7265
16:1 trans11/12	0.10 ±0.014	0.13 ±0.016	0.2017
17:1 cis9	0.53 ^a ±0.020	0.44 ^b ±0.020	0.0070
17:1 cis10	0.20 ±0.033	0.24 ±0.033	0.3682
18:1 cis12	0.27 ^b ±0.032	0.46 ^a ±0.031	<.0001
18:1 cis/trans13	0.28 ^a ±0.017	0.15 ^b ±0.016	<.0001
18:1 cis11	2.15 ±0.069	2.08 ±0.069	0.4801
18:1 cis14/trans16	0.19 ^b ±0.029	0.26 ^a ±0.029	0.0274
18:1 n9cis	34.79 ^a ±0.718	30.64 ^b ±0.718	0.0003
18:1 trans6/8	0.12 ^b ±0.012	0.20 ^a ±0.012	<.0001
18:1 trans9	0.16 ^b ±0.012	0.20 ^a ±0.012	0.0002
18:1 trans10	0.32 ^b ±0.035	0.55 ^a ±0.036	<.0001
18:1 trans11	1.00 ^b ±0.120	1.50 ^a ±0.120	0.0001
18:1 trans12	0.23 ^b ±0.022	0.44 ^a ±0.022	<.0001
19:1 cis11	0.30 ^b ±0.037	0.38 ^a ±0.038	0.0420
19:1 cis13	0.11 ±0.008	0.11 ±0.009	0.8052
19:1 cis15	0.04 ±0.003	0.04 ±0.003	0.6209
20:1 cis5	0.01 ±0.002	0.01 ±0.002	0.8261
20:1 cis8	0.04 ±0.003	0.04 ±0.003	0.5777
20:1 cis11	0.16 ±0.011	0.14 ±0.011	0.2069
22:1 n9	1.17 ±0.197	1.14 ±0.197	0.9045
ΣPUFA	6.05 ^b ±0.721	8.26 ^a ±0.721	0.0254
18:2 cis9 trans12	0.06 ±0.009	0.06 ±0.009	0.7814
18:2 n6cis	4.84 ^b ±0.650	6.93 ^a ±0.650	0.0172
18:2 n6trans	0.13 ±0.008	0.13 ±0.008	0.7219
18:2 trans8 cis13	0.05 ±0.006	0.05 ±0.007	0.6119
18:2 trans9 cis12	0.04 ^b ±0.006	0.06 ^a ±0.006	0.0185

18:3 n3	0.14 ±0.020	0.16 ±0.020	0.1630
18:3 n6y	0.03 ±0.004	0.03 ±0.004	0.3165
20:2 n6	0.04 ±0.005	0.05 ±0.005	0.2625
20:3 n3	0.01 ±0.002	0.01 ±0.003	0.5712
20:3 n9	0.11 ±0.018	0.13 ±0.018	0.4111
20:4 n3	0.03 ±0.005	0.03 ±0.005	0.8217
20:4 n6	0.05 ±0.009	0.05 ±0.009	0.9407
20:5 n3	0.011 ±0.003	0.013 ±0.003	0.9114
22:2 n6	0.006 ±0.002	0.008 ±0.002	0.5714
22:3 n3	0.03 ±0.017	0.03 ±0.016	0.8948
22:4 n6	0.14 ±0.017	0.14 ±0.017	0.9179
22:5 n3	0.33 ±0.046	0.36 ±0.045	0.6189
22:6 n3	0.06 ±0.011	0.05 ±0.011	0.7875
ΣCLA	0.049 ±0.003	0.042 ±0.003	0.1817
CLA cis9 trans11	0.025 ±0.003	0.021 ±0.003	0.2579
CLA trans10 cis12	0.021 ±0.002	0.024 ±0.002	0.1185
CLA trans8 cis10/CLA trans9 cis11	0.008 ±0.001	0.007 ±0.001	0.4628

Means in the same row followed by different letters are significantly different ($P \leq 0.05$).

N: Nelore; RGN: Rubia Gallega x Nelore; SFA, saturated fatty acid; BCFA, branched chain fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated; CLA, conjugated linoleic acid.

