UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CÂMPUS DE JABOTICABAL

PERFIL TRANSCRIPTÔMICO DO MÚSCULO *Longissimus thoracis* ASSOCIADO A CONCENTRAÇÃO DE ÁCIDOS GRAXOS EM BOVINOS NELORE TERMINADOS EM CONFINAMENTO

Gustavo Pimenta Schettini
Médico Veterinário

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Gustavo Pimenta Schettini, filho de Domingos Sávio Miranda Schettini e Sara Núbia Pimenta Schettini, nasceu em Salvador – Bahia em 12 de novembro de 1994. Iniciou o curso de Bacharelado em Medicina Veterinária na Universidade Federal da Bahia (UFBA), Escola de Medicina Veterinária e Zootecnia (EMEVZ) em maio de 2013, obtendo o título de Bacharel em Médico Veterinário em dezembro de 2018. Em março de 2019 iniciou o curso de Mestrado no Programa de Pós-Graduação em Genética e Melhoramento Animal da Universidade Estadual Paulista “Júlio de Mesquita Filho” - Faculdade de Ciências Agrárias e Veterinárias (FCAV/UNESP), câmpus Jaboticabal, sob orientação do Prof. Dr. Rogério Abdallah Curi. Foi bolsista CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) durante a realização do curso de Mestrado.

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(Albert Einstein)

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SUMÁRIO

PERFIL TRANSCRIPTÔMICO DO MÚSCULO *Longissimus thoracis* ASSOCIADO A CONCENTRAÇÃO DE ÁCIDOS GRAXOS EM BOVINOS NELORE TERMINADOS EM CONFINAMENTO

RESUMO – A carne bovina possui alto valor nutricional por fornecer nutrientes fundamentais como os ácidos graxos (AG), unidades básicas dos lipídios, que participam de vias enzimáticas e regulatórias, do armazenamento energético e de funções estruturais, além de serem fatores importantes à aceitação do produto cárneo. Os ácidos graxos saturados (AGS) estão ligados a fatores de risco associados a cardiopatias e quadros inflamatórios, já os ácidos graxos mono- (AGMI) e poli-insaturados (AGPI), como os ácidos graxos essenciais (AGE) – ácidos linoleico (AL) e alfa-linolênico (ALA), condicionam proteção. O perfil de AG na carne bovina possui caráter complexo e poligênico. A partir do sequenciamento de ácidos nucléicos foi possível identificar genes diferencialmente expressos (GDE) em regiões genômicas associados à composição de AG na carne bovina. Contudo, poucas regiões genômicas explicaram grande parte das variâncias genéticas, e os GDE quando avaliados de forma isolada**,** não foram capazes de explicar totalmente o fenótipo e os mecanismos relacionados. Neste sentido, estudos de co-expressão (COE) e co-expressão diferencial (DCO) auxiliam nas análises de GDE, bem como os estudos de associação genômica ampla (GWAS). A análise de DCO é capaz de apontar genes e fatores de transcrição (FT) melhores conectados ao fenótipo, que, em associação com GDE e COE, fomentam o conhecimento relacionado à característica estudada. Desta forma, objetivou-se identificar e mensurar o perfil transcriptômico do perfil de AG no músculo *Longissimus thoracis* em bovinos da raça Nelore terminados em confinamento sob as abordagens de GDE, COE e DCO. Para a análise de COE foram utilizados dados de 44 touros, e destes, 30 com valores extremos para cada AG. Somas e razões (totalizando 14 fenótipos) foram utilizados nas análises de GDE e DCO. Para a análise de DCO, utilizou-se duas metodologias: (i) *weighted gene co-expression network analysis* (WGCNA) para cálculo da medida de conectividade diferencial (Kdiff) e (ii) *partial correlation and information theory* (PCIT). A metodologia PCIT foi utilizada somente para os grupos dos AGE com objetivo de identificar *differential hubbing* (DH) genes e FT com maior magnitude de *regulatory impact factor*. Um total de 912 GDE foram identificados, e os grupos de somas de AGPI (n=563) e ω-3 (n=346) foram os mais representativos. A análise de COE apontou três módulos, dos quais o módulo *blue* (n=1.776) foi correlacionado com oito dos 14 fenótipos de AG. Além disso, foram listados 759 genes DCO (WGCNA), com destaque para o ácido oleico (n=358) e a soma dos AGMI (n=120). Por meio da análise que combinou PCIT+WGCNA (DCO), os DH genes *ASB5* e *ERLIN1* e o FT *NFIA* se sobressaíram por impactarem ambos os AGE (AL e ALA). Além disso, os genes *ELOVL5*, *FADS2, FASN* e *PPARG* e os FT *NFYA, NFYB* e *RORA* foram atrelados a pelo menos um dos AGE. Ao confluir os resultados de GDE+COE+DCO (WGCNA) para todas os fenótipos, os genes *ACSL3, ECI1, DECR2, FITM1* e *SDHB* foram sinalizados em pelo menos duas análises. Estes genes, relacionados ao metabolismo lipídico foram, até então, pouco explorados em estudos com bovinos. Estes achados contribuem para a melhor compreensão dos mecanismos genéticos subjacentes ao perfil de AG da carne em bovinos Nelore terminados em confinamento.

Palavras-chave: Expressão gênica, Melhoramento animal, Ácidos graxos.

TRANSCRIPTOMIC PROFILE OF *Longissimus thoracis* ASSOCIATED WITH FATTY ACID CONTENT IN NELLORE CATTLE FINISHED IN FEEDLOT

ABSTRACT – Beef has high nutritional value for providing fundamental nutrients such as fatty acids (FA), basic units of lipids, which participate in enzymatic and regulatory pathways, energy storage, and structural functions, also being important factors for meat product acceptance. Saturated fatty acids (SFA) are linked to risk factors associated with heart disease and inflammatory conditions, whereas mono- (MUFA) and polyunsaturated (PUFA) fatty acids, such as essential fatty acids (EFA) – linoleic acid (LA), and alpha-linolenic (ALA), condition protection. The FA profile in beef has a complex and polygenic character. From the nucleic acids sequencing, it was possible to identify differentially expressed genes (DEG) and genomic regions associated with the FA composition in beef. However, few genomic regions explained a large part of the genetic variances, and the DEG evaluated in isolation was not able to fully explain the phenotype and the related mechanisms. In this sense, studies of co-expression (COE) and differential co-expression (DCO) help DEG analysis, as well as genomic wide association studies. DCO analysis can identify genes and transcription factors (TF) better connected to the phenotype, which, in association with DEG and COE, provide knowledge about the traits. Thus, the objective was to identify and measure the transcriptomic profile, under the DEG, COE, and DCO approach, of the FA profile in the Longissimus thoracis muscle of Nellore cattle finished in feedlot. For the COE analysis, data from 44 bulls were used, and of these, 30, with extreme values for each FA, sums, and ratios (totaling 14 phenotypes), were used in the DEG and DCO analyzes. For the DCO analysis were used two approaches, weighted gene co-expression network analysis (WGCNA) to calculate the differential connectivity measure (K_{diff}) and partial correlation and information theory (PCIT) was used only for the EFA groups to identify differential hubbing (DH) genes and TF with a higher magnitude of regulatory impact factor. A total of 912 DEG were identified, and the PUFA sum (n = 563) and ω-3 sum (n = 346) were the most representative. The COE analysis indicated 19 modules, of which ten were related to all the traits evaluated. 759 DCO genes (WGCNA) were listed, with emphasis on oleic acid (n = 358) and MUFA sum (n = 120). On the other hand, through the analysis that combined PCIT + WGCNA (DCO), the DH genes *ASB5* and *ERLIN1* and TF *NFIA* stood out when impacting both EFA (LA and ALA), also the *ELOVL5, FADS2, FASN*, and *PPARG* genes and the TF *NFYA, NFYB*, and *RORA* linked to at least one EFA. When the results of DEG + COE + DCO (WGCNA) for all traits were combined, the *ACSL3, ECI1, DECR2, FITM1* and *SDHB* genes were signaled in at least two analyzes. These genes, related to lipid metabolism, were, until then, little explored in cattle studies. These findings contribute to the understanding of the genetic mechanisms underlying the FA profile of Nellore cattle finished in feedlot.

Keywords: Gene expression, Animal breeding, Fatty acids.

CAPÍTULO 1. Considerações gerais

1.1. Introdução

O Brasil, frente aos índices de produção e exportação da carne bovina, detém o posto de importância na cadeia produtiva da bovinocultura de corte mundial. Atualmente o Brasil possui o maior rebanho comercial de bovinos do mundo (214 milhões de cabeças) aliado à primeira e à segunda posição em termos de exportação e produção de carne bovina, respectivamente. Segue na terceira colocação em relação ao consumo interno, atingindo marcas superiores a 70% de toda a sua produção (ABIEC, 2019). Contudo, a demanda por proteína animal e o consumo *per capita* são crescentes, conforme FAO (2018).

Inserida entre as principais fontes de proteína animal, a carne bovina possui alto valor nutricional por ser capaz de fornecer micronutrientes (vitaminas e minerais) e macronutrientes (carboidratos, proteínas e lipídios) fundamentais à mecanismos regulatórios e vias metabólicas (Biesalski, 2005). Os ácidos graxos (AG), unidades básicas dos lipídios, participam de vias enzimáticas e regulatórias, no armazenamento energético e funções estruturais, além de serem fatores importantes à aceitação do produto cárneo, influenciando no sabor, suculência e maciez (Wood et al., 2008).

No entanto, o consumo elevado de ácidos graxos saturados (AGS) está ligado a fatores de risco associados à doenças como cardiopatias, aterosclerose e quadros inflamatórios (Eilander et al., 2015; Zong et al., 2016), que são atenuados e/ou eliminados a partir da ingestão adequada de ácidos graxos mono- (AGMI) e poli-insaturados (AGPI), os quais condicionam ainda proteção a doenças autoimunes e câncer (Asif, 2015; Luu et al., 2018).

Em bovinos, a composição de AG na carne é regida por efeitos de cunho genético e ambiental, o que permite observar diferentes valores entre raças e manejos. Neste sentido, manejos nutricionais e comparativos entre raças foram/são explorados em maior intensidade, de acordo com a literatura (Costa et al., 2018; Fiorentini et al., 2014; Pilarczyk e Wojcik, 2015). Por outro lado, os trabalhos com foco na identificação de genes e/ou marcadores genéticos associados ao perfil de AG em raças zebuínas encontram-se em menor número (Cesar et al., 2014; Feitosa et al., 2017; Lemos et al., 2018).

Com o advento de tecnologias como o sequenciamento de DNA e cDNA (RNA-seq) foi possível identificar regiões genômicas e genes diferencialmente expressos associados à composição de AG na carne de bovinos (Berton et al., 2016; Cesar et al., 2016; Fonseca et al., 2020; Santos Silva et al., 2019). Entretanto, a compreensão acerca da determinação do fenótipo vai além de tais identificações, e avaliá-los de forma isolada não permite inferir com precisão o produto final e os mecanismos relacionados (Hudson et al., 2012).

Esta deficiência pôde ser atenuada a partir da análise de co-expressão gênica, a qual é capaz de avaliar globalmente a expressão dos genes, não somente aqueles diferencialmente expressos, bem como suas inter-relações para a determinação do fenótipo (Hudson et al., 2012). No mesmo sentido, foi proposta a análise de co-expressão diferencial, em que se avalia o padrão de redes gênicas em situações experimentais contrastantes, permitindo a identificação de genes melhores conectados às características em estudo (Chowdhury et al., 2019; Singh et al., 2018).

1.2. Objetivo

Em decorrência do caráter complexo e poligênico do perfil de AG na gordura intramuscular bovina, tal como pela sua importância para o mercado da bovinocultura de corte e para a saúde humana, este estudo teve como objetivo identificar e mensurar o perfil transcriptômico sob abordagem das análises de genes diferencialmente expressos, co-expressão gênica e co-expressão diferencial de genes associados ao perfil de AG presente no músculo *Longissimus thoracis* em bovinos da raça Nelore terminados em confinamento. Informações geradas a partir deste estudo poderão auxiliar os programas de melhoramento genético de bovinos de corte e a seleção de animais superiores.

1.3. Revisão de literatura

1.3.1. Panorama da bovinocultura no Brasil

O Brasil é detentor do maior rebanho comercial de bovinos do mundo, com cerca de 214,69 milhões de cabeças (ABIEC, 2019), constituído sobretudo por raças zebuínas (*Bos taurus indicus*). Neste cenário, a raça Nelore predomina (Ferraz e Felício, 2010) em decorrência de características relacionadas à sua rusticidade, que vão desde adaptação às forragens de baixa qualidade e ao clima tropical, bem como a resistência a ecto- e endoparasitas (Ibelli et al., 2012). Nos setores de produção e exportação, o Brasil atinge a segunda e primeira posições no ranking mundial com valores de 10.959 e 2.205 toneladas, respectivamente (ABIEC, 2019).

Em relação aos dados de consumo interno, o Brasil atingiu a marca de 79,64% de toda a sua produção (8,75 mil toneladas), com consumo *per capita* de 42,12 kg/hab/ano, alcançando a segunda colocação no ranking mundial, atrás somente dos Estados Unidos da América (ABIEC, 2019). Tal fato encontra-se associado ao crescimento da população, desenvolvimento econômico e urbanização, conforme dados da FAO (2018). Desse modo, o desenvolvimento de conhecimento e tecnologias acerca da promoção e produção de uma dieta funcional e benéfica à saúde pública é cada vez mais desejável.

1.3.2. Aspectos e qualidade da carne bovina

A carne bovina está entre as principais fontes de proteína animal e possui um alto valor nutricional, fornecendo micronutrientes como vitaminas A, E, D e do complexo B (B1, B2, B6 e B12), além de minerais como ferro, zinco e selênio (Biesalski, 2005; Wyness et al., 2011). Ela também é fonte de diversos macronutrientes e possui aproximadamente 20 a 24g de proteína de alta digestibilidade em 100g de proteína animal (Schaafsma, 2000). Quando inserida na dieta, é capaz de fornecer por volta de oito aminoácidos essenciais (lisina, treonina, metionina, fenilalanina, triptofano, leucina, isoleucina e valina) que não são produzidos naturalmente pelo corpo humano (Williams, 2007; Wyness et al., 2011).

Como macronutrientes fornecedores de energia, destacam-se principalmente os lipídios, tendo em vista que a carne bovina não apresenta grandes quantidades de carboidratos. Aliado a isto, as unidades básicas dos lipídios, os AG, são essenciais em vias regulatórias e enzimáticas, exercem funções de armazenamento energético e estruturais, além de serem fatores

importantes à aceitação do produto cárneo, influenciando no sabor, suculência e maciez (Wood et al., 2008; Wyness et al., 2011).

As gorduras subcutânea e intramuscular atrelam-se a características ligadas à qualidade de carne, das quais a suculência, o sabor e a maciez, são as que sofrem maior interferência da quantidade de lipídios e do perfil de AG. A gordura subcutânea condiciona proteção ao encurtamento das fibras musculares pelo frio (*cold shortening*) durante as etapas de resfriamento da carcaça (Chardulo et al., 2013) e junto à maior marmorização (deposição de gordura intramuscular) estão relacionadas à menores índices de força de cisalhamento e maior suculência (Wood et al., 2008) e à aceitação da carne em países como Coréia, Japão, Austrália e Estados Unidos (Park et al., 2018).

Por sua vez, o perfil de AG está relacionado ao sabor e aos benefícios à saúde humana. Na primeira situação, está intimamente correlacionado a oxidação lipídica, aspecto de rancidez, sabor e odor anormais, bem como menor poder de atração do consumidor (Campo et al., 2006). O conjunto de AG presentes na carne apresenta também reflexos na saúde humana, em que maiores concentrações de AGPI e AGMI e menores de AGS são considerados cada vez mais importantes por condicionarem proteção ao surgimento e desenvolvimento de enfermidades (Asif, 2015).

1.3.3. Estrutura e classificação dos ácidos graxos

Na alimentação, os AG podem ser classificados de acordo com sua estrutura química em AG de cadeia longa (entre 2 a 80 carbonos) e cadeia muito longa (a partir de 12 e 20 carbonos) (Lunn e Theobald, 2006). Os AG também são classificados conforme a presença de ligações covalentes duplas em sua estrutura química, de modo que AG poli-insaturados (AGPI) apresentam mais de uma dupla ligação, monoinsaturados (AGMI) apenas uma e na ausência são denominados saturados (AGS). Nos AG insaturados (AGMI e AGPI) há um agrupamento *–cis* e um *–trans* em decorrência da configuração de ligação presente entre os átomos de hidrogênio (Nelson e Cox, 2014), tal como em virtude da posição da primeira dupla ligação, compreendendo as principais famílias ômega 3 (n3), ômega 6 (n6), e ômega 9 (n9), que possuem a primeira

dupla ligação, respectivamente, entre o carbono 3 e 4, 6 e 7 e 8 e 9 (Lunn e Theobald, 2006).

1.3.4. Metabolismo de ácidos graxos

O metabolismo lipídico pode ser, resumidamente, dividido entre os mecanismos de adipogênese, lipogênese e lipólise, permitindo o estabelecimento da homeostasia corporal acerca de atribuições que envolvem desde o fluxo energético, funções estruturais e mediadores inflamatórios, além de relação com o surgimento e desenvolvimento de doenças cardiovasculares, obesidade, diabetes e câncer (Huang e Freter, 2015; Kersten, 2001; Santos e Schulze, 2012).

A gordura animal é, para seres humanos, uma das principais fontes de AG insaturados (AGI), desde os ácidos linoleico e α-linolênico, até os seus derivados ácido araquidônico (AA, C20:4 n6), ácido linoleico conjugado (CLA), ácido docosapentaenóico (DPA, C22:5 n3) e ácido docosaexanoico (DHA, C22:6 n3) (Uauy e Castillo, 2003; Wood et al., 2008). Em mamíferos, a aquisição de AG, em especial a absorção de ácido linoleico e α-linolênico são oriundas de fontes exógenas. Estes AG são considerados ácidos graxos essenciais (AGE), os quais o organismo não é capaz sintetizar *de novo.*

1.3.4.1. Adipogênese

O termo adipogênese compreende os estágios desde a formação de pré-adipócitos a partir de precursores mesenquimais até a diferenciação e maturação do adipócito (Lefterova e Lazar, 2009). Os processos de maturação e diferenciação dos adipócitos são regidos por uma série de vias que envolvem genes como *CCAAT/enhancer-binding proteins* (*CEBP*) e *peroxisome proliferator-activated receptor gamma* (*PPARG*), além de mecanismos de angiogênese, proliferação e crescimento celular (Lefterova e Lazar, 2009; Rosen et al., 2000).

1.3.4.2. Lipogênese e lipólise

Complementarmente ao processo de adipogênese, os processos de lipogênese e lipólise são responsáveis pela síntese e degradação de lipídios, respectivamente, e juntos, determinam a homeostasia energética. Fatores nutricionais como a ingestão/consumo energético, bem como a ação de hormônios insulina/glucagon e leptina modulam tais processos. Fatores esses que são regidos pela expressão de genes como *stearoyl-CoA desaturase* (*SCD*), *fatty acid synthase* (*FASN*), *sterol regulatory element-binding transcription factor* (*SREBF*), *carbohydrate-responsive element–binding protein* (*ChREBP*), *acetyl-CoA carboxylase* (*ACACA*), *acyl-CoA synthetase short-chain* (*ACSS*) e *PPARG* (Jump e Clarke, 1999; Roberts et al., 2009).

