UNIVERSIDADE FEDERAL DO PARANÁ

GEORGIA CRISTINA DE AGUIAR

EFEITOS DA SUPLEMENTAÇÃO DE ÁCIDOS GRAXOS EM VACAS LEITEIRAS DURANTE PERÍODOS DE DESAFIO FISIOLÓGICO: TRANSIÇÃO E ESTRESSE CALÓRICO

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Orientador: Prof. Dr. Rodrigo de Almeida (UFPR)

Coorientador: Prof. Dr. João Alberto Negrão (UNESP/USP-FZEA)

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Dedico esta pesquisa aos meus amados pais, cujo apoio inabalável e amor constante foram as bússolas que guiaram minha jornada acadêmica.

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“A coisa mais bela que podemos vivenciar é o mistério. Ele é fonte fundamental de toda verdadeira arte e de toda ciência. Aquele que não o conhece e não se maravilha mais, paralisado em êxtase, é como se estivesse morto; seus olhos estão fechados”.

Albert Einstein, 1934.

“Cultivemos a Ciência por ela mesma, sem considerar aplicações no momento. Estas sempre chegam, às vezes demoram anos, e às vezes séculos. Pouco importa se uma verdade científica é utilizada pelos nossos filhos ou pelos nossos netos. O progresso teria sido prejudicado se Galvani, Volta, Faraday e Hertz, descobridores dos princípios fundamentais da ciência da eletricidade, tivessem menosprezado suas descobertas devido à falta de aplicação industrial”.

Santiago Ramón y Cajal, 1899.

RESUMO

Este estudo teve como objetivo avaliar os efeitos da suplementação lipídica durante períodos de desafio fisiológico em vacas leiteiras, focando nos ácidos graxos de cadeia média (AGCM) e nos ácidos graxos ômega-3 e ômega-6. O estudo buscou compreender as mudanças metabólicas, inflamação e estresse oxidativo, assim como a regulação de mediadores lipídicos em vacas submetidas a estresse calórico. No primeiro experimento, conduzido no Paraná, 168 vacas Holandesas foram divididas em dois grupos: Controle (sem suplementação de AGCM) e Suplemento lipídico (50 g/dia de óleo de coco e palma, 0,065% AGCM na MS). Os experimentos duraram 35 dias, abrangendo 21 dias pré-parto e 14 dias pós-parto. Foram medidas a produção e composição do leite, perfil de ácidos graxos do leite, ruminação, peso e escore de condição corporal (ECC), além dos metabólitos sanguíneos (cálcio total, cálcio iônico, glicose, GGT, AST, colesterol, bilirrubina, albumina, ácidos graxos não esterificados e beta-hidroxibutirato) em diferentes dias (-7, 0, 3, 7 e 14 d) e análise de expressão gênica (HPRT1, IL-6, SAA3, GPx3, CASP8, TLR4, MyD88, STAT1, STAT5, LPK, ACACA, FASN, LPL, SCD, SREBF1). A suplementação com AGCM não afetou significativamente a produção de leite ou sua composição ($P > 0,05$), mas influenciou a expressão gênica e o perfil de ácidos graxos no leite ($P < 0,05$). Foram observadas reduções em alguns ácidos graxos e no teor de sólidos totais no grupo tratado com AGCM ($P < 0,10$). Houve tendência de aumento na produção de proteína em vacas de segundo parto ($P < 0,10$), mas as diferenças nos metabólitos sanguíneos não foram significativas ($P > 0,05$). A dose de AGCM pode ter sido insuficiente para maiores efeitos no desempenho produtivo e metabólico. O segundo experimento, realizado no Centre de Recherche en Sciences Animales de Deschambault (CRSAD), em Quebec, Canadá, investigou os efeitos da infusão abomasal de ácidos graxos ômega-3 e ômega-6 na regulação de mediadores lipídicos durante o estresse calórico. Vinte vacas Holandesas multíparas foram distribuídas aleatoriamente em um delineamento de quadrado latino incompleto com períodos de 10 dias. Os tratamentos incluíram: Termoneutralidade com alimentação em pares + óleo de milho (TNPF/n6), Estresse térmico + óleo de milho (HS/n6) e Estresse térmico + óleo de peixe (HS/n3). Os óleos (159 g/dia) foram infundidos no abomaso em

dois bolus. Amostras de sangue foram coletadas nos dias 0, 5 e 10 para análise lipidômica por cromatografia LC-MS/MS. Os ácidos graxos ácido eicosapentaenoico (EPA), ácido docosahexaenoico (DHA), ácido docosapentaenoico (DPA) e ácido araquidônico (AA), juntamente com os oxilipídeos da via da lipoxigenase 5-HETE, 5-oxoETE, 15-HETE, 15-oxoETE, 17-HDoHe e oxilipídeos do citocromo 450: 19,20-EpDPE e 19,20-DiHDPA, aumentaram em HS/n3 em comparação com HS/n6 ($P < 0,10$). O 9-oxoODE foi reduzido em HS/n6 comparado a TNPF/n6. O tratamento HS/n3 levou a concentrações elevadas de oxilipídeos anti-inflamatórios em comparação ao HS/n6 ($P < 0,10$). Durante o estresse térmico, a disponibilidade de substrato, a via de síntese e a duração da exposição indicam o potencial de modulação exógena de mediadores lipídicos pelo ômega-3. Em resumo, a suplementação lipídica, especialmente com AGCM e ácidos graxos ômega-3, pode influenciar o metabolismo e a resposta imunológica em vacas leiteiras. Embora a suplementação com AGCM não tenha mostrado efeitos significativos na produção de leite e nos metabólitos sanguíneos, ela afetou a expressão gênica e o perfil de ácidos graxos do leite. O ômega-3 modulou mediadores lipídicos de forma benéfica durante o estresse calórico, destacando seu potencial para melhorar a saúde e a produtividade das vacas leiteiras.