ΣSFA (10:0; 11:0; 12:0; 13:0; 14:0; 15:0; 16:0; 17:0; 18:0; 19:0; 20:0; 21:0; 22:0; 23:0; 24:0); ΣBCFA (14:0 ISO; 15:0 ISO; 15:0 ANTEISO; 16:0 ISO; 17:0 ISO; 18:0 ISO); ΣMUFA (14:1 cis9; 15:1 cis10; 16:1 cis7; 16:1 cis9; 16:1 cis10; 16:1 cis11; 16:1 cis12 trans14; 16:1 trans9; 16:1 trans10; 16:1 trans11/12; 16:1 trans13; 17:1 cis9; 17:1 cis10; 18:1 cis12; 18:1 cis/trans13; 18:1 cis11; 18:1 cis14/trans16; 18:1 n9cis; 18:1 trans6/8; 18:1 trans9; 18:1 trans10; 18:1 trans11; 18:1 trans12; 19:1 cis11; 19:1 cis13; 19:1 cis15; 20:1 cis5; 20:1 cis8; 20:1 cis11; 22:1 n9); ΣPUFA (18:2 cis9 trans12; 18:2 n6cis; 18:2 n6trans; 18:2 trans8 cis13; 18:2 trans9 cis12; 18:3 n3; 18:3 n6y; 20:2 n6; 20:3 n3; 20:3 n9; 20:4 n3; 20:4 n6; 20:5 n3; 22:2 n6; 22:3 n3; 22:4 n6; 22:5 n3; 22:6 n3); ΣCLA (CLA cis9 trans11; CLA trans10 cis12; CLA trans8 cis10/CLA trans9 cis11).

No differences ($P > 0.05$) were observed for total saturated fatty acids (ΣSFA; Table 3). However, the genetic group ($P \leq 0.05$) influenced some individual FA of importance to human health, such as 14:0, 16:0 and 18:0, major saturated fatty acids present in beef. Nelore cattle presented higher concentrations of 14:0 and 16:0 (2.82 ± 0.10 % and 23.56 ± 0.38 %, respectively) when compared to RGN (2.27 ± 0.10 % and 21.91 ± 0.38 %, respectively). These FA are known to have hypercholesterolemic effect, increasing the blood levels of undesirable cholesterol, and LDL (MICHA & MOZAFFARIAN, 2010). On the other hand, 18:0 was higher in RGN (18.26%) than N (15.24%). In contrast with other SFA mentioned previously, 18:0 is known to have a neutral effect on blood cholesterol concentrations, because this FA is rapidly converted into oleic acid (18:1 cis9), which has hypocholesterolemic effects (SINCLAIR, 1993; MICHA & MOZAFFARIAN, 2010). It is noteworthy that health agencies recommend reducing the intake of SFA, particularly the FA with less than 18 carbons, due to their effects on plasma LDL and cholesterol concentration and association with the risk of obesity, hypercholesterolemia and some cancers (WILLIAMS, 2000; WORLD HEALTH

ORGANIZATION, 2003; WOOD et al., 2004), besides type 2 diabetes, and cardiovascular disease (EKMEKCIOGLU et al., 2018).

There was a tendency to higher total branched chain fatty acids (Σ BCFA) in beef from RGN cattle, besides that RGN group presented higher concentration of 17:0 ISO and 15:0 ANTEISO (0.02 ± 0.002 % and 0.15 ± 0.01 %, respectively) than N (0.01 ± 0.002 % and 0.11 ± 0.01 %, respectively). BCFA are primarily components of dairy and ruminant foods, and were absent from chicken, pork, and salmon (RAN-RESSLER et al., 2014). This type of FA is important because to induce apoptosis in human breast cancer cells, and to inhibit tumor growth in cultured cells and in a mouse model (YANG et al., 2000; WONGTANGTINTHARN, OKU, IWASAKI & TODA, 2004). In addition, BCFA have beneficial role against inflammation in the premature intestine, alter the microbiota, and increase expression of anti-inflammatory cytokines in an animal model (RAN-RESSLER et al., 2014).

Beef from N group had higher concentrations of total monounsaturated fatty acids (Σ MUFA; $P \leq 0.05$), with a mean value of 46.04 ± 0.77 %. This result is due to higher concentrations of 14:1 cis9, 16:1 cis9, 16:1 cis11, 17:1 cis9 and 18:1 n9 cis found in N cattle ($P \leq 0.05$; Table 3). According to Kwon and Choi (2015), 18:1 n9 cis (oleic acid), the main monounsaturated FA in beef, can decrease circulating serum LDL concentration in humans and even increase HDL concentration (ADAMS et al., 2010; GILMORE et al., 2011; GILMORE et al., 2013), contributing to reduction of risk factors for cardiovascular disease.