A formação e deposição de AG em forma de triglicerídeos nos adipócitos presentes no tecido mamário, sítios subcutâneos e intramusculares são regulados por mecanismos metabólicos e fatores genéticos e moleculares semelhantes (Harvatine et al., 2009). A despeito disso, o perfil de AG final encontrado nos produtos de origem animal, principalmente dos ruminantes, possuem peculiaridades devido ao metabolismo ruminal (Wood et al., 2008).

Por outro lado, o processo de lipólise ocorre em situações de maior demanda energética e envolve a reação de hidrólise completa ou parcial de triacilglicerol para a produção de trifosfato de adenosina (ATP, do inglês *Adenosine TriPhosphate*) (Lass et al., 2011). Tal processo é principalmente regido por três enzimas: *adipose triglyceride lipase* (ATGL), *hormone-sensitive lipase* (HSL) e *monoglyceride lipase* (MGL), codificadas, respectivamente, pelos genes *PNPLA2*, *LIPE* e *MGLL* (Lass et al., 2011).

Para a produção de ATP a partir da degradação lipídica existem duas vias principais, a via de oxidação de AG e a via cetogênia. Na via da oxidação de AG, estes passam por etapas de β-oxidação até a formação de acetil-CoA para então adentrar o ciclo de Krebs. Neste processo há a ação principal de proteínas FABP (*fatty acid bidind protein*), as da família SLC *(solute carrier*) e ACSL (a*cyl-CoA synthetase long chain*), tal como das enzimas CPT1 e CPT2 (Houten e Wanders, 2010). Através da via cetogênica, as moléculas de acetil-CoA seguem para o ciclo HGM-CoA (*hydroxymethylglutaryl-CoA*), desencadeando a produção de corpos cetônicos e acetato (Hocquette e Bauchart, 1999; Mcgarry e Foster, 1980). O acetato é reciclado para a formação de novas moléculas de acetil-CoA

e os corpos cetônicos seguem para a circulação sanguínea, fornecendo energia para os tecidos periféricos (Holtenius e Holtenius, 1996).

1.3.4.2.1. Metabolismo ruminal

Em bovinos, a obtenção de AGE é decorrente da alimentação ofertada e diferentemente de animais monogástricos, os poligástricos têm a absorção e deposição nos tecidos influenciadas pelo processo de bio-hidrogenação realizado pela microbiota ruminal (Wood et al., 2008). Esta por sua vez, sofre interferência do pH (Fuentes et al., 2011), da quantidade e do tipo de AG fornecido (Barletta et al., 2016), e de agentes suplementares na dieta, como o tanino (Costa et al., 2018) e a monensina (Ogunade et al., 2018).

O processo enzimático de bio-hidrogenação aliado ao de lipólise fazem parte do metabolismo lipídico no rúmen e do mecanismo de defesa frente aos efeitos tóxicos dos AGPI à microbiota (Jenkins, 1994; Wood et al., 2008). O mecanismo de lipólise é realizado por meio de lipases que hidrolisam moléculas de acilgliceróis em mono- e diglicerói s e AG livres não saturados, os quais serão biohidrogenados em seguida. Na etapa de bio-hidrogenação há saturação dos AGPI em AGMI/AGS por meio de processos de isomerização e hidrogenação, momento em que surge AG intermediários importantes à saúde humana como o CLA. Dos AG ingeridos, grande parte é absorvida no duodeno na forma de AGMI e AGS, embora pequenas quantidades de AGPI também sejam absorvidas (Jenkins, 1994; Kepler et al., 1966).

Ainda no rúmen, há a formação de ácidos graxos voláteis (AGV), acetato (C2:0), propionato (C3:0) e butirato (C4:0) que, em decorrência do tamanho de suas cadeias carbônicas, são absorvidos por difusão pelo epitélio ruminal e transportados ao tecido hepático (Hocquette e Bauchart, 1999). Contudo, a maior parte do acetato que chega ao fígado é transportado até o tecido adiposo, e lá, por meio de processos lipogênicos, há a conversão deste em ácidos graxos livres e em seguida em triglicerídeos (Hocquette e Bauchart, 1999).

1.3.4.2.2. Metabolismo no tecido adiposo

O acetato, proveniente do metabolismo ruminal e hepático, é o principal precursor na síntese de AG e triglicerídeos. O acetato que adentra os adipócitos é transformado em piruvato dentro das mitocôndrias, por meio da via oxidativa, e, em seguida, é convertido em acetil-CoA. De acordo com Shingfield et al. (2013), a partir da ação coordenada de enzimas como ACC, FAS e NADPH dá-se origem ao malonil-CoA que segue à formação de AG com 16 carbonos (ácido palmítico).

A partir de AG (≥C18) e do ácido palmítico (C16:0), oriundos, respectivamente, da circulação e do mecanismo *de novo* citado anteriormente, há a formação dos demais AGS e AGI pela a ação de dessaturases e/ou elongases. Esses processos são realizados no retículo endoplasmático e peroxissomos por enzimas elongases 1–6 (codificadas por genes da família *EVOLV 1–6*) e dessaturases (Δ^9, Δ^5 e Δ^6) (Cherfaoui et al., 2012; Jaskobsson et al., 2006). As enzimas EVOLV2, 4 e 5 tem AGPI como substratos e as EVOLV1, 3 e 6 agem sob ação de AGS e AGMI (Jakobsson et al., 2006).

As dessaturases são específicas para as diferentes classes de AG, e os genes *SCD* e *FADS* (*fatty acid desaturase*) *1* e *2* são, respectivamente, responsáveis pela codificação das enzimas Δ^9-, Δ^5- e Δ^6-dessaturases (Nakamura e Nara, 2004). A enzima Δ^9 adiciona uma dupla ligação ao ácido esteárico (C18:0), permitindo a formação do AGMI oleico (C18:1 n-9), similar ao que acontece pelas ações das Δ^5 e Δ^6 -dessaturases para a formação de EPA, DHA e AA, a partir dos AGPI linoleico (C18:2 n6) e α-linolênico (C18:3 n3) (Nakamura e Nara, 2004).

O armazenamento dos AG em forma de triacilgliceróis é realizado por meio de processos de esterificação, realizados pelas enzimas AGPAT (*acyl glycerol phosphate acyl transferase*), DGAT (*diacyl glycerol acyl transferase*) e GPAT (*glycerol-3 phosphate acyl transferase*) (Bernard et al., 2008).

1.3.4.3. Regulação endócrina

Os mecanismos regulatórios associados ao metabolismo lipídico são regidos pela expressão de uma série de fatores de transcrição como PPAR-γ, SREBP1 e ChREBP, que, por sua vez, são modulados por ação hormonal capaz de coordenar a expressão desses e de outros genes/fatores de transcrição

(Nakamura et al., 2014). A interação de hormônios como a leptina, com a insulina e glucagon concebem meios para o equilíbrio energético (Mohamed-Ali et al., 1998).

1.3.4.3.1. Leptina

A leptina é um dos hormônios mais estudados em mamíferos e possui relação com uma série de mecanismos sinalizadores no organismo. Regula a ingestão de alimentos por meio da determinação de saciedade e gasto energético, eventos estes fundamentais para o equilíbrio energético (Fasshauer e Blüher, 2015; Mohamed-Ali et al., 1998).

Complementarmente, a leptina tem a capacidade de deturpar o gasto energético, independentemente da ingestão alimentar, via mecanismos em que UCP-1 (*uncoupling protein-1*), MCH (*melanin-concentrating hormone*) e FoxO1 (*forkhead box O1*) estão envolvidos. Além disso, é capaz de interferir na homeostase da glicose a partir de vias sinalizadoras PI3K (*phosphoinositide 3-kinase*) e AMPK (*AMP-activated protein kinase*) (Münzberg e Morrison, 2015; Park e Ahima, 2015).

1.3.4.3.2. Insulina e glucagon

A homeostase glicêmica é coordenada por hormônios produzidos no pâncreas, como glucagon e insulina. Este último é produzido por células β e estimula o transporte de glicose para o interior das células, por meio de proteínas transportadores de membrana GLUT1–5, bem como inibe a síntese de energia via degradação de trigliceróis (Sasaki, 2002).

De forma contrária, age o glucagon ao promover a glicólise e gliconeogênese a partir da oxidação de AG (Lee et al., 2016). Uma maior secreção do hormônio por células α no pâncreas é identificado em situações de hipoglicemia e sob influência de GLP-1 e AMP cíclico (cAMP) (Cryer et al., 2003). O glucagon está associado à ativação de cascatas enzimáticas e vias proteicas como AMPK, MAPK (*mitogen-activated protein kinase*) e CREBBP, além de estimular a ação lipolítica da enzima HSL (Habegger et al., 2010). O glucagon age ainda sob a expressão de genes como *CPT* (*carnitine palmitoyltransferase*)

1 e 2, *ACADM* (*acyl-CoA dehydrogenase medium chain*), *DECR2* (*2,4-dienoyl-CoA reductase 2*) e *PPAR-α* no processo de oxidação de AG em hepatócitos (Longuet et al., 2008).

1.3.5. Fatores que influenciam a deposição de gordura e o perfil de ácidos graxos na carne bovina

A composição de AG presente na carne bovina é bastante variável e complexa devido ao amplo número de genes e vias metabólicas relacionadas, desde os processos de diferenciação celular, formação e maturação de adipócitos até a síntese e deposição lipídica, os quais são moldadas por fatores genéticos e ambientas.

1.3.5.1. Fatores ambientais

O manejo nutricional é capaz de interferir no metabolismo ruminal e na expressão gênica a partir da ação de moléculas bioativas provenientes da dieta, suprimindo/promovendo a produção de fatores de transcrição e enzimas relacionadas ao metabolismo energético e lipídico (Osorio e Moisa, 2019).

Em bovinos de corte, Daley e colaboradores (2010) trouxeram dados compilados de perfis de AG em raças taurinas (*Bos taurus taurus*) terminados a base de gramíneas ou grãos. Os autores evidenciaram um melhor perfil de AG em animais terminados a base de gramíneas, com menores concentrações de ácido mirístico (C14:0) e palmítico (C16:0), melhor relação n6/n3 e maiores índices de CLA. Outros trabalhos apresentaram resultados similares ao indicarem perfil de AG favorável em animais terminados a pasto em raças zebuínas, taurinas e cruzadas (Bressan et al., 2016; Horcada et al., 2016). Da Costa et al. (2013) avaliaram as raças Barrosã e Alentejana submetidas a dietas com alta/baixa concentração de volumoso. Os autores observaram maiores níveis de expressão do gene *SREBF1* no tecido adiposo subcutâneo e do gene *FABP4* nos sítios subcutâneo e intramuscular em animais submetidos a dietas ricas em concentrado. Interferências dietéticas na expressão dos genes *ACACA* (*acetyl-CoA carboxylase alpha*), *PPARA* e *LPL*, importantes ao fluxo e metabolismo lipídico, também foram observadas.

A inserção de aditivos e diferentes fontes lipídicas à dieta animal condicionam proteção aos AGPI e/ou alteraram o microbioma ruminal, afetando o fluxo intestinal e a absorção e deposição de AG nos tecidos. Neste sentido, aditivos como monensina (Ogunade et al., 2018), tanino (Costa et al., 2018) e diferentes fontes lipídicas (Fiorentini et al., 2015) foram responsáveis por modificar o perfil de AG na carne dos bovinos.

A adição de monensina e tanino na dieta foram capazes de reduzir a taxa de bio-hidrogenação via microbiota ruminal do AL (ácido linoleico) e ALA (ácido α-linolênico) (Costa et al., 2018; Ogunade et al., 2018). Este resultado é similar ao apresentado por Fiorentini et al. (2015), em que diferentes fontes lipídicas, gordura protegida e óleo de linhaça, mostraram-se associadas a concentrações de AL, ALA e CLA em sítios subcutâneo e intramuscular em bovinos da raça Nelore.

1.3.5.2. Fatores genéticos

Uma série de genes moldam o perfil de AG e a deposição lipídica nos diferentes tecidos dos bovinos. Tal complexidade torna-se evidente frente aos genes já mencionados ao longo do texto como *ACACA*, *FADS, CEBP* e *RXR,* bem como tantos outros já mencionados na literatura (Gregoire et al., 1998; Kohjima et al., 2007; Parks et al., 2012). Muitos destes atuam como fatores de transcrição e são capazes de regular e coordenar vários processos nas vias lipogênicas (Eberlé et al., 2004; Osorio e Moisa, 2019).

O maior destaque vai para os fatores de transcrição (FT) SREBPs, PPAR-α/γ/Δ, CEBP, CREBP (*cyclic AMP response element-binding protein*) e RXR/LXR, que funcionam como genes-chave no metabolismo lipídico. A expressão de SREBP-1, codificado por *SREBF1*, um dos *hubs* genes associados à adipogênese, está relacionado concomitantemente com a expressão de *PPARG* e *FASN*. O CEBP, o qual é modulado pela expressão de gene de mesmo nome, induz maior produção de PPAR-γ e esta é ativada pela interação com RXR que, por sua vez, induz a expressão do gene *ACSS*, e tem sua expressão regulada por insulina e pela cascata MAPK (Colitti e Grasso, 2014).

Complementarmente, existem mecanismos regulatórios responsáveis por modular a expressão de genes envolvidos na síntese de AG que se enquadram

principalmente nas interações epigenéticas desencadeadas por ações de microRNA (miRNA) (Muroya et al., 2019), *long noncoding* RNA (lncRNA) (Jiang et al., 2020), fenômenos de metilação da cadeia de nucleotídeos e modificações covalentes de histonas (Burdge e Lillycrop, 2014).

Ao avaliar as variações fenotípicas entre raças taurinas e zebuínas referente ao perfil de AG, foi possível identificar maiores concentrações de AGI e melhor relação n6/n3 em touros de origem zebu, como o Nelore, quando comparados a touros de raças taurinas puras, como Angus e Hereford (Lopes et al., 2012; Pilarczyk e Wojcik, 2015; Rossato et al., 2009).

Em estudos de associação genômica em bovinos, autores trouxeram indicações de regiões genômicas/genes importantes ao metabolismo lipídico. Em bovinos da raça Nelore, foram apontados genes como como *ELOVL5 (*e*longation of very long chain fatty acids protein* 5), *HMGCS2 (hydroxymethylglutaryl-CoA synthase*) e genes da família de transportadores *ABC* que participam de vias metabólicas relacionadas a síntese/degradação de AG e/ou vias do PPAR-α/γ (Cesar et al., 2014; Lemos et al., 2016).

Kelly et al. (2014) observaram, em raças de três grupos genéticos distintos (*Bos taurus indicus*, *Bos taurus taurus* e sintéticos), associação de marcadores do tipo SNP (do inglês *Single Nucleotide Polymorphism*) em genes importantes como *FASN* e *SCD*, capazes de interferir no perfil de AG. Ademais, os genes *ACSL5* (*acyl-CoA synthetase long chain family member* 5), *ACSS2 (acyl-CoA synthetase short chain family member 2*) e os da família de transportadores *SLC* também foram associados ao metabolismo lipídico em bovinos (Buchanan et al., 2016; Fernandes Júnior et al., 2016; Lemos et al., 2016).

Capazes de modificar a expressão gênica, assim como os SNP, alterações estruturais do tipo CNVs (do inglês *copy number variation*) foram associadas à vias metabólicas relacionadas a lipogênese (Lemos et al., 2018b). A biossíntese de AG, metabolismo energético e lipídico são alguns dos processos que envolvem regiões do genoma em que CNVs já foram mapeados conforme mostraram estudos nas raças Nelore (Geistlinger et al., 2018; Lemos et al., 2018b) e Angus (Stothard et al., 2011).

1.3.6. Influência da composição de ácidos graxos da carne sobre a saúde humana

Em humanos, a busca por uma alimentação saudável é crescente ao longo dos anos e o perfil dos AG carne bovina vem ganhando maior atenção (Eilander et al., 2015; Luu et al., 2018). Segundo a FAO (2010) e OMS (2018), o consumo total de gordura deve compor 15% a 35% da dieta e destes, deve-se priorizar fontes de AGPI frente aos AGS. Além disso, deve-se atentar ao fato que a relação entre AGI:n6/n3 não deve ultrapassar a razão de 4:1 (Simopoulos, 2016). Desta forma, os AGS devem encontrar-se em menores concentrações na dieta, visto que seu consumo elevado está associado à fatores de risco como o surgimento e/ou desenvolvimento de enfermidades de cunho cardiovasculares, inflamatórias e síndromes metabólicas e endócrinas (Eilander et al., 2015; Luu et al., 2018; Zong et al., 2016). Dentre os AGS provenientes da carne bovina, destacam-se pela maior concentração o ácido palmítico (C16:0) e esteárico (C18:0) e pela menor concentração o ácido mirístico (C14:0). Embora tenham a mesma classificação, tais AG possuem impactos biológicos específicos (Flock e Kris-Etherton, 2013).

O ácido palmítico é o principal AGS relacionado a desordens e está associado a resistência à insulina (Palomer et al., 2018) e leptina (Cheng et al., 2015) e aos mediadores pró-inflamatórios (Nicholas et al., 2017; Sergi et al., 2018). Estudos avaliando efeitos individuais dos ácidos esteárico e mirístico são escassos, embora sejam associados à apoptose celular e quadros inflamatórios (Anderson et al., 2012; Harvey et al., 2010). Por sua vez, a ingestão de AGI condicionam proteção às desordens acima mencionadas, bem como à processos neoplásicos (Asif, 2015; Luu et al., 2018). Na dieta humana, a carne bovina é fonte importante de AGI, fornecendo os AGE, AL e ALA, que por sua vez são precursores de AA, ácido eicosapentaenoico (EPA), DPA e DHA, além de ser fonte de CLA (Saini e Keum, 2018; Wood et al., 2008). De forma similar aos AGS, os AGI possuem funções biológicas individuais e participam de uma série de vias metabólicas relacionadas a processos anti-oxidantes, pró- e anti-inflamatórios (Saini e Keum, 2018; Simopoulos, 2016).

A partir da ação de enzimas Δ-dessaturases, elongases (*ELOVL 5* e *2*) e β-oxidases, há a formação dos principais AGPI com repercussões metabólicas à saúde humana (Saini e Keum, 2018). Oriundo do AL, o AA possui,

predominantemente, efeitos pró-inflamatórios que vão desde o aumento na agregação plaquetária e coagulação em vasos sanguíneos até vasoconstricção e influência no sistema de defesa (Das, 2018; Saini e Keum, 2018). Por sua vez, o DPA, em conjunto com EPA e DHA possuem importantes propriedades anti-inflamatórias e antineoplásicas (Drouin et al., 2019; Morin et al., 2015).

Ações similares foram observadas na administração/ingestão de CLA em quadros de doenças cardiovasculares, obesidade e processos tumorais, dado aos seus efeitos negativos sobre adesão plaquetária (Mccarthy et al., 2013), deposição de gordura (Yeganeh et al., 2017), massas tumorais (Maria et al., 2018) e mediadores pró-inflamatórios (Mohammadi et al., 2020).

Pelo exposto, o desenvolvimento de técnicas e metodologias capazes de fornecer conhecimento e direcionamento a respeito do perfil de AG na carne bovina são de grande importância. Esses conhecimentos poderão/deverão ser explorados por programas de melhoramento animal para a obtenção de produtos benéficos à saúde humana com menores concentrações de AGS e maiores de AGMI e AGPI, bem como para o estabelecimento da razão AGPI/AGS e n6/n3 ideais na dieta.