Palavras-chave: ácidos graxos de cadeia média, metabolismo lipídico, ômega-3, ômega-6, oxilipídeos plasmáticos.

ABSTRACT

This study aimed to evaluate the effects of lipid supplementation during periods of physiological challenge in dairy cows, focusing on medium-chain fatty acids (MCFA) and omega-3 and omega-6 fatty acids. The study sought to understand metabolic changes, inflammation, and oxidative stress, as well as the regulation of lipid mediators in cows subjected to heat stress. In the first experiment, conducted in Paraná, 168 Holstein cows were divided into two groups: Control (without MCFA supplementation) and Lipid supplement (50 g/day of coconut and palm oil, 0.065% MCFA in DM). The experiments lasted 35 days, covering 21 days pre-partum and 14 days post-partum. Milk production and composition, milk fatty acid profile, rumination, weight and body condition score (BCS) were measured, in addition to blood metabolites (total calcium, ionic calcium, glucose, GGT, AST, cholesterol, bilirubin, albumin, nonesterified fatty acids and beta-hydroxybutyrate) on different days (-7, 0, 3, 7 and 14 d) and gene expression analysis (HPRT1, IL-6, SAA3, GPx3, CASP8, TLR4, MyD88, STAT1, STAT5, LPK, ACACA, FASN, LPL, SCD, SREBF1). Supplementation with MCFA did not significantly affect milk production and composition ($P > 0.05$), but it influenced gene expression and the milk fatty acid profile ($P < 0.05$). Reductions in some fatty acids and total solids content were observed in the group treated with MCFA ($P < 0.10$). There was a trend towards increased protein production in second-calving cows ($P < 0.10$), but differences in blood metabolites were not significant ($P > 0.05$). The dose of MCFA may have been insufficient for greater effects on productive and metabolic performance. The second experiment carried out at the Centre de recherche en sciences animales de Deschambault (CRSAD), in Quebec, Canada, investigated the effects of abomasal infusion of omega-3 and omega-6 fatty acids on the regulation of lipid mediators during heat stress. Twenty multiparous Holstein cows were randomly distributed in an incomplete Latin square design with 10-day periods. Treatments included: Thermoneutrality with pair feeding + corn oil (TNPF/n6), Heat stress + corn oil (HS/n6), and Heat stress + fish oil (HS/n3). The oils (159 g/day) were infused into the abomasum in two boluses. Blood samples were collected on days 0, 5, and 10 for lipidomic analysis by LC-MS/MS chromatography. The fatty acids eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and arachidonic acid

(AA), together with the oxylipids of the lipoxygenase pathway 5-HETE, 5-oxoETE, 15-HETE, 15-oxoETE, 17-HDoHe and cytochrome 450 oxylipids: 19,20-EpDPE and 19,20-DiHDPA, increased in HS/n3 compared to HS/n6 ($P < 0.10$). 9-oxoODE was reduced in HS/n6 compared to TNPF/n6. HS/n3 treatment led to elevated concentrations of anti-inflammatory oxylipids compared to HS/n6 ($P < 0.10$). During heat stress, substrate availability, route of synthesis and duration of exposure indicate the potential for exogenous modulation of lipid mediators by omega-3. In summary, lipid supplementation, especially with MCFA and omega-3 fatty acids, can influence metabolism and immune response in dairy cows. Although MCFA supplementation did not show significant effects on milk production and blood metabolites, it did affect gene expression and milk fatty acid profile. Omega-3 beneficially modulated lipid mediators during heat stress, highlighting its potential to improve the health and productivity of dairy cows.

Keywords: lipid metabolism, medium chain fatty acids, omega-3, omega-6, plasma oxylipids.