On the other hand, the *longissimus* muscle from RGN showed higher concentrations for 16:1 trans9, 18:1 cis12, 18:1 cis/trans13, 18:1 cis14/trans16, 18:1 trans6/8, 18:1 trans9, 18:1 trans10, 18:1 trans11, 18:1 trans12 and 19:1 cis11 ($P \leq 0.05$). Among these MUFA, trans FA have been the most commented in studies (ALDAI et al., 2009, 2012; LEHESKA et al., 2008) due to their beneficial or harmful relation to human health. The implication of each 18:1 trans isomers in human health is not yet known. According to Scollan et al. (2006) and Lim et al. (2014), vaccenic acid (18:1 trans11) is the 18:1 trans isomer of highest concentration in beef. Nevertheless, this FA has beneficial potential for human health.

The genetic group influenced the total polyunsaturated fatty acids (Σ PUFA; $P \leq 0.05$). Beef from RGN cattle had higher concentration (8.26 ± 0.72 %), when compared to N cattle (6.05 ± 0.72 %; Table 3). Consequence of the higher concentration of 18:2 n6cis and 18:2 trans9 cis12 fatty acids in beef from RGN when compared to N ($P \leq 0.05$).

Aldai et al. (2006) observed that Asturiana de los Valles homozygous cattle of myostatin had lower MUFA proportions and higher PUFA proportions, the same behavior was obtained for RGN animals in the present study, concluding that this mutation has a measurable impact on FA composition. The lower total lipids content observed in RGN group may account for fewer and smaller adipocytes containing fewer triglycerides, accompanied by a relative increase in the proportion of phospholipids and thus a higher concentration of PUFA (DE SMET et al., 2000; LABORDE et al., 2001; NÜRNBERG et al., 1999).

The groups did not differ with regard to total CLA (Σ CLA), either for individual CLAs ($P > 0.05$; Table 3). RGN animals were expected to have a higher concentration of CLA cis9 trans11 due to the higher 18:1 trans11 concentration observed in these animals, as 18:1 trans11 is known to be the precursor of this CLA (BAUCHART et al., 2007). However, this behavior was not observed, probably due to the lower activity of the $\Delta 9$ desaturase enzyme in this group (Table 4).

Regarding the total n6 (Σ n6), RGN had higher concentration of Σ n6 ($P \leq 0.05$), with an average value of 7.46 ± 0.66 %, while N cattle presented 5.34 ± 0.66 % (Table 4). However, the total n3 (Σ n3) was not affected by the genetic groups ($P > 0.05$; Table 4). Omega 3 (n3) are important for embryo growth and development, have cardioprotective effects and reduce the risk of certain mental illnesses (EUROPEAN FOOD SAFETY AUTHORITY, EFSA, 2010; RIEDIGER, OTHMAN, SUH, & MOGHADASIAN, 2009).

Table 4. Means of $\sum n6$, $\sum n3$, $n6/n3$ ratio, PUFA/SFA ratio, $\Delta 9$ desaturase 14, 16 and 18 activity, health index (HI), atherogenicity index (AI), thrombogenicity index (TI) and Hypocholesterolemic/Hypercholesterolemic (h/H) of *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle

Variable	Genetic Group		P value
	N	RGN	
$\sum n6$	5.34 ^b ± 0.660	7.46 ^a ± 0.660	0.0206
$\sum n3$	0.55 ± 0.057	0.62 ± 0.057	0.3829
$n6/n3$	8.74 ± 0.687	10.28 ± 0.687	0.1233
PUFA/SFA	0.13 ± 0.015	0.17 ± 0.016	0.0674
$\Delta 9$ DESATURASE 14	17.47 ^a ± 0.577	13.15 ^b ± 0.559	<.0001
$\Delta 9$ DESATURASE 16	10.40 ^a ± 0.277	8.99 ^b ± 0.277	0.0010
$\Delta 9$ DESATURASE 18	69.57 ^a ± 0.739	61.99 ^b ± 0.764	<.0001
HI	1.51 ^b ± 0.046	1.64 ^a ± 0.046	0.0500
AI	0.66 ± 0.018	0.62 ± 0.017	0.1174
TI	1.52 ± 0.050	1.60 ± 0.050	0.3205
h/H	1.59 ± 0.065	1.53 ± 0.053	0.4056

Means in the same row followed by different letters are significantly different ($P \leq 0.05$).