1.3.7. Análises de transcriptoma aplicadas ao perfil de ácidos graxos

1.3.7.1 Metodologias empregadas nas análises de transcriptoma

Com o avanço nas áreas da genética e biotecnologia, tornou-se possível o estudo e análise do conjunto de transcritos. Ao longo dos anos, surgiram metodologias como a técnica de *Northern blot* (Alwine et al., 1977), primeira capaz de identificar RNA mensageiro (mRNA) de interesse por hibridização com sondas de DNA complementar (cDNA), e, assim, realizar a sua quantificação relativa pela intensidade das bandas eletroforéticas (Trayhurn, 1996). A partir da técnica desenvolvida por Mullis et al. (1986), a reação em cadeia da polimerase (PCR), obteve-se uma capacidade semelhante de quantificação, embora fosse necessário adição de etapas para conversão das moléculas de RNA em cDNA (transcrição reversa - RT), a técnica (RT-PCR) ainda apresentava-se simples e pouco dispendiosa (Dean et al., 2002).

Alguns anos depois, Higuchi et al. (1993) apresentaram a metodologia da PCR em tempo real ou RT-qPCR, aprimorando a técnica convencional (RT-PCR), reduzindo riscos de contaminação e o tempo empregado por meio da eliminação das etapas relacionadas à corrida eletroforética. Tal metodologia permitiu quantificar a expressão gênica, tanto de forma absoluta quanto de forma relativa, a partir do uso de intercalantes de DNA (SYBR®) ou sondas de hidrólise (TaqMan™).

Contemporaneamente, surgiram os microarranjos de DNA (Schena et al., 1995), o que permitiu a identificação de grande número de genes expressos concomitantemente em uma única reação. Somado ao potencial quantitativo, o método parte do princípio da hibridização, em que moléculas de mRNA extraídas dos tecidos são convertidas em cDNA, marcadas com fluoróforos e então ligadas, por paridade de bases, com as sequencias gênicas de DNA previamente conhecidas plotadas no poços dos microarranjos, emitindo luminescência (Schena et al., 1995).

No entanto, os microarranjos possuem limitações que favoreceram o desenvolvimento e o uso de outras técnicas como o sequenciamento de cDNA proveniente de RNA (RNAseq), principalmente em situações de muito alta ou muito baixa expressão gênica, em que ocorre, respectivamente, a saturação e a ausência de captação de luminescência. Microarranjos apresentam também desvantagens na detecção de genes/variantes gênicas restritas somente às plotadas, impossibilitando a quantificação de genes desconhecidos, bem como a identificação de novas isoformas (Wang et al., 2009). Essas limitações não estão presentes na metodologia de RNAseq, que se utiliza do sequenciamento de próxima geração ou *next-generation sequencing* (NGS) (Wang et al., 2009).

1.3.7.2. Análise de genes diferencialmente expressos

A análise de genes diferencialmente expressos (GDE) nos permite avaliar e comparar, de forma global, a expressão gênica entre duas ou mais amostras e, portanto, indicar potenciais genes associados aos fenótipos avaliados. O desenvolvimento de ferramentas capazes de realizar esta análise auxiliaram pesquisadores na busca de padrões voltados a características relacionadas à produção de leite (Raven et al., 2016), eficiência alimentar (Higgins et al., 2019),

resistência à doenças parasitárias e infecciosas (Dewangan et al., 2015; Sbardella et al., 2019), bem como características voltados à qualidade de carne (Fonseca et al., 2020; Santos Silva et al., 2020).

Quanto ao perfil de AG intramuscular, Berton et al. (2016) identificaram uma série de GDE em animais Nelore, dentro os quais podemos destacar o *acetyl-CoA acetyltransferase 1* (*ACAT1*), *FABP7*, *PPARA/D*, *acyl-CoA synthetase medium chain family member* (*ACSM 1* e *3*), *DGAT2* e *ACSS1*. Esses genes estão envolvidos em processos do metabolismo lipídico, além de genes transportadores *LPL* e da família SLC. Dentro de raças taurinas (Wagyu, Piemontês e Holandês) foi possível observar GDE similares, bem como os genes *SREBF1*, *SCD, FASN*, *ELOVL5* e *6* (Huang et al., 2017; Hudson et al., 2014).

Cesar et al. (2016) avaliou GDE entre bovinos da raça Nelore agrupados em alta/baixa concentração quanto aos AG palmítico, esteárico, oleico, LA, CLA, EPA e DHA presentes no músculo *Longissimus dorsi* (LD). No grupo de alta concentração de ácido oleico observaram maior expressão e/ou relação de genes como *SCD*, *SREBF1*, *peroxisome proliferator-activated receptor gamma coactivator 1 alpha* (*PPARGC1A*) e *beta* (*PPARGC1B*), *FOXO1* e *FOXO3*. No grupo CLA foram apontados GDE como *signal transducer and activator of transcription 2* (*STAT2*) e *nuclear factor NF-kappa-B 1A* (*NFKB1A*), além de compartilhar *SREBF1* e *FOXO1* com o grupo do ácido oleico.

Genes já descritos anteriormente nesta revisão foram apontados por Jeong e colaboradores (2012) como correlacionados positivamente à deposição intramuscular de gordura em bovinos de raças Coreanas, com destaque maior para aqueles responsáveis pela esterificação dos AG (*DGAT1* e *2*, *AGPAT1* e *GPAT1*) e envolvidos em etapas de captação de lipídios (*LPL*, *CD36* e *fatty acid transport protein 1 – FATP1*) e lipogênese (*ACACA* e *FASN*). No mesmo trabalho, os autores apontaram genes relacionados à lipólise (*ATGL* e *MGLL*) e oxidação de AG (*VLCAD –very long-chain acyl-CoA dehydrogenase* e *ACADM*) negativamente correlacionados com as mesmas características. Tal fato, fomenta a relação e a co-expressão desses genes regulatórios para a deposição de gordura intramuscular, bem como do perfil de AG.

1.3.7.3. Análise de co-expressão gênica

Aliadas a estudos de GDE, análises de co-expressão gênica (COE) são utilizadas a fim de melhor entender a interação e o perfil de expressão dos genes associados aos fenótipos. Estas análises apontam GDE identificados em estudos prévios, bem como outros genes que não são diferencialmente expressos, mas que estão relacionados à característica (Hudson et al., 2012). Existem diversas ferramentas que auxiliam neste tipo de análise, contudo os pacotes WGCNA (Langfelder e Horvarth, 2008) e PCIT (Watson-Haigh et al., 2010) são os mais difundidos nos trabalhos científicos.

De forma breve, as análises de COE utilizam genes como ‘nós’ e a similaridade entre seus perfis de expressão como arestas, as quais são determinadas por meio da correlação entre os genes (Langfelder e Horvarth, 2008; Reverter e Chan, 2008). Em seguida, por meio do pacote WGCNA, são formados os módulos gênicos pelo uso da medida de sobreposição de topologia e dos métodos de clusterização inseridos no pacote. Por fim, ambos os pacotes (WGCNA e PCIT) possuem ferramentas que auxiliam na seleção e visualização de genes, além de apresentar recursos que possibilitam exportar os resultados para softwares de enriquecimento funcional e de construções gráficas de redes.

Sob a metodologia de análise de co-expressão WGCNA, estudos nas raças Hanwoo (Lim et al., 2013) e Nelore (Gonçalves et al., 2018; Silva-Vignato et al., 2019) identificaram módulos gênicos significativos que continham genes, por vezes, não identificados como diferencialmente expressos. Na raça Nelore, Diniz et al. (2019), a partir de dados de RNA-seq oriundos do músculo LT, encontraram módulos gênicos inseridos em vias relacionadas à sinalização AMPK, PPAR e glucagon/insulina. Dentre os módulos gênicos, foram pontuados genes já citados aqui anteriormente como *EVOLV5* e *6*, *FASN*, *FABP4*, *ACACA* e *LIPE*, mas também genes, até então, não citados em análises de GDE como *PLIN1* (*perilipin 1*), *PDE3B* (*phosphodiesterase 3B*), *COL4A1 e 2* (*collagen type IV alpha 1 chain* e *alpha 2 chain*).

Silva-Vignato et al. (2019) ao avaliarem o perfil de COE para as características de área de olho de lombo e cobertura de gordura subcutânea em bovinos Nelore, sinalizaram os genes *EIF2AK2* (e*ukaryotic translation initiation factor 2 alpha kinase 2*) e *HADHA*/*HADHB* (*hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha* e *beta*), além de genes já bem conhecidos como *ACSL1*, *ACAT1* e a família de genes *SLC*.

Análises de COE também mostraram genes codificadores de miRNA em associação a mRNA para a formação de grupos gênicos relativos a vias metabólicas importantes na determinação do fenótipo (Gonçalves et al., 2018; Oliveira et al., 2019). Os miRNA identificados em módulos relacionados a sinalização de AMPK, MAPK e insulina apresentaram padrão de co-expressão e regularam a expressão de uma série de genes-alvo como *SCD*, *CDS2* (*CDP-diacylglycerol synthase 2*), *FAR2* (*fatty acyl-CoA reductase 2*) e *PRKAG2* (*protein kinase AMP-activated non-catalytic subunit gamma 2*) (Oliveira et al., 2019).

1.3.7.4. Análise de co-expressão gênica diferencial

A análise de co-expressão gênica diferencial (DCO) surgiu para ir além de apenas combinar análises de co-expressão em condições experimentais divergentes. A análise de DCO permite a identificação de padrões não apontados nas análises isoladas de GDE e COE. Evidencia mudanças no perfil de expressão das redes gênicas em situações contrastantes, avaliando o surgimento de sub-redes, bem como a identificação de grupos gênicos melhores conectados ao fenótipo (Chowdhury et al., 2019; Hudson et al., 2012; Singh et al., 2018).

Em humanos, há uma série de estudos experimentais a respeito de processos tumorais (Gov e Arga, 2017; Kori et al., 2019; Lin et al., 2015) identificaram genes associados ao desenvolvimento dessas enfermidades por meio da abordagem de DCO. Em bovinos, Alexandre et al. (2015) analisaram amostras de tecido hepático de animais da raça Nelore avaliados quanto à características relacionadas à eficiência alimentar e encontraram 463 genes diferencialmente co-expressos voltados ao sistema imune e metabolismo lipídico. Contudo, estudos em animais de produção encontram-se ainda escassos.

A despeito deste fato, o uso da análise de DCO apresenta potencial, principalmente, para características complexas, de difícil mensuração e de caráter quantitativo como a deposição de gordura intramuscular e o perfil de AG na carne bovina. Desta forma, constitui-se em mais um meio para o melhor entendimento da fisiologia por trás de características de importância econômica, apontando genes, mecanismos regulatórios e moduladores que ainda não foram descritos. A partir disso, poderá contribuir para a obtenção de produtos cárneos

mais saudáveis e de qualidade superior para o mercado consumidor e auxiliar programas de seleção e melhoramento genético no Brasil.

1.4. Referências

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CAPÍTULO 2 – Transcriptomic profile of *Longissimus thoracis* associated with fatty acid content in Nellore beef cattle finished in feedlot

ABSTRACT – The beef fatty acids (FA) profile has the potential to impact human health, and displays polygenic and complex features. This study aimed to identify the transcriptomic FA profile in the *Longissimus thoracis* muscle in the Nellore beef cattle finished in feedlot. Forty-four young bulls were sampled to assess the beef FA profile by considering 14 phenotypes and including differentially expressed genes (DEG), co-expression (COE), and differentially co-expression genes (DCO) analyses. All samples (*n=44*) were used for COE analysis, whereas 30 samples with extreme phenotypes for the beef FA profile were used for DEG and DCO. A total of 912 DEG were identified, and the polyunsaturated (*n=563*) and unsaturated ω-3 (*n=346*) FA sums groups were the most representatives. The COE analyses pointed out three modules, of which the blue module (*n=1,776)* was correlated with eight of fourteen FA phenotypes. Also, 759 DCO genes were listed, and the oleic acid (*n=358*) and monounsaturated fatty acids sum (*n=120*) were in higher numbers. Furthermore, were enriched, respectively, 243 and 13, 319 and 7, and 173 and 12 gene ontology terms and KEGG pathways for the DEG, COE and DCO analyses. Crossing the results, we highlight the unexplored *GIPC2*, *ASB5* and *PPP5C* genes in cattle. Besides, *LIPE* and *INSIG2* genes in COE modules. But also, the *ACSL3, ECI1, DECR2, FITM1* and *SDHB* genes signaled in at least two analyzes. These findings contribute to understand the genetic mechanisms underlying the beef FA profile in Nellore beef cattle finished in feedlot.

KEY WORDS: Co-expression, RNA-seq, DEG, *Bos taurus indicus*, *Longissimus thoracis*, fatty acid profile

2.1. Introduction

Beef has a high nutritional value, being a rich source of macronutrients for regulatory mechanisms and metabolic pathways (Biesalski, 2005). The fat content is an essential fatty acid (FA) source, in which we can highlight the linoleic (C18:2 ω-6) and alpha-linolenic (C18:3 ω-3) (Wood et al., 2008). Further, the beef fat content provides conjugated linoleic acid (CLA) and monounsaturated fatty acids (MUFA), which contribute to the energetic and hormonal metabolism (Dilzer & Park, 2012; Palomer et al., 2018). However, the unbalanced consumption may lead to detrimental effects on human health since it is also a saturated fatty acids (SFA) source, which has been associated with obesity risk factors and cardiovascular disease development (Blekkenhorst et al., 2015; Eilander et al., 2015). In cattle, breed/genetic, diet and ruminal metabolism affect the FA profile (da Costa et al., 2013; Fiorentini et al., 2015; Osorio & Moisa, 2019), and studies have disclosed that the indicine (*Bos taurus indicus*) cattle displayed a healthier FA profile in subcutaneous adipose tissue with lower SFA content than did the taurine (*Bos taurus taurus*) cattle breeds (Huerta-Leidenz et al., 1996; Perry et al., 1998). It corroborated with Rossato et al. (2010) in Nellore cattle breed, in which the intramuscular fat (IMF) of the *Longissimus thoracis* muscle appointed higher MUFA, CLA, and polyunsaturated fatty acids (PUFA) concentrations, and a better ω-6/ω-3 ratio than did Angus cattle finished in pasture.

Nevertheless, genome wide association studies (GWAS) display difficulties in identifying genomic regions capable of explaining a large proportion of the genetic variance regarding intramuscular FA profile in different cattle breeds (Cesar et al., 2014; Kelly et al., 2014; Lemos et al., 2016; Zhu et al., 2017). This issue was mitigated by adding information from at least one of the transcriptomic analyzes. Yan et al. (2020) indicated genes related to ketosis in Holstein cattle using COE and GWAS. Further, Deng et al. (2019) evaluated milk yield of buffaloes using information from both DEG, COE, and GWAS analyses.

The RNA sequencing (RNA-seq) technology provided better understanding of pathways and biological process involved in lipid metabolism (Berton et al., 2016; Cesar et al., 2016; Fonseca et al., 2020; Santos Silva et al., 2019). Although the differentially expressed genes (DEG) analysis indicated important genes in Nellore cattle FA profile (Berton et al., 2016; Cesar et al.,

2016), this analysis does not allow us to fully understand this complex trait since it only pinpoints differentially expressed genes. Therefore, the co-expression gene analysis (COE) is presented as an approach to explain the variation of this trait since it is possible to evaluate the interrelationships between global gene expression, not only DEG, and to indicate sub-connections of co-expressed genes (Hudson et al., 2012). Additionally, the differential co-expression (DCO) analysis has the aim to evaluate the genetic networks patterns, in which it is possible to identify better connected genes to phenotypes (Chowdhury et al., 2019; Hudson et al., 2012; Singh et al., 2018). These approaches have been used in feed efficiency (Alexandre et al., 2015), fat deposition (Cesar et al., 2015; Silva-Vignato et al., 2019), and FA profile (Oliveira et al., 2019) studies in beef cattle.

However, studies on FA profile in zebu cattle that have been integrated information from transcriptomic analysis (DEG, COE, and DCO) are still incipient, especially evaluating a high number of FA. The present study, in an innovative way, evaluated a large part of the FA profile, including the individual FA content, sums and ratios. Besides, given the GWAS studies limitations, the integrated transcriptomic analyzes results can be used to aid in indicating the potential genes and genomic regions related to arduous and expensive measuring traits, such as the beef FA profile. Hence, this study aimed to identify potential regulatory genes associated with the beef FA profile in the transcriptomic profile of the *Longissimus thoracis* muscle of Nellore beef cattle finished in feedlot through the integrated analysis of DEG, COE, and DCO.

2.2. Materials and methods

2.2.1. Animals and sampling

Samples from the *Longissimus thoracis* muscle were obtained from 44 young Nellore bulls, sons of six sires, belonging to the Capivara farm (São Paulo state, Brazil), which participates in the Nelore Qualitas breeding program. Animals are selected based on growth, finishing, and sexual precocity traits. These animals were finished with nutritional management previously detailed by Berton et al., (2016).

Animals were slaughtered in commercial slaughterhouses following the Brazilian legislation (Instruction n°3 of January 17th, 2000 – Brazilian Ministry of Agriculture) when attaining an average of 24 months of age and 550kg of liveweight. Samples from the *Longissimus thoracis* muscle were collected 48 hours *post-mortem* between the 12th and 13th ribs (left half carcass). Subsequently, the samples were placed in airtight plastic bags and stored at -80°C for subsequent analyses.

All procedures performed involving animals were in approved by the Ethics Committee on Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences, Sao Paulo State University (UNESP), Jaboticabal, Brazil, under Protocol Number 18340/16.

2.2.2. Lipid extraction

The quantification of the lipid fraction was performed at the Animal Product Technology Laboratory in the Technology Department of FCAV/UNESP (Jaboticabal, São Paulo, Brazil) according to the method described by Bligh and Dyer (1959). Approximately 3g of raw and ground samples of the *Longissimus thoracis* muscle were subjected to lipid extraction. The samples were transferred to a 250 mL flask, and chloroform (10 mL), methanol (20 mL) and distilled water (8 mL) were added. The solutions were then homogenized with glass rods, and centrifuged for 30 min on a horizontal shaker table (HITACHI High-Speed Micro Centrifuge model CF16RN himac). Following, an additional 10 mL of chloroform and 10 mL of an aqueous sodium sulfate solution (1.5%) were added, and the solution was agitated for more 2 minutes. This final solution was transferred to a 50 mL falcon tube and centrifuged (1000 g-force) at room temperature. The supernatant was discarded and the remaining solution was filtered into measuring cylinders (25 mL) through a paper filter to separate the meat fragments from the solution with the extracted lipids. From this last solution, 5 mL were transferred to a 50 mL pre-weighed beaker, dried in an oven, and cooled in a desiccator for at least 24 hours. Afterwards, the beaker with the solution was placed in an oven at 110°C for the complete evaporation of the solvent, cooled in a desiccator (O/N), and weighed. The differences between the initial and final

weight of the beaker were used to determine the lipid concentration of the samples.

2.2.3. Fatty acids profile quantification

The FA profile was determined for each sample using the extraction method described by Folch et al. (1957), and grounded muscle samples (~100g) were used. The lipids were extracted and isolated by homogenizing a solution of chloroform and methanol in 2:1 ratio and subsequent addition of a saline solution of 1.5% NaCl. The methylation process of the isolated lipids was carried out as proposed by Kramer et al (1997), and the methyl esters were formed. The FA profile composition was quantified using a gas chromatography (GC-2010 Plus - Shimadzu AOC 20i auto-injector) with a SP-2560 capillary column (100 m × 0.25 mm in diameter with 0.02 mm thickness, Supelco, Bellefonte, PA). With an initial temperature of 70°C, under continuous heating of 13°C per minute, an intermediate temperature of 175°C was established and then maintained for 27 minutes. Thereafter, at a heating speed of 4°C per minute, the temperature was increased to 215°C and maintained for more 31 minutes.