N: Nelore; RGN: Rubia Gallega x Nelore.

$\sum n6$ (18:2 n6 trans; 18:2 cis9 trans12; 18:2 trans9 cis12; 18:2 n6 cis; 18:3 n6y; 20:2 n6; 20:4 n6; 22:2 n6; 22:4 n6); $\sum n3$ (18:3 n3; 20:3 n3; 20:4 n3; 20:5 n3; 22:3 n3; 22:5 n3; 22:6 n3);

$\Delta 9$ desaturase 14 (100*(14:1 cis9/(14:1 cis9+14:0)));

$\Delta 9$ desaturase 16 (100*(16:1 cis9/(16:1 cis9+16:0)));

$\Delta 9$ desaturase 18 (100*(18:1 cis9/(18:1 cis9+18:0)));

HI: Health Index (MUFA+PUFA)/(4*14:0+16:0);

AI: Atherogenicity Index (12:0+(4*14:0)+16:0)/($\sum n6$ + $\sum n3$ + $\sum$ MUFA);

TI: Thrombogenicity Index (14:0+16:0+18:0)/((0.5* $\sum$ MUFA)+(0.5* $\sum n6$)+(3* $\sum n3$)+ $n3/n6$);

h/H: Hypocholesterolemic/Hypercholesterolemic (18:1 n9cis+18:2 n6cis+20:4 n6+18:3 n3+20:5 n3+22:5 n3+22:6 n3)/(14:0+16:0).

There was no difference between genetic groups for $n6/n3$ ratio, ($P = 0.1233$; Table 4). This proportion has been increasing in human food during the last years (SIMOPOULOS, 2008) and this increase of $n6/n3$ ratio has been associated with risk to diseases such as prostate cancer (WILLIAMS et al., 2011) and obesity (SIMOPOULOS, 2016). The British Department of Health recommends a ratio of 4:1 for human health (BRITISH DEPARTMENT OF HEALTH, 1994). However, some authors reported higher values of $n6/n3$ ratio when compared to this study. Higher values of $n6/n3$ commonly are found in finished feedlot animals (WOOD et al., 2008), this explains ours results.

There was a tendency for PUFA/SFA ratio between the genetic groups ($P = 0.0674$; Table 4), it was observed a increasing of PUFA/SFA in beef from RGN cattle (0.17 ± 0.016) than N (0.13 ± 0.015). These results corroborate with others authors who reported that beef from double-musced cattle has a more favorable PUFA/SFA ratio than meat from other genotypes (ALDAI et al., 2006; FIEMS, 2012). It is noted that the values found in the present study were lower than the recommended for human health (≥ 0.45 ; BRITISH DEPARTAMENT OF HEALTH, 1994). Bispo et al. (2010) also observed

lower values (average of 0.19) in beef from Rubia Gallega cattle fed in intensive or semi-extensive system. According to Scollan et al. (2006), studies are performed to increase the proportion of PUFA/SFA, however the strategies used may cause changes in the n6/n3 ratio, however the PUFA/SFA ratio is not increased and normally values from 0.10 to 0.15 are observed in beef.

Genetic groups affected activity of delta 9 desaturase enzyme for FA 14:0, 16:0 and 18:0 ($P \leq 0.05$). N cattle had higher activity of desaturation to 14:0, 16:0, and 18:0 when compared to RGN cattle. According to Huerta-Leidenz et al. (1993; 1996), *Bos indicus* cattle showed higher activity of delta 9 desaturase when compared to *Bos taurus*. In addition, Pitchford et al. (2002) related a higher enzymatic activity to higher intramuscular fat content, corroborating the results observed in this study, in which higher total lipids content, MUFA and activity of delta 9 desaturase were found in *longissimus* muscle from N cattle.