The FA identification was performed by comparing the retention time of the methyl esters of the samples with standards C4-C24 (F.A.M.E mix Sigma®), vaccenic acid C18:1 trans-11 (V038-1G, Sigma®) C18:2 trans-10 cis-12 (UC-61 M 100 mg), CLA e C18:2 cis-9, trans-11 (UC- 60 M 100 mg), and tricosanoic acid (Sigma®). Then, the FA were quantified by normalizing the area under the methyl esters curve using the GS solution 2.42 software, and were expressed as a percentage of the total FA methyl ester. The FA profile determination was performed at the Meat Science Laboratory (LCC) in the Department of Animal Nutrition and Production at FMVZ/USP.

Among the quantified FA, we selected those in higher concentration as well as important for human health: myristic (C14:0), palmitic (16:0), stearic (C18:0), oleic (C18:1 n9), linoleic (C18:2 n6), alpha-linolenic (C18:3 n3), and CLA (C18:2 *cis*9-trans11). The sum of SFA (C4:0–C24:0), MUFA (C14:1 + C15:1 *cis*10 + C16:1 + C17:1 *cis*10 + C18:1 n7 + C18:1 *trans*11 + C18:1 *cis*9 + C18:1 *trans9* + C20:1 *cis*11 + C22:1 n9 + C24:1), PUFA (C18:2 n6 + CLA – *cis*9,*trans*11

+ C18:2 *trans*10,*cis*12 + C18:3 n3 + C18:3 n6 + C20:3 n3 *cis*11,*cis*14,*cis*17 + C20:3 n6 *cis*8,*cis*11,*cis*14 + C20:4 n6 + C20:5 n3 + C22:6 n3), ω-6 (C18:2 n6 + C18:3 n6 + C20:3 n6 *cis*8,*cis*11,*cis*14 + C20:4 n6), and ω-3 (C18:3 n3 + C20:3 n3 *cis*11,*cis*14,*cis*17 + C22:6 n3 + C20:5 n3). Furthermore, the ratio between the sums of ω-6/ω-3 and PUFA/SFA were also determined, reaching out a total of 14 traits.

2.2.4. RNA-seq extraction

Total RNA extraction from the bovine *Longissimus thoracis* muscle (~100 mg) was performed using the TRIzol® (Life Technologies, Carlsbad, CA, USA) extraction protocol. The RNA integrity number (RIN) was verified using the Agilent 2100 Bioanalyzer® (Agilent, Santa Clara, CA, EUA) equipment, and RIN values higher than eight were used. The preparation of the cDNA libraries followed the TruSeq® RNA Sample Preparation v2 protocol (Illumina, San Diego, CA, USA), and a total of 2 µg of RNA from each sample was used. The libraries were quantified using the KAPA Library Quantification kit® (KAPA Biosystems, Foster City, CA, USA). Thereafter, barcode sequences were added for individual sample identification and samples were subjected to sequencing. Paired-end with 2x100 bp was performed on the Illumina HiSeq2500 (Illumina, San Diego, CA, USA), and the TruSeq kits PE Cluster kit v3-cBot-HS and TruSeq SBS kit v3-HS (Illumina, San Diego, CA, USA) were used. The sequencing analysis was performed at the Genome Center at ESALQ (Piracicaba, São Paulo, Brazil).

2.2.5. Reads alignment and gene count

Raw sequencing data generated from the HiSeq platform were converted into FASTQ files and separated into individual libraries using the Casava v.1.8.2 software (Illumina, San Diego, CA, USA). Quality control was performed to remove adapters and PCR primers together with low-quality and small sequences (< 36 bp) using the Trimmomatic v.0.32 (Bolger et al., 2014). Reads were then aligned to the *Bos taurus taurus* genome assembly ARS-UCD1.2 using the STAR v.2.7.0 program (Dobin et al., 2013). After alignment, a second quality control was performed using SAMtools v1.09 (Li et al., 2009) to remove low-

quality alignments, secondary alignments, and PCR duplicates on the binary format (BAM) files. The QualiMap software (García-Alcalde et al., 2012) was used for the visualization and comparison of the alignment quality across all samples. Gene counts were performed by the HTSeq Python package (Anders et al., 2015), and were used for subsequent gene expression analyses (DEG, COE, and DCO).

2.2.6. Differentially expressed genes analysis

To perform the DEG analyses, animals (*n=30*) were grouped based on their FA concentration into two extreme phenotype values groups (highest and lowest FA concentration). Each group harbored 15 samples, and aimed to identify potential genes responsible for phenotypic variation by removing samples with intermediate values. DEG were identified for each FA including their sums and ratios (*n=14*), totaling 28 groups (two extreme groups for each FA). Different animals composed the extreme phenotype groups among the FA since the same animal does not necessarily have the highest concentrations for a wide range of FA. A t-test (Gosset, 1908) was performed, and a significant ($p<0.0001$) difference was observed between the means of the highest and lowest FA concentration groups (Supplementary File 1). Genes counts were normalized, and those below one count per million (cpm) in 90% of the samples for each FA were filtered out. The R package DESeq2 (Love et al., 2014) was used to perform the DEG analysis. The p-values for the DEG were adjusted by False Discovery Rate ≤ 0.1 (Benjamini & Hochberg, 1995). These genes were selected mainly based on the highest Log_2FoldChange (LFC) genes due to the higher gene expression differences between FA groups.

2.2.7. Co-expression analysis

All animals (*n=44*) and FA (*n=14*) were analyzed together using the R package for weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath, 2008). Prior COE analyses, a quality control was applied on the gene expression data, and genes counts <1 cpm in 90% of the samples were filtered out. A default quality control provided by the WGCNA package was

also applied on the gene expression data aiming to eliminate samples lacking gene counts, as well as genes with very low counts. After quality control, a total of 44 samples and 10,739 genes were retained for COE analyses.

The connectivity values (K) were calculated among the genes of the entire network. This measure represents the degree of connection between each pair of genes in the network, and it is obtained by the sum of the Pearson's correlations raised to the soft power threshold (β=4), which should approach the topology criterion without a scale ($R^2 \geq 0.8$) (Zhang & Horvath, 2005). For the gene clustering and module formation, the unsigned step-by-step method was used by considering a minimum size of 30 genes and joining of modules with a similarity of 80%. The topological overlap measure (TOM) between genes was calculated, in which a value of zero represents the absence of neighbors (genes) in common and a value of one denotes the total sharing of neighbors. Genes were clustered into modules and signaled by colors using the dissimilarity matrix (1-TOM) (Yip & Horvath, 2007) and hierarchical clustering algorithms (dynamic tree-cutting) (Langfelder et al., 2008).

The modules were built together with their respective eigengene (ME), a measure that corresponds to the representation of the first principal component of the expression profile of the entire module and the Pearson's correlation was used to link these modules and the phenotypes assessed in this study (Langfelder & Horvath, 2008). Significant modules ($p \leq 0.05$) with a person correlation above |0.4| were selected for further functional enrichment analysis.

2.2.8. Differential co-expression analysis

For the DCO analyses, the same samples (*n=30*) and extreme phenotype values groups (*n=28*) assessed for DEG were used. Each group was submitted to COE analyses with the same thresholds as early described, except for the β parameter that varied in each analysis due to the divergent gene expression values presented between the groups (Supplementary File 2). The K values for each gene were obtained for both extreme phenotype values groups, which were then divided by the maximum connectivity in each network. Values from the high ("K_H") and low ("K_L") FA concentrations groups were used to compute the

difference between the connectivity ("K_{diff}") for each FA, according to the formula below:

$$K_{diff} = K_H - K_L$$

Genes with K_{diff} ≥ | 0.6 | were considered differentially connected and selected for functional enrichment analysis.

2.2.9. Functional enrichment

Using the list of genes retrieved from the DEG, COE, and DCO analyses and the *Bos taurus taurus* annotation file as a background, the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 tool (Huang et al., 2009a, 2009b) was used to identify overrepresented (FDR<0.05) gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. All analyses were performed separately and restricted to the list of genes retrieved from each FA groups (DEG and DCO) and to each significant module (COE).

2.3. Results

2.3.1. Differentially expressed genes analysis

The DEG analysis identified genes in all contrasting FA groups, except for palmitic acid, CLA, and ω-6/ω-3 ratio. A total of 912 DEG were described within the studied FA (Supplementary File 3). The extreme groups for PUFA, alpha-linolenic, ω-3, and myristic FA displayed the highest number of DEG (563, 352, 346, and 216, respectively), whereas the SFA sum, oleic, and stearic FA exhibited the lowest (1, 3, and 15, respectively). Positive Log2FoldChange (LFC) values represent upregulated genes in high concentration groups, while negative values determine downregulated genes in these groups. Table 1 describes the top five DEG with the highest |LFC| values.

Table 1. Five differentially expressed genes with the highest Log2FoldChange (|LFC|) values within the extreme phenotype fatty acid groups.

Gene	Fatty acid	*LFC*	*P-value*	*P-adjusted*[1]
GIPC2	Stearic	2.21	0.000	0.035
	PUFA	-2.21	0.000	0.019
	Linoleic	-2.21	0.000	0.042
	ω-6	-2.21	0.000	0.042
BDH1	PUFA	2.15	0.000	0.034
	Linoleic	2.11	0.000	0.054
	ω-6	2.11	0.000	0.054
	PUFA:SFA	2.08	0.000	0.073
	ω-3	1.89	0.001	0.079
ACTC1	PUFA	-2.05	0.006	0.098
ACSS1	PUFA	1.58	0.000	0.034
	PUFA:SFA	1.54	0.000	0.075
	ω-3	1.52	0.001	0.074
	Linoleic	1.50	0.000	0.071
	ω-6	1.50	0.000	0.071
ENSBTAG00000050626	ω-3	-1.82	0.000	0.071
	Palmitic	1.49	0.001	0.082
	Alpha-linolenic	-1.47	0.004	0.086
	PUFA	-1.29	0.006	0.098

[1] The p-values were adjusted by False Discovery Rate (FDR < 0.1).

Among the top 5 |LFC| DEG identified, we can highlight the GIPC PDZ domain containing family member 2 (*GIPC2*) and *ENSBTAG00000050626* with higher expressions in SFA high concentration groups, stearic and palmitic, respectively. These genes also showed reduced expression in groups with a high concentration of unsaturated FA, such as the actin alpha cardiac muscle 1 (*ACTC1*) gene in these FA. On the other hand, the 3-hydroxybutyrate dehydrogenase 1 (*BDH1)* and synthetase short-chain family member 1 (*ACSS1*) genes were more expressed in unsaturated FA high concentration groups.

2.3.2. Co-expression analysis

From 22 modules detected, three were significantly correlated ($p \leq 0.05$) with $r < |0.4|$ at least one phenotype (Figure 1, Supplementary File 4). The largest modules were the turquoise (*n=1,802* genes) and blue (*n=1,776* genes), while the smallest one was the tan (*n=262* genes).

The blue, tan, and turquoise modules were found to contain the highest number of evaluated phenotypes significantly correlated with their respective eigengenes module (Figure 1). Among them, the tan module showed the highest

positive correlation (r=+0.46) with the sum of MUFA. Conversely, the blue module was negatively correlated (r=-0.44) with the ω-6 sum and the Linoleic FA.

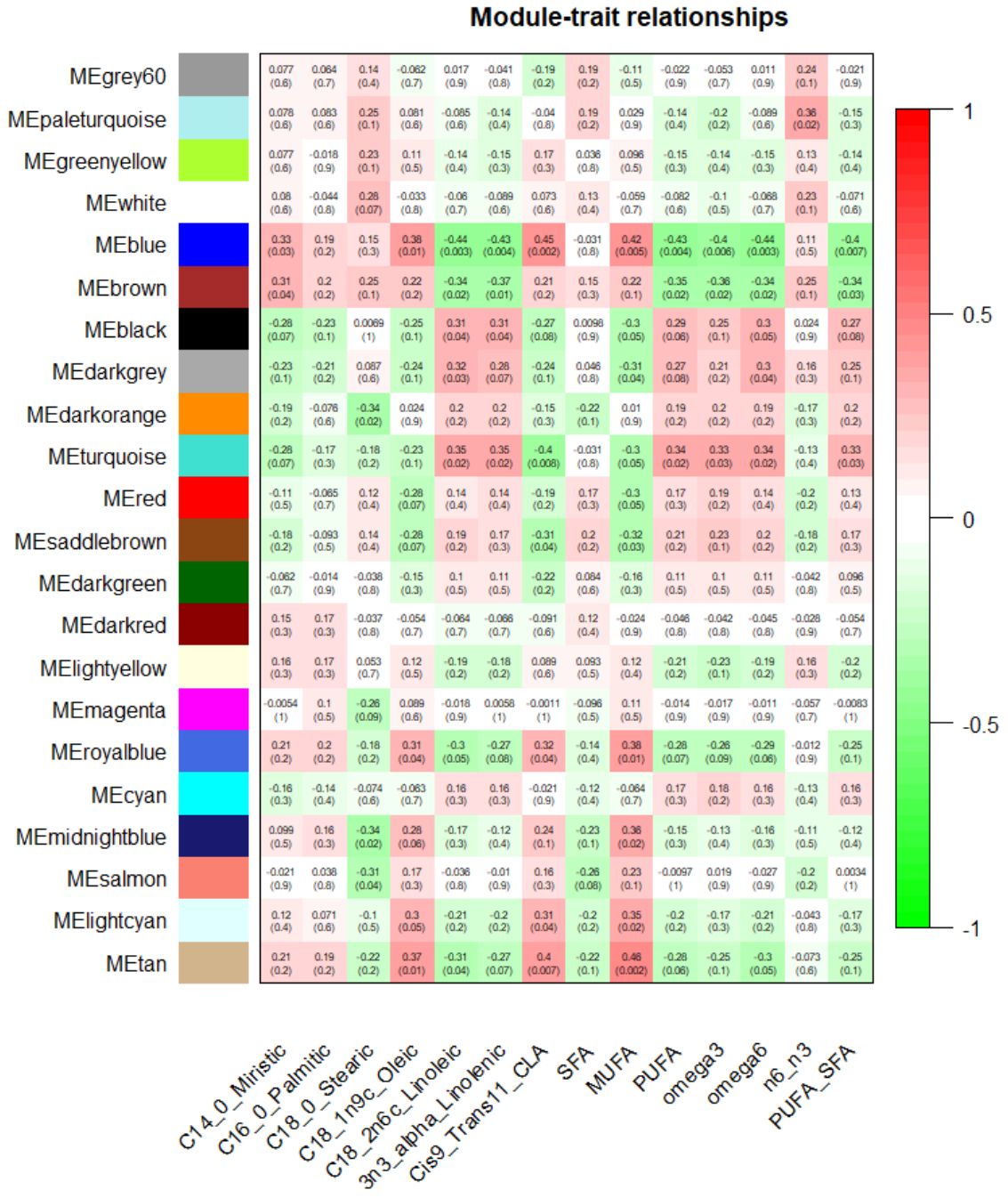

Figure 1. Correlations between the eigengene modules and the evaluated phenotypes of the fatty acid profile for the Nellore beef cattle.

2.3.3. Differential co-expression analysis

The DCO identified genes in all contrasting FA groups, totaling 759 genes (Supplementary File 5). Genes with Kdiff values > +0.6 and < -0.6 indicate better

connectivity with the groups of highest and lowest FA concentrations, respectively. Among the identified genes, 229 were exclusively better connected to groups with the highest FA concentration, 460 with the lowest FA concentration, and 70 genes were identified in situations of connectivity in between both FA concentration groups.

We can highlight the genes such as protein phosphatase 5 catalytic subunit (*PPP5C*), ankyrin repeat and SOCS box containing 5 (*ASB5*), NADH Dehydrogenase (Ubiquinone) Complex I, Assembly Factor 3 (*NDUFAF3*), ATP binding cassette subfamily A member 6 (*ABCA6*) and erythrocyte membrane protein band 4.1 like 3 (*EPB41L3*) with the highest modular Kdiff values (Table 2). All of them presented a similar pattern, they were better connected to the lowest concentration groups of unsaturated FA, except *EPB41L3* gene that pointed out a connectivity with the highest concentration groups of ω-6 sum and linoleic acid. Moreover, the oleic acid showed the highest number of DCO genes (*n=358*), followed by MUFA sum (*n=120*).

Table 2. List of the five differentially co-expressed genes with the highest Kdiff ≥ | 0.6 | values within the extreme phenotype fatty acid groups.

Gene	**Fatty acid**	**K_{diff}**
PPP5C	Oleic	-0.93429
	MUFA	-0.84057
ASB5	Alpha-linolenic	-0.93201
	ω-6	-0.89478
	Linoleic	-0.89478
	ω-3	-0.83049
	PUFA:SFA	-0.82298
	PUFA	-0.81610
	Myristic	+0.68775
NDUFAF3	Oleic	-0.92544
	MUFA	-0.77802
ABCA6	Oleic	-0.92093
	MUFA	-0.69957
EPB41L3	Oleic	-0.90548
	MUFA	-0.62544
	ω-6	+0.63245
	Linoleic	+0.63245

2.3.4. Functional analysis

The enrichment analysis was performed individually for each analysis (DEG, COE, and DCO) on DAVID tool to obtain a broad functional insight about the predicted gene networks.

When considering the DEG identified, a total of 243 GO terms – 116 biological processes (BP), 103 cellular component (CC), and 24 molecular function (MF) – and 13 KEGG pathways were identified as enriched (FDR<0.05, Supplementary File 6). With the exception of the groups of myristic, stearic, CLA and oleic acids, and SFA sum and ω-6:ω-3 ratio, all other phenotypes had DEG enriched in GO:Terms (Supplementary File 6). We can highlight the alpha-linolenic acid and PUFA sum, with 102 and 95 enriched BP, respectively. Among the biological processes, the “mitochondrion organization” (GO:0007005, *n=71* genes) stands out for exhibiting the highest frequency (*n=6*) among the evaluated FA groups, followed by the and “oxidation-reduction process” (GO:0055114, *n=66* genes), enriched in four phenotypes (Figure 2a). The “oxidative phosphorylation” (bta00190, *n=33*) appears as the most frequent KEGG pathway (Figure 3a). With a smaller number of related phenotypes, the “PPAR signaling pathway” (bta03320, *n*=9 genes) was also enriched in the PUFA sum contrasting groups. Regarding this pathway, we can highlight the acetyl CoA acyltransferase 1 (*ACAA1*), acyl-CoA oxidase 2 (*ACOX2*), and stearoyl-CoA desaturase 5 (*SCD5*) genes.