Beef from RGN showed better health index ($P = 0.05$) (1.64 ± 0.05) than N cattle (1.51 ± 0.05), and this result was due to the high concentrations of 14:0 and 16:0 present in beef from N. On the other hand, there were no differences between genetic groups for atherogenicity index, thrombogenicity index and hypocholesterolemic/hypercholesterolemic ratio ($P > 0.05$). These index deserve attention, since they allow a better nutritional assessment, indicating whether the food is harmful or not to human health, inferring the risks of coronary and cardiovascular diseases, such as atherosclerosis and stroke (HOFFMAN et al., 2005; HOCQUETTE et al., 2012; TROY et al., 2016).

Thus, despite the differences found between genetic groups, for some FA, it is important to emphasize that, depending on the focus on human health, some FA were undesirable, for example the major SFA, 14:0 and 16:0, which were N beef. While, the beef from RGN would be more desirable its consumption, due to higher health index.

3.2. Biochemical and Metabolic Parameters

The genetic group did not influence serum concentrations of glucose, triglycerides and VLDL ($P > 0.05$; Table 5). However, differences in total cholesterol, HDL and LDL concentrations were observed ($P \leq 0.05$; Table 5).

Table 5. Means of serum concentrations of biochemical and metabolic parameters of Nelore and Rubia Gallega x Nelore cattle finished in feedlot

Variable	Genetic Group		P value
	N	RGN	
Glucose mg/dl	99.35 ± 5.71	107.48 ± 5.64	0.1105
Triglycerides mg/dl	31.00 ± 3.83	28.44 ± 3.83	0.4179
Total cholesterol mg/dl	242.12 ^a ± 8.11	150.67 ^b ± 8.11	<0.0001
VLDL mg/dl	6.20 ± 0.77	5.69 ± 0.77	0.4179
HDL mg/dl	67.92 ^a ± 3.27	49.66 ^b ± 3.27	0.0005
LDL mg/dl	160.69 ^a ± 6.47	93.43 ^b ± 6.25	<0.0001
Insulin ng/ml	2.94 ^a ± 0.701	1.65 ^b ± 0.714	0.0231
Leptin ng/ml	0.011 ± 0.002	0.012 ± 0.002	0.6999
GH ng/ml	0.0581 ± 0.0011	0.0584 ± 0.0011	0.7578
IGF-1 ng/ml	0.23 ^b ± 0.005	0.25 ^a ± 0.005	0.0181

Means in the same row followed by different letters are significantly different ($P \leq 0.05$).

N: Nelore; RGN: Rubia Gallega x Nelore.

In the present study, the animals received the same diets *ad libitum*, this fact contributed to similarity in the results of this parameter, not being observed difference between the genetic groups. However, glucose is captured by stimulation of the hormone insulin in peripheral tissues, contributing to glucose homeostasis. Insulin is involved in fat synthesis, being able to increase lipogenesis or reduce lipolysis and, therefore, serum insulin concentration is correlated positively with carcass adiposity (RHOADES et al., 2007). Thus, the higher total lipid concentration observed in N cattle can be explained by the insulin concentration, since they had higher serum insulin concentrations when compared to RGN ($P \leq 0.05$).

Regarding triglycerides, their synthesis usually occurs in the liver and adipose tissue, from glycerol and fatty acids (KRIS-ETHERTON & ETHERTON, 1982). There were differences for the concentration of total cholesterol, HDL and LDL between the genetic groups ($P \leq 0.05$). RGN cattle presented lower serum total cholesterol, HDL and LDL concentrations (150.67 ± 8.11 , 49.66 ± 3.27 and 93.43 ± 6.25 mg/dl, respectively) when compared to N cattle (242.12 ± 8.11 , 67.92 ± 3.27 and 160.69 ± 6.47 mg/dl, respectively). According to Claesson and Hansson (1956), serum total cholesterol concentration is directly related to lipid metabolism of cattle, besides that is a good indicator of liver function (CAVESTANY et al., 2005). This increase may be a consequence of the increased demand of cholesterol to triglyceride transport by chylomicrons (NESTEL et al., 1978). Thus, this result reflects the higher concentrations

of HDL and LDL present in N cattle. According to our results, N cattle showed higher hepatic function due to higher plasma LDL and HDL concentration, contributing to the deposition of fat in tissues, as seen in the total lipid results.

For the leptin and GH hormones, no differences were observed between the genetic groups ($P > 0.05$). Leptin is associated with body fat percentage, and it is very common to observe differences in animals with extreme weights and scores (MINTON et al., 1998). This hormone is produced specifically by adipocytes, has its function mediated through neuropeptide Y, being responsible for the body's energy homeostasis, which causes changes in food consumption and body energy expenditure (MINER, 2004). In the present study, it is suggested that leptin exerted its function by falling into the bloodstream, reaching basal levels and equalizing between genetic groups due to the function of energy homeostasis.

On the other hand, the IGF-1 hormone concentration was higher ($P = 0.0181$) in RGN (0.25 ± 0.005 ng/ml) than in N cattle (0.23 ± 0.005 ng/ml). The somatotrophic axis, also known as the GH-IGF system, consists of peptide hormones, cell surface receptors and binding proteins (DENLEY et al., 2005). This axis acts mainly in the regulation of postnatal growth, muscle development and skeletal muscle differentiation (DUAN & XU, 2005; DUAN, REN & GAO, 2010). Thus, muscle growth is mediated by activation, proliferation and differentiation of muscle cells, and is also modulated by the mitotic and myogenic activity of IGF-1 produced by muscle cells (PHILIPPOU et al., 2007). Therefore, IGF-1 stimulates cell proliferation and the metabolic process associated with protein synthesis, this is more evident in RGN group due to double muscled characteristic. In addition, research has reported that locally produced IGF-1 in muscle plays a key role in myofiber regeneration and hypertrophy (ISGAARD, 1992; PHILIPPOU et al., 2007).

This result is in accordance with the final live weight, hot carcass weight, carcass yield, and loin area results which were significantly higher for RGN cattle (516.11 kg, 290.79 kg, 57.46%, 90.19 cm², respectively) than N (480.58 kg, 275.51 kg, 56.14%, 76.44 cm², respectively) (data not yet published).

3.3. Gene Expression

The higher total lipid content found in N when compared to RGN characterized the two contrasting groups for intramuscular fat deposition (Table 3). This fact can be explained by intrinsic characteristics of the breed. Thus, it is possible that there are differences regarding the signaling for intramuscular fat deposition between the two

genetic groups studied. Therefore, these differences in total lipids content as well as fatty acid profile may be due to adipogenesis and / or intramuscular lipogenesis. Adipogenesis occurs as consequence of adipocyte hyperplasia, which involves pre-adipocyte differentiation (TAN et al., 2012). On the other hand, it may also be due to adipocyte hypertrophy without alteration in adipogenesis (ALBRECHT et al., 2011; HUFF et al., 2004; YAMADA et al., 2009).

For transcription factor *C/EBP α* , RGN cattle tended to show higher levels of transcript of this gene when compared to N ($P = 0.0569$; Figure 1). Expected to find lower levels of *C/EBP α* transcripts in RGN cattle due to lower total lipid concentration observed in this group. According to Wegner, Albrecht and Ender (1998), double muscled cattle have less developed adipose cells in muscle tissue, lower adipocyte growth rate and, consequently, smaller adipocytes in marbling, when compared to cattle without the presence of double muscle.

On the other hand, it is suggested that the highest total lipid content observed in N cattle may be related to adipocyte hypertrophy, ie, the higher adipocyte growth rate, rather than hyperplasia. In addition, adipocyte differentiation is a complex process that is regulated by a cascade of transcription factors, including factor *C/EBP α* , which acts in conjunction with *PPAR* to stimulate differentiation of mesenchymal cells to pre-adipocytes, mature adipocytes, and finally in adipocytes (ROSEN et al., 2002; WEI et al., 2005). Rosen and MacDougald (2006) demonstrated that *C/EBP α* regulates adipose tissue development in mice by binding to several specific genes of fat that are required for the synthesis, absorption and storage of long chain fatty acids. Therefore, the difference found in the expression of this single gene cannot be directly related to the total lipids result.

Duarte et al. (2013) compared intramuscular fat deposition of Wagyu and Angus cattle. The authors showed that increasing marbling in Wagyu was associated with higher expression of early markers adipogenic (*ZFP423*) and late (*PPAR γ* and *C/EBP α*) together, indicating that the increased expression of these genes involved in adipogenesis was probably responsible for the higher ether extract content in muscle from Wagyu compared to Angus.