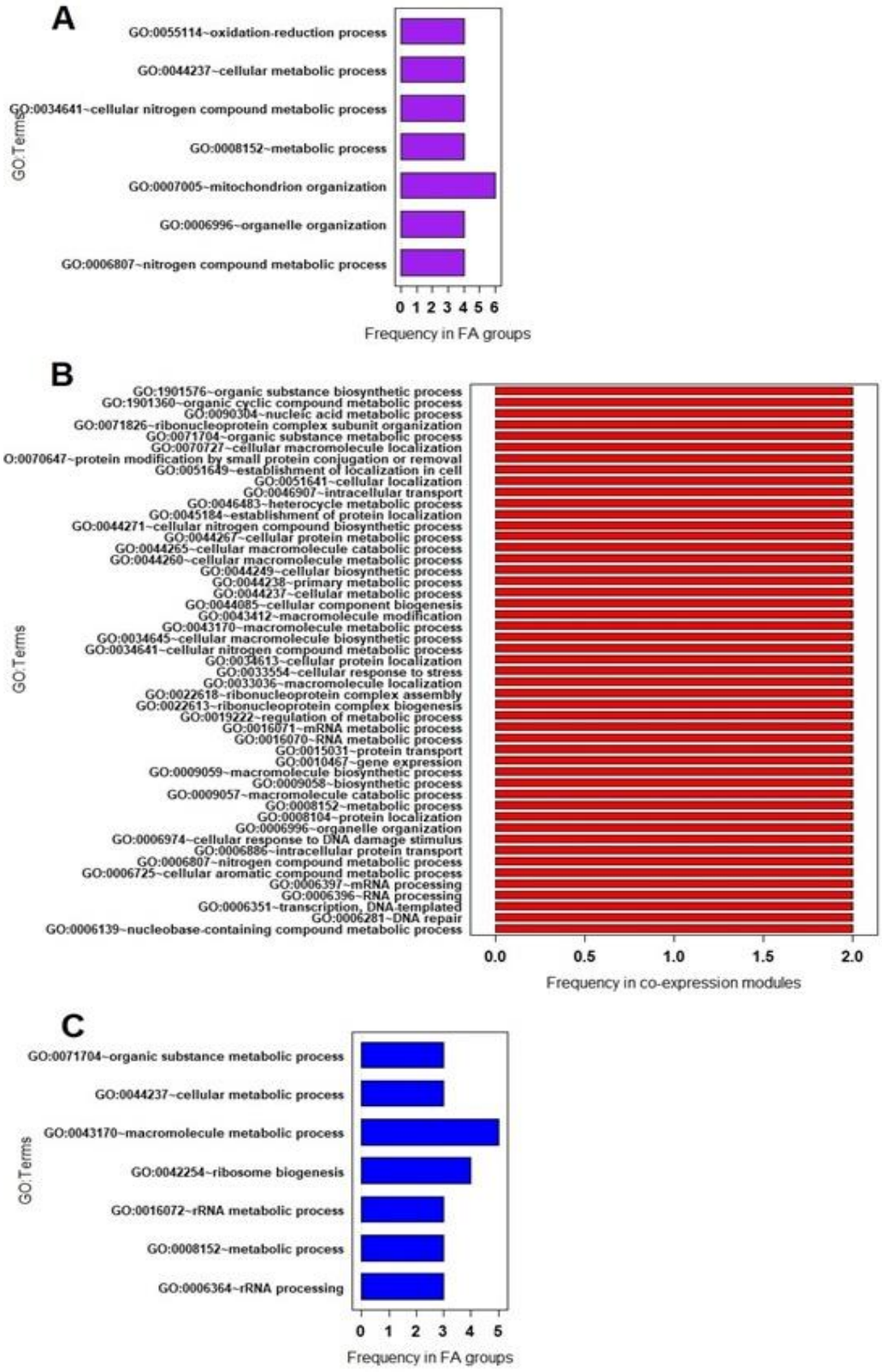

Figure 2. Most frequent Biological Process retrieved from the list of differentially expressed genes (a), co-expressed gene modules (b) and differentially co-expressed genes (c).

The genes inserted in the three significant modules ($p<0.05$, $r>|0.4|$) in the COE analysis were enriched in 319 GO terms – 182 BP, 96 CC, and 41 MF – and 7 KEGG pathways (Supplementary File 7). The most frequent biological processes were the “metabolic process” (GO:0008152, *n=1,578* genes) and “gene expression” (GO:0010467, *n=850* genes (Figure 2b). In the blue module, the adiponectin receptor 1 (*ADIPOR1*), 2,4-dienoyl-CoA reductase 2 (*DECR2*), and insulin receptor substrate 2 (*IRS2*) genes inserted in the “metabolic process” (GO:0008152), as well as the lipase hormone-sensitive (*LIPE*) and CREB regulated transcription coactivator 1 (*CRTC1*) genes in the “gene expression” (GO:0010467) should be highlighted. In the turquoise module, we can underscore the 2,4-dienoyl-CoA reductase 1 (*DECR1*) gene enriched in the “metabolic process” (GO:0008152) as well as the insulin induced gene 2 (*INSIG2*) in the “gene expression” (GO:0010467). We can also highlight the KEGG pathway “Spliceosome” (bta03040) and “RNA transport” (bta03013), in which the turquoise and blue modules presented, respectively to each pathway, 28 and 32, and 20 and 26 genes inserted (Figure 3b).

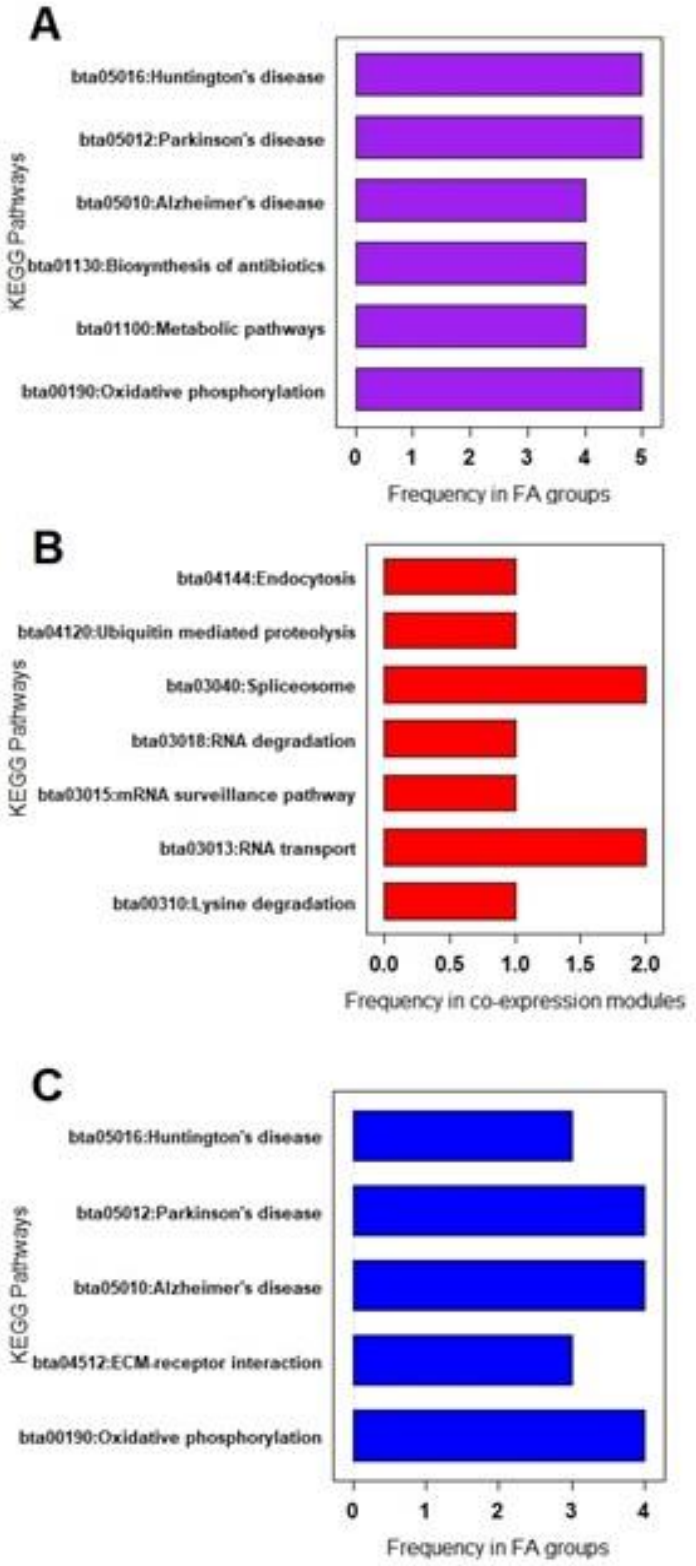

Figure 3. Most frequent KEGG Pathways retrieved from the list of differentially expressed genes (a), co-expressed gene modules (b) and differentially co-expressed genes (c).

The DCO analysis identified 173 GO terms – 68 BP, 91 CC, and 14 MF – and 12 KEGG pathways enriched (FDR<0.05, Supplementary File 8). The “macromolecule metabolic process” (GO:0043170, *n=104* genes) was present in five out of 14 phenotypes evaluated (palmitic, myristic, linoleic, and ω-3 and ω-6 sums). It was followed by the “ribosome biogenesis” (GO:0042254, *n=12* genes), described for the alpha-linolenic, linoleic, and ω-3 and ω-6 sums. Further, the “metabolic process” (GO:0008152, *n=89*) was pointed out among the myristic, linoleic, and ω-6 sum groups. Within these biological processes, we can highlight the carnitine palmitoyl transferase 2 (*CPT2*) and ER lipid raft associated 1 (*ERLIN1*) genes. The KEGG pathway “oxidative phosphorylation” (bta00190, *n=21* genes) was the most frequent (Figure 3c), and it was described for the unsaturated fatty acids, linoleic, oleic, and MUFA and ω-6 sums. Besides, the succinate dehydrogenase complex iron sulfur subunit B (*SDHB*) gene, identified within this pathway, should be highlighted.

2.3.5. Overlapping genes among the expression analysis

By evaluating the genes overlapping among the results of the three analyses (Figure 4, Supplementary File 9), it was possible to observe the complementarity among them. Unique genes were identified in each expression analysis, however, 771 genes were common in at least two of them, reinforcing the importance of such genes in determining the phenotype. Interestingly, common genes between DEG and COE (*n=461*, Supplementary File 10), DCO and COE (*n=304*, Supplementary File 11), and DEG and DCO (*n=178,* Supplementary File 12) were identified. Genes such as the *DECR2,* acyl-CoA synthetase long-chain family member 3 *(ACSL3)*, and fat storage inducing transmembrane protein 1 (*FITM1*) were common to DEG and COE analyses. The enoyl-CoA delta isomerase 1 (*ECI1*) and succinate dehydrogenase complex iron sulfur subunit B (*SDHB*) genes were common to DEG and DCO results.

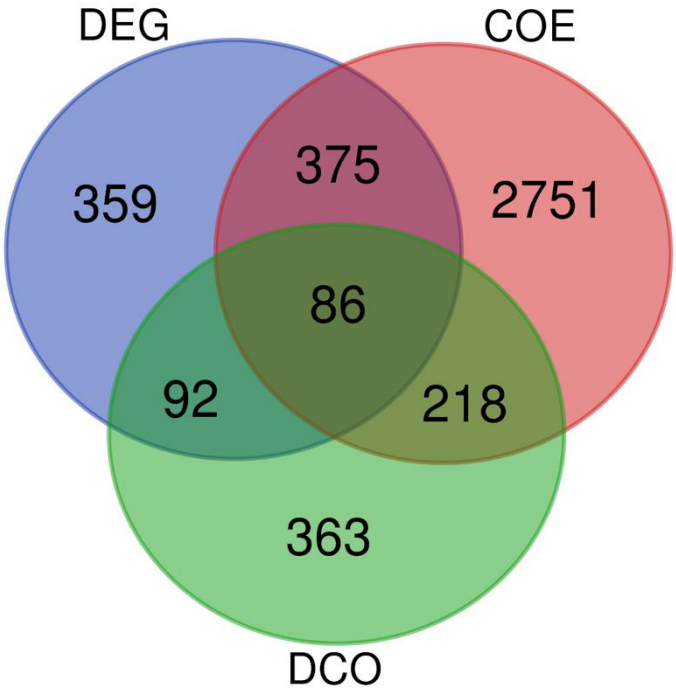

Figure 4. Venn diagram of the genes in common among the three different expression analysis. DEG: Differentially expressed genes analysis; COE: Co-expression analysis, and DCO: Differential co-expression analysis

2.4. Discussion

2.4.1. Differentially expressed genes

Among the top five DEG with the highest |LFC| values, the ENSBTAG00000050626 (LncRNA, 23:24,559,298-24,573,333 bp) gene codes for two miRNA (bta-mir-206 and mir-133b), and have gene targets related to the muscle development and metabolism in cattle (Sun et al., 2013; Zhang et al., 2016). The second gene worth to be highlighted is the *GIPC2*. Its expression increases upon high estrogen concentrations (Mercier et al., 2009), and this hormone has been linked to the resolution of inflammatory conditions induced by a high concentration of SFA in humans (Miller et al., 2012). Interestingly, the *GIPC2* gene was upregulated in groups of high concentration of stearic acid and downregulated in high groups of PUFA, linoleic, and ω-6 sum (Table 1). These findings allow us to hypothesize the existence of a relationship between the *GIPC2* gene and the stearic acid, which suggests the participation of *GIPC2* in inflammatory pathways triggered by estrogen SFA induced.

The *ACTC1* gene (LFC |2.05| for PUFA) has functions related to muscle development and growth (Bertola et al., 2008) as well as to meat quality traits (Bongiorni et al., 2016) in cattle. Bongiorni et al. (2016) identified such gene downregulated in groups of higher tenderness in two Italian cattle breeds. Muñoz et al. (2018) evaluated the intramuscular fat content (IMF) of the *Longissimus dorsi* muscle in pigs and observed a lower expression of the *ACTC1* gene in groups with high IMF content. This information corroborates with our results, in which the *ACTC1* gene was downregulated in the high PUFA group, suggesting a relationship with fat content, and consequently, fatty acids concentration.

The *ACSS1* and *BDH1* genes showed a similar expression pattern, with greater expression in the high concentration of unsaturated FA groups (Table 1). They were described enriched in the “metabolic process” (GO:0008152) and “metabolic pathway” (bta01100). The *ACSS1* gene participates in acetyl-CoA synthesis from acetate for FA formation (Xu et al., 2018), which justifies its upregulation in groups with a high FA concentration. However, although the *BDH1* gene acts on the degradation of ketone bodies (Nakamura et al., 2014), it was found more expressed in high marbling groups in Nellore beef cattle (Fonseca et al., 2020), which allows us to infer that there is a relationship with higher concentrations of FA.

The most frequent BP in the DEG analysis (“mitochondrion organization”, GO:0007005) aggregates genes related to mitochondrial synthesis and oxidative functions. It should be noted that most FA oxidation occurs inside the mitochondria through successive enzymatic dehydrogenation processes (Houten & Wanders, 2010). The “oxidation-reduction process” (GO:0055114) is also related to the FA synthesis, which is regulated by the PPAR family genes (i.e., *PPARA* and *PPARG* genes), responsible for modulating the expression of genes linked to mitochondrial β-oxidation processes (Rakhshandehroo et al., 2010; Nakamura et al., 2014). Therefore, these BP can highly impact the beef FA profile, and it is not surprising that they were the most frequent ones among the significant terms identified. The PPAR family also interacts with several important genes related to energy and lipidic metabolism, such as sterol regulatory element binding transcription factor 1 (*SREBF1*), acetyl-CoA carboxylase alpha (*ACACA*), CCAAT/enhancer-binding proteins (*CEBP*), and retinoid X receptor (*RXR)* (Lefterova & Lazar, 2009; Sevane et al., 2013). Hence, we can also

highlight the “PPAR signaling pathway” (bta03320), one of the main pathways that coordinate the lipid metabolism (Walczak & Tontonoz, 2002). The *ACAA1*, *ACOX2* and *SCD5* genes were identified within this pathway, and were upregulated in the high PUFA group.

Notwithstanding, the *ACAA1* is a target gene regulated by the *PPARA* (Rakhshandehroo et al., 2010), an important gene related to the oxidative process. The role of the ACAA1 gene in our results can be justified due to its function in the final stages of PUFA biosynthesis in cattle (Cherfaoui et al., 2012). Nevertheless, higher IMF rates were related to a higher expression of genes such as the *ACOX2* in Hanwoo cattle (Lim et al., 2015). Although both genes are related to FA oxidation steps, they are also linked to fat deposition as well as FA profile, especially for PUFA. This allows us to suggest that this occurs as a way of controlling high concentrations of PUFA in the muscle. On the other hand, the *SCD5* (Δ9-desaturase) and *SCD* genes act mainly on SFA (Igal & Sinner, 2021) and can interfere with the SFA:unsaturated FA balance. Thus, in a study by Waters et al. (2009) evaluating the *Longissimus* muscle of Limousin and Charolais cattle, the highest expression of Δ9-desaturases was related to higher concentrations of PUFA n-6. Also, SNP-like variations in the *SCD5* gene were associated with higher concentrations of PUFA in the milk of Holstein cows (Rincon et al., 2012), corroborating our results.

The “oxidative phosphorylation” (bta00190) was also enriched in the DEG analysis. This pathway, with some gene members of NADH:ubiquinone oxidoreductase and cytochrome c oxidase families, have been related to energy metabolism through the FA oxidation. This pathway was also pointed out by Silva-Vignato et al. (2019) in a COE analysis as negatively correlated to ribeye area in Nellore cattle. It may indicate the relationship of this pathway with the regulation of lipid deposition and the muscle FA profile in cattle, corroborating with the present study that pointed it out among the contrasting groups of different FA categories.

2.4.2. Co-expression analysis

The COE analysis allowed us to point out gene networks enriched in BP and pathways related to fat metabolism and FA. The blue and tan modules showed a similar pattern of correlations, i.e., positively correlated with CLA and MUFA (Figure

1). Also, the blue module was negatively correlated with linoleic and alpha-linolenic acids as well as PUFA, ω-3 and ω-6 sums, and PUFA:SFA ratio. This fact might be due to the presence of genes such as the *ADIPOR1*, *DECR2*, and *IRS2* in the blue module, enriched in the most frequent term ("metabolic process", GO:0008152). Also in the blue module, the *LIPE*, and *CRTC1* genes were enriched in the BP "gene expression" (GO:0010467).

The three genes highlighted within the "metabolic process" (GO:0008152) trigger the FA oxidation steps in different ways. The *ADIPOR1* gene was related to the higher FA oxidation via AMPK signaling in rodent models (Saha et al., 2014); the *IRS2* gene promotes the oxidation via insulin signaling (Wakil & Abu-Elheiga, 2009); and the *DECR2* acts on the FA oxidation in peroxisomes (Rakhshandehroo et al., 2010). Notwithstanding, the *ADIPOR1* gene was related to severe insulin resistance in knockout mice, leading to disruptions in the energetic metabolism (Nakamura et al., 2014; Yamauchi et al., 2007). On the other hand, the *DECR2* gene was upregulated in mice' hepatocytes when submitted to glucagon treatment or prolonged fasting, triggering FA oxidation via gluconeogenesis (Longuet et al., 2008). This set of genes related to the oxidation processes present in the blue module supports the wide negative correlations with the FA evaluated, mainly with the PUFA.

The "gene expression" (GO:0010467) involves production, maturation, or translation RNA processes. The *LIPE* and *CRTC1* genes act in different ways to control the lipid content, regulated by the PPAR family (i.e., *PPARA* and PPARG genes) (Hu et al., 2021; Rakhshandehroo et al., 2010). The hormone-sensitive lipase (HSL) is encoded by the *LIPE* gene, and it acts in the lipolytic process by hydrolyzing triglycerides into diglycerides and diglycerides into monoglycerides, as well as producing non-esterified fatty acids (NEFA) that can participate as ligands of lipid metabolism regulator genes, and ATP synthesis by β-oxidation (Lass et al., 2011). Also, HSL was positively correlated (r=+0.49) with IMF in Korean steers (Jeong et al., 2012) and had different SNP genotypes associated with the FA profile, especially to higher MUFA sum and total FA content in the *Longissimus dorsi* of Chinese Simmental cattle (Fang et al., 2017). In this regard, the *LIPE* gene and the intramuscular FA content relationship could be justified by a lipid homeostasis mechanism to which it is

related, corroborating with our wide correlation coefficient identified among the phenotypes.

The *CRTC1* gene, a member of the cAMP-responsive element binding (*CREB*) family, has a regulatory potential on lipid metabolism due to its relationship with the *PPARG* gene in white adipose mice tissue (Hu et al., 2021) as well as fatty acid synthase *(FASN)* and sterol regulatory element binding transcription factor 1 (*SREBF1)* genes in hepatic mice cell culture (Kim, 2016). Since the *CRTC1* gene acts activating the *CREB1* gene, the *CRTC1* gene promote indirectly *CREB1* target genes. The overexpression of the *CREB1* gene in goat mammary epithelial cells promoted upregulation of *SREBF1* and *ELOVL6*, which is related to MUFA synthesis (Jakobsson et al., 2006; Yao et al., 2020). This reaffirms our results when the blue module was correlated to MUFA sum (+0.42, *p= 0.005*).

The turquoise module negatively correlated with CLA (-0.40, *p=0.008*), and the genes inserted within this module were also enriched in “metabolic process” (GO:0008152) and “gene expression” (GO:0010467). In the “metabolic process” (GO:0008152), we can highlight the *DECR1* gene related to FA oxidation processes (Cotter et al., 2013; Houten & Wanders et al., 2010). The *DECR1* gene is regulated by *PPARA* gene on different unsaturated fatty acids oxidation steps, mainly on PUFA (Rakhshandehroo et al., 2010; Weeghel et al., 2012). The *INSIG2* enriched in “gene expression” (GO:0010467) interacts with the complex among *SREBF1* and SREBP cleavage-activating protein (*SCAP*) genes, and the maintenance of this interaction inhibits the FA synthesis, and therefore, triggers higher FA oxidation (Colliti et al., 2014; Howe et al., 2016). Regarding that, the unsaturated fatty acids were able to stabilize the complex INISG/SCAP/SREBP, blocking the signaling and gene regulation cascade from the *SREBF1* gene (Ye & DeBose-Boyd, 2011). Further, it supports the negative correlation of the turquoise module with traits related to the unsaturated fatty acids concentration, such as MUFA sum.

The KEGG pathways “RNA transport” (bta03013) and “Spliceosome” (bta03040), enriched in blue and turquoise modules, refer to steps of the transcription and translation RNA processes essential to gene expression. The RNA transport involves different steps to transport the RNA from the nucleus to the correct site where this RNA should be, in the case of mRNA, translated into a functional protein (Kindler

et al., 2005). Concomitant, the spliceosome, a complex molecule formed by a wide protein subunit, acts in maturing RNA molecule processes, mainly through intron removal mechanisms, called splicing and exon rearrangement (Will & Luhrmann et al., 2011). Both pathways support the BP "gene expression", also identified in the two modules, being fundamental for the gene expression machinery related to cellular metabolism, including those related to lipid and FA metabolism.

2.4.3. Differential co-expression analysis

The DCO analysis indicated genes better connected to the evaluated FA, which were not necessarily identified as differentially expressed. Among them, we can highlight those with the highest K_{diff} value, including the *PPP5C, ABCA6, NDUFAF3, EPB41L3*, and *ASB5* genes.

The *PPP5C* gene was described enriched in the MF “binding” (GO:0005488), and it is related to fat deposition and FA synthesis. It binds to the *PPARG* gene (Hinds et al., 2011), which indicates that the *PPP5C* is involved in a potential regulatory mechanism in the low oleic acid (K_{diff}=-0.93) and MUFA (K_{diff}=-0.84) groups. The *PPP5C* gene is a catalytic subunit of the PPP5 protein. In an experiment carried out with mouse cell cultures, knockout animals for the *PPP5* showed lower lipid deposition as well as lower FA synthesis (Hings et al., 2011), reiterating the relationship of this gene with the FA profile.

The *ABCA6* gene*,* also inserted in the MF “binding” (GO:0005488), belongs to a gene family with functions in the plasma membrane of macrophages by transporting substrates, and in particular lipids and cholesterol molecules (Schmitz, Kaminski, & Orsó, 2000). This gene is involved in the intracellular cholesterol flow (Wenzel et al., 2007), a function closely linked to the action of genes such as the *SREBF1* and its target genes in cholesterol control, impacting the intracellular FA deposition (Howe et al., 2016). It justifies the greater connectivity of the *ABC6* gene with the low oleic acid (K_{diff}=-0.92) and MUFA (K_{diff}=-0.69) concentration groups.

The *NDUFAF3* gene was described enriched in the CC “mitochondrion” (GO:0005739). This gene was better connected to groups with low oleic acid concentration (K_{diff}=-0.92) and MUFA sum (K_{diff}=-0.78). The *NDUFAF3* gene is inserted in a large gene family that encodes subunits for a complex mitochondrial enzyme

formation responsible for energy production (Gabaldón et al., 2005). Although there is no clear explanation of the relationship between the genes of this family and the FA, Silva-Vignato et al. (2019) in a co-expression study identified them in a negatively correlated module with ribeye area in Nellore cattle. This result was reinforced by Cesar et al. (2015), in which the *NDUFB11* gene was related to intramuscular fat deposition in Nellore cattle.

Involved in cell structure and proliferation, the *EPB41L3* gene was also linked to pro-inflammatory situations of cell stress with the production of reactive oxygen species (ROS) (Olszewska et al., 2012). ROS are stimulated by high concentrations of PUFA, especially ω-6 (Liu et al., 2019; Wahli & Michalik, 2012), and lower of MUFA, such as oleic acid (Wahli & Michalik, 2012), corroborating our results.

The *ASB5* gene, enriched in “metabolic process” (GO:0008152), is associated with cell growth and acts on vascular and muscle development. Together with genes related to lipid metabolism, it was identified as upregulated in low marbling groups in Nellore cattle (Fonseca et al., 2020) as well as upregulated in the intramuscular fat of crossed cattle (Wang et al., 2009). These facts may suggest the relationship of the *ASB5* gene in the FA determination.

Besides the genes and terms previously described, we can also point out the *CPT2* and *ERLIN1* genes. Such genes were found enriched in the “macromolecule metabolic process” (GO:0043170) and “metabolic process” (GO:0008152), respectively, and were chosen due to their relationship with the FA profile determination and fat deposition (Houten & Wanders, 2010; Huber et al., 2013).

The *CPT2* gene acts on the FA oxidation since its protein product is located on the inner membrane of the mitochondria and it is responsible to FA uptake (Houten & Wanders, 2010). The *CPT2* gene is related to members of the *PPAR* family, in particular to *PPARA* gene, involved to FA oxidation steps (Mandard et al., 2004; Rakhshandehroo et al., 2010). Unsaturated fatty acids, such as linoleic acid (C18:2 ω-6), act as *PPARA* agonists and this interaction promotes the *CPT2* gene expression (Brown et al., 2018; Wahli & Michalik, 2012). Thus, our findings that there is higher connectivity of the *CPT2* gene in the high linoleic acid (+0.66) and PUFA:SFA ratio (+0.69) groups are justified.

The *ERLIN1* gene was identified as the better connected in low concentration groups of linoleic (-0.81), alpha-linolenic (-0.76), PUFA (-0.74) and PUFA:SFA ratio (-0.78), as well as in the high concentration group of myristic acid (+0.65). The *ERLIN1* gene acts synergistically with the *ERLIN2, SCAP*, and *INSIG1/2* genes in modulating the *SREBF1* gene expression, responsible for the cholesterol homeostasis (Howe et al., 2016). According to Huber et al. (2013), the *ERLIN1* gene was also responsible for controlling lipid deposition, impacting the rate of free FA and the formation of triglycerides in cell culture. This fact justifies the relationship of this gene both in the high group of myristic acid and in the low groups related to PUFA.

The second most frequent biological process, “ribosome biogenesis” (GO: 0042254), involves a set of genes producing ribosome functional parts, which participates in the translation processes since recognition and binding to cap-structures from mRNA until protein release (Ingolia et al., 2019). This process impacts other gene expressions and, therefore, indirectly modulates the protein expression related to the FA profile.

The “oxidative phosphorylation” pathway (bta00190) represents a regulatory mechanism of energetic balance that is associated with FA β-oxidation in mitochondria (Wang et al., 2010). The higher unsaturated fatty acids uptake and derivates promote the oxidative-related gene expression and, in this way, triggers FA profile changes (Clarke, 2004; Wahli & Michalik, 2012). The *SDHB* gene, enriched in this pathway, encodes a hydrophilic enzyme core-forming subunit of the succinate dehydrogenase (SDH), which participates in the ATP synthesis through the Krebs cycle (Rutter, Winge, & Schiffman, 2010). The SDH enzyme was also linked to intramuscular fat deposition, in which the lower SDH activity was associated with higher IMF in Korean cattle Longissimus dorsi (Jeong et al., 2013). Notwithstanding, in Nellore cattle *Longissimus dorsi muscle*, Silva-Vignato et al. (2019) identified this pathway in a gene co-expression module correlated to the ribeye area, a trait related to muscularity and backfat thickness, a carcass indicator of fat deposition. Cesar et al. (2016) also identified the "oxidative phosphorylation" pathway in Nellore steers when evaluating DEG among contrasting oleic acid concentration groups. Thus, this corroborates the functional enrichment of this pathway in contrasting FA groups, especially for the oleic acid.

2.4.4. Overlapping genes among the expression analysis

The complementarity of these analyzes (DEG, COE, and DCO) allows us to indicate potential genes linked to the FA profile in the intramuscular fat of Nellore cattle finished in feedlot. By crossing the results, we were able to point out five genes (*ACSL3, DECR2, ECI1, FITM1*, and *SDHB*) with outstanding functions present in at least two analyzes.

Identified in DEG and COE analyses, the *ACSL3, FITM1,* and *DECR2* genes are related to the synthesis/degradation of fatty. These genes are regulated by the PPAR family, reiterating their influence in the FA profile modulation (Rakhshandehroo et al., 2010; de la Rosa Rodriguez et al., 2017). The *ACSL3* gene has a regulatory potential in the lipogenesis process due to its relationship in the FA uptake and by providing substrate for the triglyceride synthesis and FA oxidation (Rakhshandehroo et al., 2010; Yan et al., 2015). This gene was downregulated in the ALA (*LFC = -0.42, p-adj = 0.069)* and PUFA sum (*LFC = -0.44, p-adj = 0.068*) higher groups. It was inserted in the tan module, which was positively correlated to MUFA sum (r = +0.46, p = *0.002*) and CLA (r = +0.40, p = *0.007*). These findings suggest a regulatory function to maintain a lipidic homeostasis, mainly for unsaturated FA.

The *FITM1* gene was correlated with lipid deposits in muscle cells (Gross et al., 2011) and higher fat accumulation in cell cultures (Kadereit et al., 2008). This relationship with lipidic deposition reinforces the *FITM1* gene influence on FA concentration and, consequently, on FA profile. Although it is not possible to specify the influence on specific FA, this corroborates with our results that pointed out the *FITM1* gene more expressed in the ALA-H group (*LFC = +0.35, p-adj = 0.079)* and inserted in the blue module, correlated with several FA phenotypes.

The *DECR2* gene acts on the FA oxidation process in peroxisomes regulated by *PPARA* (Rakhshandehroo et al., 2010). In our study, *DECR2* gene was upregulated in unsaturated fatty acid higher groups (ω-6 sum, PUFA sum, and Linoleic). Further, it was inserted in the blue module, which was correlated with eight out of 14 FA evaluated, especially to unsaturated fatty acids. In this way, it is possible to suggest that the *DECR2* gene optimizes the oxidation steps of unsaturated fatty acids in peroxisomes.

Crossing the DEG and DCO results, we can also point out the *ECI1* and *SDHB* genes. Both of them are responsible for the FA oxidation steps (Houten & Wanders, 2010; Wang et al., 2010) and are mainly regulated by the fat concentration and gene activation through different FA classes (Janssen & Stoffel, 2002; Jeong et al., 2013). The *ECI1* gene codes the 3,2-trans-enoyl-CoA isomerase enzyme, which plays a fundamental role in β-oxidation of unsaturated FA, especially the oleic acid (Janssen & Stoffel, 2002; Houten & Wanders, 2010). The *ECI1* gene overexpression was also associated with a higher concentration of intramuscular fat in swine as well as related to the oleic acid concentration (Lu et al., 2017). These findings support the relationship of the *ECI1* gene better connected to the low oleic group (K_{diff} = *-0.68*) and upregulated in the higher PUFA sum group (*LFC = +0.33, p-adj = 0.099*).

The *SDHB* gene, already discussed in the previous section, was better connected to the MUFA sum (K_{diff} = *-0.60)* and oleic (K_{diff} = *-0.73)* low groups, and upregulated in PUFA sum (*LFC = +0.27, p-adj = 0.068)* and alpha-linolenic acid (*LFC* = +0.29, *p-adj = 0.062*). This gene was inversely associated with IMF, in which its lower expression triggered higher fat deposition in Korean cattle (Jeong et al., 2013). It corroborates our result that pointed out that DCO was better connected to groups with lower FA concentration. Notwithstanding, it also supports the hypothesis that the *SDHB* gene was upregulated in high concentration groups in DEG analysis due to the higher FA concentration in an attempt to maintain lipid homeostasis.

In summary, these methodologies allowed us to point out potential genes as biomarkers, as well as biological processes and KEGG pathways involved in determining the FA profile. The three analyzes were complementary since unique genes were identified in each one, but also some genes were identified in more than one analysis. Part of these genes was corroborated by studies that evaluated a smaller number of FA and correlated traits, such as intramuscular and subcutaneous fat deposition in cattle. However, unexplored genes associated with energy and lipid metabolism as well as transcriptional and translational machinery were pointed out.

2.5. Conclusion

With the combination of DEG, COE and DCO analyzes, we were able to point out gene groups with the ability to modulate the beef FA profile. Among the genes identified in each analysis, we can highlight the *GIPC2,* with the highest |LFC| in DEG analysis, but also *LIPE* and *INSIG2* inserted in COE modules, and the *PPP5C* and *ASB5* genes*,* with highest K_{diff} values in DCO analysis. Also, the *ACSL3, DECR2, ECI1, FITM1*, and *SDHB* genes were identified overlapping in two of the three analyzes as potential regulatory genes of the FA profile in the *Longissimus thoracis* of the Nellore cattle finished in feedlot. Furthermore, the most frequent biological processes and pathways (GO:0055114, GO:0010467, bta00190, and bta03320) remind us of the pivotal role of the PPAR family, as well as the oxidative mechanisms and transcriptional processes linked to energy and lipid metabolism that coordinate the FA composition in the musculature.

Our results contribute in identifying potential biomarkers for complex and late phenotypes, such as the beef FA profile. It would support genomic predictions, not yet commonly performed, in the early selection of animals with healthier meat, by providing *a priori* information on gene groups with higher impact on the phenotype. Such information can be used to weight markers located in genomic regions in which they are inserted/near to the genes found in this study.

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CAPÍTULO 3 – Regulatory genes related to the essential fatty acid profile (Linoleic and Alpha-linolenic acids) of *Longissimus thoracis* in Nellore cattle finished in feedlot

ABSTRACT – Beef is a source of essential fatty acids (EFA) – linoleic (LA) and alpha-linolenic (ALA), which protect against inflammatory and cardiovascular diseases in humans. However, the intramuscular EFA profile in cattle is a complex and polygenic trait. Thus, this study aimed to identify potential regulatory genes to the essential fatty acids profile in *Longissimus thoracis* of Nellore cattle finished in feedlot. Forty-four young bulls clustered in four groups of fifteen animals with extreme values for each FA were evaluated through the differentially expressed genes analysis and two differential co-expression methodologies (WGCNA and PCIT). We highlight the *ECHS1, IVD, ASB5, ERLIN1* and *NFIA* genes to both FA, and *NFYA, NFYB, PPARG, FASN* and *FADS2* to LA, and RORA and *ELOVL5* to ALA. Furthermore, the functional enrichment analysis pointed out several terms related to fatty acids metabolism. These findings contribute to understanding the genetic mechanisms underlying the beef EFA profile in Nellore cattle finished in feedlot.

Key words: co-expression, differential co-expression, Regulatory Impact Factor, Differential hubbing genes, PCIT, WGCNA

3.1. Introduction

Beef is a source of fats, proteins, vitamins and minerals that take place in several metabolic pathways in humans (Biesalski, 2005). Among the fatty acids (FA), the basic unit of lipids, the beef provides essential fatty acids (EFAs) such as the linoleic (LA – C18:2 n6) and alpha-linolenic (ALA – C18:3 n3). These FA are not naturally synthesized by mammals and have been associated with lower incidences of inflammatory and cardiovascular diseases in humans (Eilander et al., 2015). The lipid absorption and deposition in cattle is influenced by the effects of genetic and ruminal metabolism (Costa et al., 2018; da Costa et al., 2013; Fiorentini et al., 2015; Osorio & Moisa, 2019). Given this, the Nellore beef cattle (*Bos taurus indicus*) displayed higher LA and ALA concentrations deposited in the *Longissimus thoracis* muscle than Angus cattle (*Bos taurus taurus*) pasture-finished (Rossato et al., 2010).

Although the genomic-wide association studies (GWAS) have assisted in the understanding of complex traits, i.e., FA profile and intramuscular fat (IMF) deposition in cattle (Cesar et al., 2014; Fonseca et al., 2020; Lemos et al., 2016), this methodology displayed some difficulties to point out genomic regions that explain a high proportion of genetic variance as well the cause-and-effect relationship among the genotype and EFA profile. In an attempt to mitigate these limitations, the results generated by transcriptomic analyzes such as differentially expressed genes (DEG), co-expression (COE) and differential co-expression (DCO) are used.

Notwithstanding the DEG analysis has been widely used to achieve a better understanding of genetic mechanisms, it is not sufficient to fully identify the gene groups related to complex traits since it does not allow the identification of potential genes that are not differentially expressed (Hudson et al., 2012). Hence, the DCO emerges due to the capacity to evaluate the globally gene expression and its interrelationships to indicate sub-networks and better connected genes to the phenotype (Chowdhury et al., 2019; Hudson et al., 2012; Singh et al., 2018).

The combined use of two co-expression algorithms, weighted gene co-expression network analysis (WGCNA) and partial correlation information theory (PCIT), are able to point out potential gene groups related to complex traits through different ways (Langfelder & Horvath, 2008; Reverter & Chan, 2008). The WGCNA

group genes in co-expression modules based on gene expression patterns between the samples, indicating gene groups correlated to the trait evaluated (Langfelder & Horvath, 2008). Otherwise, the PCIT incorporates theoretical information about transcription factors, which allows, through different metrics, the indication not only of hub genes but also the identification of transcription factors responsible for modulating the expression of differentially expressed genes between contrasting groups (Hudson et al., 2012). Moreover, the DCO can also assists the GWAS to indicate genomic regions with higher accuracy in integrated -omics studies (Deng et al., 2019; Yan et al., 2020).

Studies evaluating the beef FA profile of EFAs in indicine cattle (*Bos taurus indicus*) are incipient, especially incorporating different methodologies of differential co-expression analysis. Therefore, we aimed to identify potential regulatory transcription factors and hub gene groups related to the EFAs profile in the *Longissimus thoracis* muscle of Nellore cattle finished in feedlot based in two methodologies, WGCNA and PCIT.

3.2. Materials and methods

3.2.1. Animals and sampling

A total of 44 young Nellore bulls, sons of six sires, belonging to the Capivara farm (São Paulo state, Brazil), which participated in the Nelore Qualitas breeding program. Animals were selected based on growth, finishing, and sexual precocity traits. The nutritional management was according previously reported by Berton et al., (2016).

The animals were slaughtered in commercial slaughterhouses when attained with approximately 550 kg of liveweight and an average age of 24 months. Samples of the *Longissimus thoracis* (LT) muscle were collected 48h *post-mortem* from the left half carcasses (at least 3.0 kg, including muscle and bone) between the 12th and 13th ribs. Samples were properly identified and immediately subjected to liquid nitrogen. Subsequently, they were stored at -80°C until further analysis.