Figure 1- Transcript levels of *C/EBPα* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.0569$)

In studies about transcription factors of adipogenesis process, *ZFP423* has been defined as a marker factor for early stages of the process, which promotes adipogenesis by increasing *PPARγ* expression (GUPTA et al., 2010; GUPTA et al., 2012). However, in the present study, there was no difference in the relative expression of the *ZFP423* gene ($P = 0.2046$; Figure 2) between the genetic groups. Similarly, Martins et al. (2015) when evaluating Angus and Nelore cattle also did not observe differences in gene expression of *ZFP423*, even animals having contrasting phenotypes for intramuscular fat. Thus, we can infer that is necessary a joint action of transcriptional factors, such as *ZFP423*, *PPARγ* and *C/EBPα*, to justify the differences found in intramuscular fat content between the genetic groups.

Figure 2- Transcript levels of *ZFP423* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.2046$)

The lipogenic enzyme, Acetyl CoA carboxylase (*ACC*), is associated with *de novo* synthesis of lipids performed in the adipose tissue from ruminant (SMITH, WITKOWSKI & JOSHI, 2003). According to Ward et al. (2010), intramuscular fat content is positively related to the gene expression *ACC* that encodes this enzyme, together with fatty acid synthase (*FAS*), responsible for the synthesis of fatty acids. However, no difference was observed in the levels of *ACC* transcripts between the evaluated genetic groups ($P = 0.2224$; Figure 3), even with higher total lipid content in beef from N cattle. Martins et al. (2015) also found no difference in *ACC* gene expression when evaluating contrasting subspecies to intramuscular fat content (Angus and Nelore).

Figure 3- Transcript levels of *ACC* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.2224$)

However, the genetic group influenced the relative expression of *LPL* ($P = 0.0098$; Figure 4). RGN animals showed higher levels of *LPL* transcripts when compared to N. The *LPL* enzyme is responsible for the hydrolysis of triglycerides from several organs to the blood circulation in free fatty acids and glycerol (HOCQUETTE, GRAULET, OLIVECRONA, 1998), which are used by muscle for energy production or stored by adipose tissue (BONNET et al., 2000). The activity of *LPL* gene in adipose tissue and the subsequent increase in triglyceride deposition are promoted by insulin (WANG & ECKEL, 2009). However, despite the higher *LPL* expression observed in RGN cattle, insulin concentration was lower when compared to N animals. Authors report that *LPL* gene activity in muscle tissue appears to be insulin dependent (RHOADES et al., 2007; RHOADES et al., 2009).

Figure 4- Transcript levels of *LPL* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.0098$)

Teixeira et al. (2017) evaluated Nelore and Angus cattle and reported higher levels of *LPL* transcripts in muscles from Nelore. In this sense, our results suggest that when comparing breeds with different levels of total lipids, *LPL* can be more expressed in tissue with lower total lipids content, as is the case of RGN. According to Berton et al. (2016), high *LPL* expression was associated with low concentration of 16:0, 18:1 cis9, n3 and PUFA/SFA. Kersten (2014) reported that PUFA has the ability to increase transcription of the genes encoding the *LPL* enzyme and fatty acid binding transporter 4 (*FABP4*). Moreover, Oliveira et al. (2014) found that *LPL* and *FABP4* levels are correlated positively with stearic acid (18:0). Therefore, our results, such as, the low 16:0 and 18:1 cis9, and high PUFA and 18:0 concentrations found in muscle from RGN animals may be associated with high transcript *LPL* level, corroborating with previously cited authors.

Despite Oliveira et al. (2014) and Kersten (2014) correlated *LPL* and *FABP4* with the same fatty acids, we did not find difference between genetic groups for the relative expression of *FABP4* gene ($P = 0.1337$; Figure 5). According to Hoashi et al. (2008), the high expression *FABP4* is associated with high palmitoleic acid (16:1) content in intramuscular fat. In this sense, we expected to find greater *FABP4* in Nelore cattle. However, fatty acid composition is a polygenic trait and researches has shown that the

fatty acid composition of cattle is regulated by many potential candidate genes (SACHEZ et al., 2007; ABE et al., 2008), the effect of each gene may be rather small.