The experiment was approved by the Animal Use Ethics Committee of the Faculty of Agricultural and Veterinary Sciences (FCAV), Unesp, Jaboticabal/SP (certificate number 18340/16).

3.2.2. Lipid extraction

The lipid fraction quantification was performed at the Animal Product Technology Laboratory in the Technology Department of FCAV/UNESP (Jaboticabal, São Paulo, Brazil) according to Bligh and Dyer (1959). Approximately 3g of raw and ground samples of *Longissimus thoracis* muscle were subjected to lipid extraction, in which, after being transferred to a 250 mL flask, chloroform (10 mL), methanol (20 mL) and distilled water (8 mL) were added. After that, the solutions were homogenized, and centrifuged for 30 min on a horizontal shaker table (HITACHI High-Speed Micro Centrifuge model CF16RN himac). Then, an additional 10 mL of chloroform added together with 10 mL of an aqueous sodium sulfate solution (1.5%) and agitated for 2 minutes. This solution, in a 50 mL falcon tube, was centrifuged (1000 g-force) at room temperature. The supernatant was discarded and the rest was filtered into measuring cylinders (25 mL) through a paper filter in order to separate the extracted lipids solution. From this last solution, 5 mL were transferred to a 50 mL beaker, dried in an oven and cooled in a desiccator for at least 24 hours. Afterwards, the beaker with the solution was placed in an oven at 110 °C for the evaporation of the solvent, cooled in a desiccator (O/N) and at the end weighed. The differences between the initial and final weight of the beaker were used to determine the lipid concentration of the samples.

3.2.3. Fatty acid profile identification

The FA profile was determined for each sample according to Folch et al., 1957. Approximately 100g of the ground muscle samples were used. The lipids were extracted and isolated by homogenizing a chloroform and methanol solution in 2:1 ratio and subsequent addition of a 1.5% NaCl solution. After that, as proposed by Kramer et al (1997), the methylation process of the isolated lipids was carried out and with this resulted in the formation of methyl esters and from these FA profile compositions it was possible to be quantified using a gas chromatography (GC-2010 Plus - Shimadzu

AOC 20i auto-injector) with a SP-2560 capillary column (100 m × 0.25 mm in diameter with 0.02 mm thickness, Supelco, Bellefonte, PA). With an initial temperature of 70°C, under continuous heating of 13°C per minute, an intermediate temperature of 175°C was established and, when reached, was maintained for 27 minutes. Thereafter, at a heating speed of 4°C per minute, the temperature was increased to 215°C and maintained for 31 minutes.

The FA identification was performed by comparing the retention time of the methyl esters of the samples with standards C4-C24 (F.A.M.E mix Sigma®), vaccenic acid C18:1 trans-11 (V038-1G, Sigma®) C18:2 trans-10 cis-12 (UC-61 M 100 mg), CLA e C18:2 cis-9, trans-11 (UC- 60 M 100 mg), and tricosanoic acid (Sigma®). Then, using the GS solution 2.42 software, the FA were quantified, by normalizing the area under the methyl esters curve, and expressed as a percentage of the total FA methyl ester. This step was performed at the Meat Science Laboratory (LCC) in the Animal Nutrition and Production Department at FMVZ/USP. Among the quantified FA, we selected the fatty acids linoleic (C18:2 ω-6) and alpha-linolenic (C18:3 ω-3) due their importance for human health.

3.2.4. RNA extraction

The extraction of total RNA from bovine LT muscle samples (~100mg) was performed using the extraction protocol via TRIzol® (Life Technologies, Carlsbad, CA, USA). Only samples that obtained RNA integrity number (RIN) ≥ 8, measured by the Agilent equipment 2100 Bioanalyzer® (Agilent, Santa Clara, CA, USA), were used. The preparation of the cDNA libraries followed the TruSeq® RNA Sample Preparation v2 protocol (Illumina, San Diego, CA, USA) and their quantifications were performed using the KAPA Library Quantification kit® (KAPA Biosystems, Foster City, CA, USA). Subsequently, barcode sequences were added for individual identification of the samples, which were subjected to sequencing. The method used for sequencing was the paired-end, which produces fragments of 2x100bp. The HiSeq 2500 equipment (Illumina, San Diego, CA, USA) and the TruSeq PE Cluster kit v3-cBot-HS and TruSeq SBS kit v3-HS kits (Illumina, San Diego, CA, USA) were used for its realization. The sequencing analysis was performed at the Genome Center at ESALQ/USP (Piracicaba, São Paulo, Brazil).

3.2.5. Reads alignment and gene count

HiSeq platform raw sequencing data were converted into FASTQ files and separated into individual libraries using the Casava v.1.8.2 software (Illumina, San Diego, CA, USA). These files were submitted to the software FastQC v. 0.11.9 [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/] and Trimmomatic v. 0.32 (Bolger et al., 2014) to perform quality control, to identify and remove PCR adapters and primers, as well as low quality and/or small sequences (<36 bp). The reads were then aligned to the *Bos taurus taurus* genome assembly ARS-UCD1.2 using the STAR v.2.7.0 program (Dobin et al., 2013). Subsequently, from the SAMtools v1.09 software (Li et al., 2009), a second quality control was performed to remove low-quality alignments, secondary alignments and PCR duplicates. After, the gene counts were performed by the HTSeq Python package (Anders et al., 2015), and it were used for subsequent gene expression analyses (DEG and DCO).

3.2.6. Differentially expressed genes analysis

For the differentially expressed analysis (DEG), animals were grouped based on their concentrations for each selected FA (linoleic, LA – C18:2 n6 and alpha-linolenic, ALA – C18:3 n3). Two groups were formed by considering those samples with the 15 highest (high - H) values/phenotypes and the 15 lowest (low - L), totaling four main groups: LA-H, LA-L, ALA-H, and ALA-L. A significant ($p<0.0001$) difference was observed, by t test (Gosset, 1908), between the means of the highest and lowest FA concentration groups (Supplementary File 1). Genes counts were normalized, and those below one count per million (cpm) in 90% of the samples for each FA were filtered out. The R package DESeq2 (Love et al., 2014) was used to perform the DEG analysis. The p-values for the DEG were adjusted by False Discovery Rate ≤ 0.1 (Benjamini & Hochberg, 1995).

3.2.7. Differential gene co-expression: Partial correlation and information theory (PCIT)

For the differential co-expression analyzes (DCO), the partial correlation and information theory (PCIT) algorithm implemented in R software (Reverter & Chan, 2008; Watson-Haigh et al., 2010) was used (DCO-PCIT). The quality control carried out by DESeq2 package (Love et al., 2014) resulted in 10,739 genes for the DCO analysis. The correlation matrices for the gene expression values were performed separately for each of the four FA groups evaluated (LA-H, LA-L, ALA-H, and ALA-L) using the PCIT package (Watson-Haigh et al., 2010). From these matrices, only significant correlations ≥|0.9| were used to perform the differential hubbing (DH) analysis. The measurement of DH values is based on the difference in the quantity of significant correlations ≥|0.9| of each gene between the contrasting groups. Genes were then ranked based on the top five DH values (highest and lowest) for each FA.
The regulatory impact factor (RIF) scores were calculated as described by Reverter et al. (2010). These scores reflect the co-expression of each transcription factor (TF), previously informed, and its potential target genes, i.e., differentially expressed genes identified among contrasting EFA groups. There are two different ways to calculate the RIF measurements. In the first one (RIF1), the RIF score is strongly influenced by large differences in the correlation values between TF and DEG between the divergent groups. In the second one (RIF2), the RIF score is affected mainly by the magnitude of the potential target genes (DEG) expression induced by TF expression. The positive values for both scores indicate a better connection with the groups with high concentrations of the assessed fatty acid and, therefore, the opposite situation reveals a relationship with the low groups. For the TF selection, 740 TF were considered, which were expressed among the FA groups and were deposited in the Animal transcription factor database (AnimalTFDB) v.3.0 (Hu et al., 2019). Only the TF which had their scores with ± 2.58 standard deviation (99%) in relation to the mean were selected for functional and network analysis.

3.2.8. Differential gene co-expression: Weighted gene co-expression network analysis (WGCNA)

For the differential co-expression analysis (DCO), the weighted gene co-expression network analysis (WGCNA) package implemented in R software

(Langfelder & Horvath, 2008) was used (DCO-WGCNA). The four FA groups (LA-H, LA-L, ALA-H, and ALA-L) were analyzed individually, and the connectivity values were calculated among all the genes in the network. The connectivity measure represents the degree of connection between each pair of genes in the network, and it is obtained through the sum of Pearson's correlations raised to the soft power threshold (β). It should be noted that β is individual for each analysis, and it should approach the criterion of scale-free topology (R2 ≥ 0.8) (Zhang & Horvath, 2005). The values of β=9 and 8 were used for LA-H and ALA-H and β=13 and 10 for LA-L and ALA-L, respectively. For the genes clustering and modules formation, the unsigned step-by-step method was used implemented in the hierarchical clustering algorithm (dynamic tree-cutting) package (Langfelder et al., 2008). A minimum size of 30 genes per module and a dissimilarity of >0.20 among the modules were applied (Langfelder et al., 2008; Yip & Horvath, 2007).

Each module has an eigengene measurement (ME), which corresponds to the representation of the first main component of the expression profile of the entire module (Langfelder & Horvath, 2008). This measure was correlated with the evaluated traits in the study, and modules that showed correlations of ≥ |0.4| as significant (p ≤ 0.1) were selected for further analysis.

3.2.9. Functional enrichment

Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 tool (Huang et al., 2009a, 2009b) was used to identify overrepresented gene ontology (GO) terms and KEGG pathways using the list of genes from the differential analyzes and the *Bos taurus taurus* annotation file as a background. The list of genes identified in the DEG and both DCO (PCIT and WGCNA) analyzes were subjected separately to functional enrichment. The p-values were adjusted by False Discovery Rate (FDR) (Benjamini & Hochberg, 1995), and significant GO:Terms – biological process (BP), molecular function (MF), and component cellular (CC) – and KEGG pathways were considered when p-adj ≤0.05.

3.3. Results

3.3.1. Differentially expressed genes analysis

DEG were identified among the contrasting groups for LA and ALA. The LA groups encompassed 151 genes differentially expressed enriched in 36 GO:Terms (2 BP, 2 MF, 34 CC) and six KEGG pathways, while the ALA groups displayed 352 DEG in 207 GO:Terms (102 BP, 18 MF, 87 CC) and 10 KEGG pathways (Supplementary Files 2 and 3). Among the genes identified, we can highlight the acyl-CoA oxidase 2 (*ACOX2*) and 3-hydroxybutyrate dehydrogenase type 1 (*BDH1*), listed as upregulated in the LA-H group and enriched in the term “oxidation-reduction process” (GO:0055114) and “metabolic pathways” (bta01100). The genes succinate dehydrogenase complex iron sulfur subunit B (*SDHB*), enriched in the term “oxidation-reduction process” (GO:0055114) and “oxidative phosphorylation” (bta00190), and the fat storage inducing transmembrane protein 1 (*FITM1*), inserted in the “metabolic process” (GO:0008152), were found upregulated in the ALA-H group. Besides, the gene the acyl-CoA synthetase long-chain family member 3 (*ACSL3*), also enriched in the GO:0008152, was found downregulated in the ALA-H group. Furthermore, the enoyl-CoA hydratase, short chain 1 (*ECHS1*) and isovaleryl-CoA dehydrogenase (*IVD*) genes, involved in the “oxidation-reduction processes” (GO:0055114) and “metabolic pathways” (bta01100), were upregulated in both groups (LA-H and ALA-H).

3.3.2. Differential co-expression analysis (PCIT – DH)

The correlation matrices for each FA group were performed using the PCIT algorithm and only the correlations ≥ |0.9| were used for the identification of DH genes between the contrasting groups. A total of 5,649 genes inserted in 732 GO:Terms (419 BP, 115 MF, 198 CC) and 68 KEGG pathways were identified between LA-H and LA-L groups (Supplementary Files 4 and 6). For the ALA-H and ALA-L, a total of 5,559 differential hubbing genes and 774 GO terms (454 BP, 106 MF, 214 CC) and 73 KEGG pathways were described (Supplementary Files 5 and 7). Of these, we can underscore the genes WD repeat domain (*WDR43*) – “regulation of catalytic activity” (GO:0050790) and “RNA binding” (GO:0003723), ankyrin repeat and SOCS box

containing 5 (*ASB5*) – “protein ubiquitination” (GO:0016567), ER lipid raft associated 1 (*ERLIN1*) – “regulation of gene expression” (GO:0010468), and TRAF-type zinc finger domain containing 1 (*TRAFD1*), which presented a high number of connections in the LA-H and ALA-H groups (Supplementary File 6-7 and Table 1). However, when considering the low concentration groups, no common genes were found between LA and ALA.

Table 1. List of extreme differential hubbing genes for the linoleic acid (LA – C18:2) and alpha-linolenic (ALA – C18:3) fatty acids.

Groups	Gene symbol	DH	Groups	Gene symbol	DH
LA-H	*WDR43*	68	ALA-H	*ASB5*	40
	ASB5	63		*TRAFD1*	35
	ORC4	60		*WDR43*	31
	ERLIN1	57		*NAA15*	30
	TRAFD1	50		*ERLIN1*	30
LA-L	*FSTL1*	-70	ALA-L	*DEK*	-91
	FN1	-66		*PRPF38B*	-89
	DAB2	-63		*KMT2E*	-77
	LRP1	-62		*USP8*	-77
	MMP14	-60		*SEC62*	-75

LA-H: Samples with the 15 highest values/phenotypes for the linoleic acid; LA-L: Samples with the 15 lowest values/phenotypes for the linoleic acid; ALA-H: Samples with the 15 highest values/phenotypes for the alpha-linoleic acid; ALA-L: Samples with the 15 lowest values/phenotypes for the alpha-linoleic acid.

3.3.2. Differential co-expression analysis (PCIT – RIF)

In the RIF analysis, 20 potential TF were listed for each FA (Supplementary Files 8 and 9). Such TF were enriched in 86 GO:Terms (52 BP, 28 MF, 6 CC) to LA and 81 GO:Terms (38 BP, 31 MF, 12 CC) to ALA, in none of the fatty acids there were enriched KEGG pathways (Supplementary File 10). Six TF were common to both fatty acids, in which we can highlight the nuclear factor IA (*NFIA)* and histone H4 transcription factor (*HINFP*), both enriched in the term “regulation of gene expression” (GO:0010468), as well as the zinc finger protein 473 (*ZNF473*), which was ranked with the more expressive RIF scores (Tables 2 and 3).

Table 2. List of genes with the highest and lowest regulatory impact factor (RIF) scores (RIF1 and RIF2) between the contrasting linoleic acid (LA – C18:2) groups.

Groups	Gene symbol	RIF1	RIF2
RIF1 (+)	*ZNF134*	4.91	-1.01
	NFIA	4.50	-0.39
	NFYA	4.07	-0.84
	NFAT5	4.02	0.78
	ZNF584	3.54	0.07
RIF2 (+)	*ZBTB43*	-0.46	2.95
	ZNF473	-0.09	2.83
	SLC2A4RG	-0.34	2.77
	BACH1	-0.17	2.73
	NFYB	2.87	2.68
RIF2 (-)	*HINFP*	1.02	-2.70
	WIZ	-0.63	-2.65

More specifically to the LA, the enriched TF nuclear transcription factor Y subunit alpha (*NFYA*) and beta (*NFYB*) - “gene expression” (GO:0010467), as well as the BTB domain and CNC homolog 1 (*BACH1*) and nuclear factor of activated T cells 5 (*NFAT5*) – “regulation of biological process” (GO:0050789) positively impacted the target genes (DEG) expression in the LA-H group (Supplementary File 10; Table 2). The presence of the TF zinc finger protein 134 (*ZNF134),* zinc finger protein 584 (*ZNF584*), zinc finger and BTB domain containing 43 (*ZBTB43*), and SLC2A4 regulator (*SLC2A4RG*) were also pointed out in the RIF analysis (Table 2). On the other hand, impacting more the DEG in LA-L, we highlight the TF WIZ zinc finger (*WIZ*) - “regulation of biological process” (GO:0050789) (Supplementary File 10; Table 2).

Table 3. List of genes with the highest and lowest regulatory impact factor (RIF) scores (RIF1 and RIF2) between the contrasting alpha-linoleic acid (ALA – C18:3) groups.

Groups	Gene symbol	RIF1	RIF2
RIF1 (+)	*NFIA*	7.43	-1.05
	DNAJC1	5.11	0.67
	RORA	4.94	0.46
	NFX1	4.80	2.27

	MITF	4.75	1.14
RIF1 (-)	*FOXS1*	-3.18	0.15
	UBP1	-2.81	0.14
	GZF1	-2.68	0.73
RIF2 (+)	*TSC22D2*	-0.46	2.95
	ZNF800	-0.09	2.83
	ATF6	-0.34	2.75
	HIVEP2	-0.17	2.73
	ZNF473	2.87	2.68
RIF2 (-)	*NR2C1*	0.16	-3.57
	HINFP	-0.32	-2.68
	ZFPM1	0.19	-2.65

With the emphasis on the ALA, the TF RAR related orphan receptor A (*RORA*), nuclear transcription factor X-box binding 1 (*NFX1*), melanocyte inducing transcription factor (*MITF*), and activating transcription factor 6 (*ATF6*) – “gene expression” (GO:0010467) – and HIVEP zinc finger 2 (*HIVEP2*) impacted the expression of DEG to the ALA-H group (Supplementary File 10; Table 3). The DnaJ heat shock protein family (Hsp40) member C1 (*DNAJC1*), TSC22 domain family member 2 (*TSC22D2*), and zinc finger protein 800 (*ZNF800*) were also ranked, although they were not listed within a significant GO:Terms (Supplementary File 10; Table 3). In contrast, the TF forkhead box S1 (*FOXS1*), upstream binding protein 1 (*UBP1*) and nuclear receptor subfamily 2 group C member 1 (*NR2C1*) - “gene expression” (GO:0010467), GDNF inducible zinc finger protein 1 (*GZF1*) - “DNA binding” (GO:0003677), and the zinc finger protein FOG family member 1 (*ZFPM1*) were indicated with the lowest values for RIF1 and RIF2 (Supplementary File 10; Table 3).

3.3.3. Differential co-expression analysis (WGCNA)

The DCO-WGCNA identified 521 genes inserted in six modules significantly correlated ($p \leq 0.1$) with values higher than |0.4| in the LA-H group (Supplementary Files 11 and 12). However, only the lightgreen (-0.59, *p = 0.02, n = 123* genes) and

skyblue (-0.47, *p =0.*08, *n = 126* genes) modules had genes enriched in some GO:Term. It pointed out seven unique component cellular terms, but none KEGG pathway, biological processes or molecular function (Supplementary File 19). Among these terms, we highlight the “intracellular” (GO:0005622), which the ATP binding cassette subfamily A member 6 (*ABCA6*) gene – lightgreen module – was inserted.

Under the same parameters, five modules were identified harboring 1,682 co-expressed genes in the LA-L group (Supplementary Files 13 and 14) enriched in unique 245 GO:Terms, 174 BP, 15 MF, 56 CC (Supplementary File 19) and 9 unique KEGG pathways. We can highlight the SREBF chaperone (*SCAP*) gene in darkseagreen4 module (-0.46, *p = 0.08, n = 248* genes) enriched in “intracellular” (GO:0005622), as well as the fatty acid desaturase 1 (*FADS1*) and 2 (*FADS2*), fatty acid synthase (*FASN*), and peroxisome proliferator activated gamma receptor (*PPARG*) genes in purple module (0.45, *p=0.10, n = 800* genes) enriched in the term “cell differentiation” (GO:0030154) (Supplementary File 19).

For the ALA, a total of 852 genes were listed in six modules (ALA-H, Supplementary Files 15 and 16) enriched in 111 GO:Terms (69 BP, 5 MF, 37 CC) and 17 KEGG pathways (Supplementary File 19), and 247 genes were inserted in two modules (ALA-L, Supplementary Files 17 and 18) enriched in 19 GO:Terms (1 BP, 18 CC). Among them, we can emphasize the ELOVL fatty acid elongase 5 (*ELOVL5*) – “single-organism metabolic process” (GO:0044710) in the dark olive-green module (-0.49, *p*=0.07, *n = 474* genes, ALA-H) and the acetyl-CoA carboxylase beta (*ACACB*) and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*) inserted in the floral-white module (-0.44, *p*=0.10, *n = 177* genes, ALA-L) enriched in the term “intracellular” (GO:0005622) (Supplementary File 19).

3.4. Discussion

3.4.1. Differentially expressed genes

Among the DEG described for the LA, we can highlight the *ACOX2, BDH1, ECHS1* and *IVD* genes, enriched in the term “oxidation-reduction process” (GO:0055114), with higher expression in the LA-H group. The *ACOX2* gene

corroborate with results from Lim et al. (2015), in which it was also identified in Hanwoo cattle with high marbling score. This gene was also associated with the *BDH1* gene overexpression in the Nellore cattle (Fonseca et al., 2020), which suggests a relationship with higher concentrations of FA. This relationship can still be justified by the regulatory action of such genes due to the high concentration of unsaturated fatty acids, as it can be seen with the oxidative action genes *ECHS1* and *IVD* (Houten and Wanders, 2010; Schulz, 2002), which were also pointed out as differentially expressed between ALA groups.

Particularly to the ALA-H group, the *SDHB* gene, enriched in the BP “oxidation-reduction process” (GO:0055114), was identified as upregulated. This gene encodes a subunit that forms the SDH enzyme, responsible for ATP synthesis steps through the Krebs cycle (Rutter, Winge, & Schiffman, 2010). Moreover, this same enzyme was related to FA concentration in cattle. According to Jeong et al. (2013), the SDH enzyme was overexpressed in samples of *Longissimus dorsi* muscle in Korean cattle after castration, which had a higher rate of IMF compared to non-castrated animals. This fact reiterates the relationship of some genes with the oxidative action and FA profile (i.e., *SDHB* gene), and it suggests a possible regulatory mechanism associated with a high FA concentration.

The *ACSL3* and *FITM1* genes, enriched in the “metabolic process” (GO:0008152), were identified as differentially expressed between the ALA groups. The *ACSL3* gene has regulatory potential in the lipogenesis process due to its relationship in the intracellular uptake of FA, as well as the enzymatic action triggered by its protein product by providing substrate for the triglyceride synthesis and β-oxidation steps (Rakhshandehroo et al., 2010; Yan et al., 2015). This gene was downregulated in the ALA-H group, a finding justified by the high FA rate. On the other hand, the *FITM1* gene was correlated with small deposits of lipids in muscle cells (Gross et al., 2011) and higher fat accumulation in cell cultures (Kadereit et al., 2008). This fact corroborates our results that pointed it more expressed in the ALA-H group and suggests a relationship with the high FA concentration.

3.4.2. Differential co-expression analysis (PCIT – DH)

For the DCO analysis aiming to identify DH genes (PCIT-DH), we highlighted *WDR43, ASB5, TRAFD1* and *ERLIN1* genes due to their implications in the LA-H and ALA-H groups. The *WDR43* gene acts in the transcriptional control by binding to RNA polymerase II modulating proteins and promoter regions for the mRNA formation (Bi et al., 2019). Members of the same gene family were related to the FA profile in Nellore cattle (Cesar et al., 2014; Lemos et al., 2016; Oliveira et al., 2019), as well as in marbling score in Hanwoo cattle (Lim et al., 2013). The *ASB5* gene was associated with cell differentiation and proliferation in muscle tissue (Jensen et al., 2012), besides being related to the marbling score in the Nellore (Fonseca et al., 2020) and crossbreed Wagyu × Hereford cattle (Wang et al., 2009). Although the function of the *TRAFD1* gene is not clear, it regulated the DEG expression for IMF content in Iberian swine (Munõz et al., 2018). The appointment of these genes as DH in the ALA-H and LA-H groups is justified not only by their potential function by regulating gene expression but also because of their impact on the lipid metabolism. A similar explanation can be attributed to the *ERLIN1* gene, enriched in the term “regulation of gene expression” (GO:0010468), due to its relationship with the ER lipid raft associated 2 (*ERLIN2*), *SCAP*, and insulin induced gene 1 (*INSIG1*) and 2 (*INSIG2*) for the modulation of the *SREBF1* gene, and consequently, to the cholesterol homeostasis establishment (Howe et al., 2016). This fact is also related to fat deposition, as observed by Huber et al. (2013), when indicating the *ERLIN1* gene as responsible for the triglyceride formation in a cell culture study.

The *ORC4* gene - “DNA metabolic process” (GO:0006259) and *NAA15* - “positive regulation of gene expression” (GO:0010628) were pointed as one of the most expressive DH in the LA-H and ALA-H groups, respectively. The protein encoded by the *ORC4* gene belongs to a protein complex associated with the DNA replication (Bell, 2002). Complementary, the *NAA15* gene was associated with cell proliferation in tumor cells (Fluge et al., 2002), indicating a possible relationship between these genes and the mechanisms underlying cell proliferation and differentiation in the high groups due to the high cell activity in the fat synthesis and storage.

Although no common DH genes were identified between the LA-L and ALA-L groups, the *FN1, MMP14, FSTL1, LRP1* and *DAB2* genes are able to modulate the gene expression in the LA-L group. Among them, the *FN1* and *MMP14* are closely

related to the extracellular matrix formation, a fundamental step for cell proliferation and fat deposit formation in muscle tissue (Carmeli et al., 2004; Wang et al., 2009). The *FN1* gene was overexpressed in pre-adipocytes in cell culture (Urs et al., 2004), besides being differentially expressed in Nellore cattle with contrasting ribeye muscle areas (Santos Silva et al., 2020). The *FSTL1* gene, enriched in the term “response to starvation” (GO:0042594), acts promoting adipogenesis and fat accumulation in pre-adipocytes and adipocytes differentiated in cell culture, as well as upregulated in humans’ non-obese patients (Flanagan et al., 2009). The relationship of these genes with the LA-L group can be justified by the low FA concentration, likewise hypothesizing that the animals in this group are later than those in the high group. The *DAB2* gene - “cellular response to lipid” (GO:0071396) and *LRP1* - “positive regulation of gene expression” (GO:0010628) are related to fat deposition in tissues since they act intimately in the cholesterol homeostasis (Tao et al., 2016a; Terrand et al., 2009). According to Tao et al. (2016b), the *DAB2* gene acts in pre-adipocyte differentiation stages, which is shared by the *LRP1* gene, as presented by Masson et al. (2009) in a cell culture study. This association with the early stages of adipocyte formation suggest that the animals in the LA-L group are in prior stages to those in the high group.

The *DEK* and *KMT2E* genes, enriched in the term “gene expression” (GO:0010467), were identified as the DH genes in ALA-L group. Although the *DEK* gene is related to tumor processes in humans, it is involved in stages of cell differentiation (Yi et al., 2015) and complementarity to the *KMT2E* gene, which acts on cell proliferation (Zhang et al., 2017), allows us to reinforce the possibility that ALA-L animals are less precocious. The identification of the *PRPF38B, SEC62,* and *USP8* genes as majors DH in the ALA-L group does not indicate a direct association with the FA phenotype due to functions related to the protein complex involved in general transcriptional processes (Daguenet et al., 2015; Lakkaraju et al., 2012; Niendorf et al., 2007).

3.4.3. Differential co-expression analysis (PCIT – RIF)

Common to both FA, the TF *NFIA* and *ZNF473* displayed the highest positive RIF1 and RIF2 values, respectively. The *NFIA* gene was related to the adipogenic processes and cell differentiation since it could regulate the expression of genes such

as *PPARG* in non-differentiated adipocyte of cell cultures (Waki et al., 2011) and in mice brown fat (Hiraike et al., 2017). The transcription factor *ZNF473* was identified as one of the potential regulators of feed efficiency in pigs (Ramayo-Caldas et al., 2019). Further, this TF was related to energy and lipid metabolism in a genomic-wide association and gene co-expression studies in the Nellore cattle (Alexandre et al., 2015; Brunes et al., 2020). These facts corroborate with our results, indicating a relationship of these TF with the EFA metabolism. Besides, the LA and ALA also shared the TF *HINFP* with lower RIF2 scores. The TF *HINFP* can regulate the lipid metabolism due to its interaction with the SREBP2 protein, which regulates cholesterol levels and lipid deposition (Li & Liu, 2012). In this way, the TF *HINFP* suggests a negative regulatory action under the FA biosynthesis.

Among the TF ranked with the highest RIF1 and RIF2 scores for the LA, we highlight the *NFYA* and *NFYB*, which modulates the *FASN* expression in mouse adipocytes, and interacts with the SREBP1 protein for the FA synthesis and cholesterol homeostasis (Nishi-Tatsumi et al., 2017). Besides, the identified TF *NFAT5* and *SLC2A4RG* were related to adipogenic processes linked to glucose uptake and cell differentiation (Lee et al., 2019; Ma et al., 2011). This last trait was also linked to the TF *BACH1* in a study carried out by Nishizuka et al. (2002) in cell cultures, which indicated it as a potential regulator in adipocyte differentiation. These studies corroborate the potential relationship of these TF with the DEG expression and, consequently, fat deposition and EFA profile, especially for the LA. Moreover, the TF *ZNF134*, *ZNF584*, and *ZBTB43* in RIF1, and *WIZ* (RIF2) also were highlighted. Although these genes have an unclear relationship with the lipid metabolism, the zinc finger family members act in the gene expression of several metabolic processes (Laity et al., 2001), and therefore, these TF can be crucial in regulating DEG expression.

When considering the RIF analysis for the ALA, the TF with the highest RIF1 (*MITF*) and RIF2 (*ATF6* and *HIVEP2*) scores were enriched in the term “gene expression” (GO:0010467). The TF *MITF* indication may have been due to its relationship with the Stearoyl-CoA desaturase (*SCD*) gene, which acts in the unsaturated fatty acids synthesis. According to Vivas-García et al. (2020), the FT *MITF* promoted a higher expression of the *SCD* gene and, consequently, a higher concentration of unsaturated fatty acids in tumor cells. The TF *ATF6* and *HIVEP2* share

a similar potential to regulate adipogenic processes since both interact with the *PPARG* gene. According to Lowe et al. (2012), the *ATF6* gene knockout significantly reduced the *PPARG* expression in cell culture. The TF *HIVEP2*, also known as *Schnurri-2*, acts as a co-activator in the expression of the *PPARG* gene, and in knockout situations, restricted the *PPARG* expression during adipocyte differentiation (Jin et al., 2006). These relationships reinforce the regulatory potential by these TF under DEG expression in the ALA-H group, and consequently, EFA profile.

With the ability to impact the lipid deposition, the TF with higher RIF1 values (ALA, *RORA*, and *NFX1*) were described enriched in the term “gene expression” (GO:0010467). Such impact may be due to the interrelation between these TF and lipogenesis promoting genes. According to Lau et al. (2004), the TF *RORA* has regulatory potential in lipid homeostasis, given that in knockout situations, this TF induced a lesser expression of genes such as *FASN* and *SCD*, crucial for FA synthesis. The FT *NFX1* encodes a protein with the same name that binds to the NF-Y protein, which regulates the *FASN* expression, and, consequently, impacts FA synthesis (Nishi-Tatsumi et al., 2017; Reith et al., 1994). A relationship was also observed by Fernandes Júnior et al. (2016) when pointing to the *NFX1* gene as a candidate gene to explain a part of the genetic variance for backfat thickness in Nellore cattle.

The TF *DNAJC1* (RIF1), *TSC22D2* (RIF2), and *ZNF800* (RIF2) for the ALA act mainly in the cell growth control. The *DNAJC1* and *TSC22D2* genes were related to a large number of genes to suppress cell proliferation in tumor processes and adverse conditions (Huang et al., 2014; Liang et al., 2016). The *ZNF800* gene has a similar function, in which it can inhibit cell proliferation by blocking the AKT/mTOR pathway (Lu et al., 2020). These TF with higher positive scores suggests a potential regulatory action to control cell proliferation in animals in the ALA-H group.

With lower RIF1 scores for the ALA, TF such as the *FOXS1* and *UBP1* - “gene expression” (GO:0010467) and *GZF1* - “DNA binding” (GO:0003677) were identified. The FT *FOXS1* is associated with several cell proliferation steps (Lei et al., 2020), and its overexpression inhibited tumor cells proliferation. The FT *GZF1* is also involved in the cell proliferation process, however, it was related to cell proliferation promotion in cell culture (Dambara et al., 2007). The FT *UBP1* acts in angiogenesis by stimulating neovascularization, an important step in pre-adipocytes to promote growth and

differentiation cellular as well as extracellular matrix (Borges et al., 2006; Kotarba et al., 2018). These TF allow us to hypothesize that animals in the ALA-L group are more delayed than those in the ALA-H, indicating early stages of adipocytes formation, and consequently, less lipid deposition.

With low RIF1 scores for the ALA, the TF *ZFPM1* and *NR2C1* are worth to be highlighted. The FT *ZFPM1* (alias name: *FOG1*) acts as a co-regulator in early adipocyte differentiation stages since it was overexpressed in pre-adipocytes and showed a lower expression in mature adipocytes in cell culture (Jack & Crossley, 2010). Besides, the *ZFPM1* was pointed out as a DH gene in a low genomic value (GEBV) group for IMF in the Nellore cattle (Cesar et al., 2015). The *NR2C1* gene encodes the TR2 protein, that when interacting with the TR4 protein product, is related to pathways opposite to those presented by the *PPARG* gene, and also suppress the expression Retinoid X Receptors (Lin et al., 2017). These findings corroborate with our results, by showing that these TF are related to a lower FA concentration.

3.4.4. Differential co-expression analysis (WGCNA)

The WGCNA methodology employs a different algorithm from that used by the PCIT regarding the gene networks formation as well as the gene clustering, complementing each other. This complementarity allowed us to identify other potentially DCO genes to improve the understanding of the mechanisms underlying the phenotypes.

The DCO analysis through the WGCNA package identified gene groups important to FA profile development. In the LA-H group only two modules had GO:Terms enriched, but we highlight the lightgreen module (-0.59, $p = 0.02$), which the *ABCA6* gene was inserted and pointed out in the component cellular “intracellular” (GO:0005622). According to Wenzel et al. (2007), the *ABCA6* gene is related in the cholesterol uptake, which is closely linked to *SREBF1* gene and its target gene functions in the cholesterol homeostasis, impacting EFA profile (Howe et al., 2016). This fact justifies the *ABCA6* in a significant module correlated to LA concentration. For the LA-L group, the *SCAP* gene, enriched in “intracellular” (GO:0005622) and clustered in darkseagreen4 module (-0.46, $p = 0.08$), also interacts with *SREBF1* gene, activating it (Howe et al., 2016). The protein complex SCAP:SREBP1 promotes, in low

cholesterol conditions, higher synthesis of FA and cholesterol (Howe et al., 2016), reinforcing the relationship with EFA profile. Justifying the *SCAP* co-expression in a module negatively correlated to LA-L group. Notwithstanding, the purple module (0.45, *p = 0.10*) pointed out clustered hub genes for the FA phenotype, i.e., *PPARG*, *FASN*, *FADS1* and *FADS2*, enriched in “cell differentiation” (GO: 0030154). The *PPARG* and the *FASN* genes act in crucial cell proliferation and differentiation stages in adipocytes (Lefterova & Lazar, 2009; Roh et al., 2006; Sevane et al., 2013). The *PPARG* impacted the lipid deposition in the Hanwoo cattle since it was overexpressed in high marbling score groups (Lim et al., 2015). Similarly, the *FASN* gene was correlated (0.44) with the IMF (*Longissimus dorsi*) in Korean cattle (Jeong et al., 2012), and it was overexpressed in Iberian pigs with high IMF content (Muñoz et al., 2018). Further, it explained part of the genetic variance for the FA profile in Angus and crossbred cattle musculature (Chen et al., 2015). The *FADS1* and *FADS2* genes act in the FA desaturation process. According to Ralston et al. (2015), in adipocyte cell cultures, the highest LA concentration triggered a higher expression of *FADS1* and *FADS2*. Besides, SNP-type mutations in the *FADS2* gene were associated with higher marbling in the Holstein breed (Matsumoto et a., 2014). These studies corroborate our results that indicate a positive correlation of the purple module with the LA concentration.

Regarding the ALA, gene groups related to the FA synthesis were identified. The dark olive-green module (-0.49, *p=0.07*, ALA-H) encompassing the *ELOVL5* gene - “single-organism metabolic process” (GO:0044710) and the floral white module (-0.44, *p = 0.10*, ALA-L) harboring the *ACACB* and *HMGCS1* genes - “intracellular” (GO:0005622) should be underscored. The *ELOVL5* gene acts in stages of unsaturated fatty acids elongation (i.e., C18-20 chains), especially the ALA and eicosatrienoic acid (C20:3 n3) (Leonard et al., 2000). This result corroborates with a negative correlation of the dark olive-green module with the ALA concentration. On the other hand, the *ACACB* and *HMGCS1* genes act in the FA oxidation stages (Wakil & Abu-Elheiga, 2009). The *ACACB* gene was related to lower lipid deposition in cattle since it was identified by Zhang et al. (2017) as downregulated in castrated animal groups, which shows a high lipid deposition, compared to non-castrated animals. The *HMGCS1* gene, besides participating in the formation of ketone bodies, is also involved in cholesterol homeostasis and FA uptake together with the same family member, 3-

hydroxy-3-methylglutaryl-coenzyme reductase (*HMGCR*) (Cotter et al., 2013; Howe et al., 2016). These results corroborate the module correlation with the ALA concentration, and suggest that the *ACACB* and *HMGCS1* gene co-expression are linked to a lower FA concentration.

In summary, the combination of two complementary differential co-expression methodologies, WGCNA and PCIT, with the differentially expressed genes analysis, allowed us to identify hub genes already described in the literature. But also, unexplored genes and transcription factors able to modeling the EFA profile in zebu meat cattle were identified. Revealing regulatory mechanisms linked to lipid and energy metabolism, as well as, cell proliferation and differentiation.

3.5. Conclusion

The DEG and DCO analyzes combined allowed us to point out potential unexplored genes/transcription factors and biological processes underlying the EFA (LA and ALA) profile in the *Longissimus thoracis* muscle of Nellore cattle finished in feedlot. Among them, we can highlight the differentially expressed genes *ECHS1* and *IVD*. The hubbing genes *ASB5* and *ERLIN1,* and the TF *NFIA*, since these genes displayed an outstanding capacity to strongly impact both EFA, linoleic and alpha-linolenic acids. Furthermore, the TF *NFYA*, *NFYB*, *FASN*, and *PPARG* and *FADS2* genes associated with the LA, as well as, the TF *RORA* and *ELOVL5* gene to ALA, were able to directly impact the phenotypes.

Our findings contribute to the identification of the potential biomarkers for this complex and late-measuring trait. Also, our results provide information that will help in a better understanding of the genetic mechanisms about the essential fatty acid profile in beef. Besides, it can support -omics studies and assist in the early animal selection in breeding programs. Providing knowledge about transcription factor or gene groups with higher impact on the trait, that are able to regulate gene expression and pathways related.

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