Figure 5- Transcript levels of *FABP4* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.1337$)

RGN cattle tended to present higher levels of *LEP* transcripts when compared to N ($P = 0.0943$; Figure 6). The *LEP* gene encodes the hormone leptin and is responsible for suppressing appetite and reducing food intake in mammals, specifically modifying adipocyte metabolism to inhibit lipid accumulation and promote lipid mobilization (KERSHAW & FLIER, 2004; HARRIS, 2014). Despite this result in *LEP* gene expression, the concentration of serum leptin hormone was not influenced by genetic group. Several regulatory processes occur after mRNA expression, such as post-transcriptional, translational and protein degradation regulation, so not always, what has been expressed as mRNA, will come to final production of proteins, enzymes or hormones (VOGEL & MARCOTTE, 2012).

Figure 6- Transcript levels of *LEP* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.0943$)

There was no difference in expression of the stearoyl CoA desaturase (*SCD*) gene between genetic groups ($P = 0.1101$; Figure 7). The *SCD* gene is responsible for coding the enzyme $\Delta 9$ desaturase, the main enzyme that acts on the conversion of SFA to MUFA (MATSUISHI, FUJIMORI & OKITANI, 2001) and is also associated with the conversion of vacenic acid (C18:1 trans-11) to CLA (C18:2 cis9 trans11; SMITH et al., 2006). Higher levels of *SCD* transcripts were expected in N cattle, due to these animals presented higher values of Σ MUFA and enzymatic activity of $\Delta 9$ desaturase, however, no difference in CLA concentration was observed. However, Grygiel-Gorniak (2014) concluded that there is no strong association between the expression patterns of *SCD* gene involved in desaturation and desaturase indices ($\Delta 5$, $\Delta 6$, $\Delta 9$), making less informative its use to infer enzymatic expression/activity. Besides that, according to Sevane et al. (2014), leaner animals, such as both genetic groups studied, typically present low *SCD* activity.

Figure 7- Transcript levels of *SCD* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.1101$)

For the acyl-CoA oxidase (*ACOX*) gene, RGN cattle tended to present higher transcript level of this gene when compared to N ($P = 0.0998$; Figure 8), suggesting higher catabolism of FA by β -oxidation in these animals. *ACOX* is the first enzyme in peroxisomal fatty acid β -oxidation and plays a key role in fatty acid metabolism and fat deposition (MORAIS et al., 2007; JIAO et al., 2011). According to Teixeira et al. (2017), beef cattle with greater expression of genes related to synthesis (*ACACA* and *SCD1*), absorption (*FABP4* and *LPL*) and degradation (*CPT2*) present greater lipid *turnover*, which may be responsible for less intramuscular fat. In this sense, we suggest that RGN cattle display greater lipid *turnover* due to higher transcript levels of *LPL* and *ACOX*, evidenced by less total lipids.

Figure 8- Transcript levels of *ACOX* gene expression in *longissimus* muscle from Nelore and Rubia Gallega x Nelore cattle finished in feedlot ($P = 0.0998$)

Besides that Gruffat et al. (2013) also observed higher *ACOX* expression in muscles from Blond d'Aquitaine and Limousin bulls, with lower total lipid concentration when compared to Angus bulls. This suggest that animals with double muscle trait present increase in transcript level of this gene.

4. CONCLUSION

The beef from Rubia Gallega x Nelore cattle has better attributes for human consumption, with low total lipid content, high concentrations of polyunsaturated fatty acids and branched chain fatty acids, besides to present PUFA/SFA ratio and health index more favorable to human health. However, it is important to highlight that both breeds are producers of lean beef with low fat content.

The high muscle growth rate and low fat deposition related to Rubia Gallega cattle and their crosses were supported by high serum IGF-1 and low insulin concentration observed in Rubia Gallega x Nelore animals of this study.

Fatty acid composition is a polygenic trait, which is regulated by several potential candidate genes, the effect of each gene may be rare. Wherefore, we must be careful to observe an individually gene and characterize it as a marker. Our results displayed higher transcript levels of LPL and *ACOX* and lower total lipids content in

Rubia Gallega x Nelore cattle, indicating that these animals should have greater lipid turnover compared to Nelore.

Further analysis investigating the interaction of the genetic factors in the biochemical pathway with respect to fatty acid metabolism will bring new insight into the fat-related in beef cattle with low fat deposition.

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