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LISTA DE ABREVIAÇÃO

AA	Ácido Araquidônico
ACACA/ACC	Acetil CoA Carboxilase Alfa
AG	Ácidos Graxos
AGCM	Ácidos Graxos de Cadeia Média
AGI	Ácidos Graxos Insaturados
AGMI	Ácidos Graxos Monoinsaturados
AGNE	Ácidos Graxos Não Esterificados
AGPI	Ácidos Graxos Poli-insaturados
AGS	Ácidos Graxos Saturados
ALA	Ácido Alfa-Linolênico
BHB	β-hidroxibutirato
C16:0	Ácido Palmítico
C18:0	Ácido Esteárico
C18:1	Ácido Oleico
C18:2	Ácido Linoleico
C18:3	Ácido Linolênico
CLA	Ácido Linoleico Conjugado
COX	Cicloxigenase
CYP450	Citocromo 450
DGL	Depressão da Gordura no Leite
DHA	Ácido Docosahexaenoico
EPA	Ácido Eicosapentaenoico
ERO	Espécies Reativas de Oxigênio
FABP	Proteína Ligadora de Ácidos Graxos
FAS	Ácido Graxo Sintase
FGF21	Fator de Crescimento de Fibroblastos 21
GH	Hormônio do Crescimento
GPCR	Receptor Acoplado à Proteína G
HETE	Hidroxieicosatetraenoico

HODE	Hidroxioctadecadienoico
HS	Heat Stress/ Estresse calórico
Ig	Imunoglobulina
IL	Interleucina
ITU	Índice de Temperatura e Umidade
LA	Ácido Linoleico
LBP	Proteína Ligadora de Lipopolissacarídeo
LOX	Lipooxigenase
LPL	Lipoproteína Lipase
LPS	Lipopolissacarídeo
MS	Matéria Seca
NF-kB	Fator Nuclear Kappa-B
oxoODE	Oxooctadecadienoico
PAMPs	Padrões Moleculares Associados a Patógenos
PGD2	Prostaglandina D2
PGE2	Prostaglandina E2
PGF2a	Prostaglandina F2 alfa
PLA2	Fosfolipase A2
PPAR-γ	Receptor Ativado por Proliferador de Peroxissomo γ
SCD	Dessaturase de Ácidos Graxos
SREBP1	Elemento Regulatório de Ligação a Esterol 1
TLR-4	Receptor Toll Like 4
TNF-α	Fator de Necrose Tumoral alfa
TNPF	Termoneutralidade in Pair Feeding
VLDL	Lipoproteína de Muito Baixa Densidade
Δ5D	Delta-5-Dessaturase
Δ6D	Delta-6-Dessaturase

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1 INTRODUÇÃO

A pecuária leiteira enfrenta diversos desafios fisiológicos que afetam diretamente o desempenho produtivo e a saúde das vacas. Entre esses desafios, dois dos mais proeminentes na atualidade são o período de transição e o estresse calórico (LeBlanc et al., 2006; Schleussner et al., 2016). Durante o período de transição, que engloba as 3 semanas que antecedem o parto e as 3 semanas seguintes ao parto, as vacas experimentam mudanças fisiológicas e metabólicas marcantes (Drackley, 1999). O aumento da demanda energética devido à crescente produção de leite e as adaptações hormonais inerentes ao início da lactação podem desencadear processos inflamatórios, estresse oxidativo e outros distúrbios metabólicos (Chew et al., 1979; Agrawal et al., 2017).

Paralelamente, o estresse calórico, intensificado pelas alterações climáticas, representa um desafio adicional para toda a cadeia de produção, prejudicando o desempenho produtivo, reprodutivo e o bem-estar dos animais (Mattachini et al., 2013; Das et al., 2016). O estresse térmico expande os enterócitos intestinais, permitindo a entrada de bactérias na corrente sanguínea, e os lipopolissacarídeos (LPS) presentes na parede bacteriana ativam centros de resposta imunológica, desencadeando inflamação (Mueller et al., 2004; Munford, 2016). Além disso, vacas expostas ao estresse calórico por períodos prolongados tornam-se mais suscetíveis a desencadear distúrbios metabólicos, originados pelo desequilíbrio fisiológico, através da inflamação sistêmica (West, 2003).

Nesse cenário desafiador, a suplementação lipídica surge como estratégia promissora para mitigar os efeitos adversos do período de transição e do estresse calórico em vacas leiteiras. Os ácidos graxos de cadeia média apresentam propriedades benéficas, podendo ser uma fonte eficaz de energia para vacas em transição, auxiliando na adaptação metabólica e na redução de problemas associados, como a cetose e a lipidose hepática (Fukomori et al., 2013). Já a inclusão de ácidos graxos ômega-6, durante o estresse térmico, levanta questionamentos sobre seu potencial pró-inflamatório agravante, enquanto a introdução de ácidos graxos ômega-3 pode ser explorada como uma abordagem anti-inflamatória para favorecer o conforto térmico e a saúde dos animais (Calder, 2010).

Além disso, a pesquisa busca investigar o uso de oxilipídeos como biomarcadores inflamatórios, visando compreender os mecanismos moleculares envolvidos nos processos inflamatórios desencadeados pelo estresse calórico. Os oxilipídeos são ácidos graxos poliinsaturados oxidados, que desempenham papéis cruciais na regulação do início, desenvolvimento e resolução da inflamação (Sordillo & Mavangira, 2014). Identificar e analisar esses biomarcadores pode oferecer entendimentos valiosos sobre o status inflamatório das vacas leiteiras, possibilitando intervenções mais precisas e eficazes. Assim, a literatura respalda que a suplementação de ácidos graxos poliinsaturados pode modificar a biossíntese do perfil oxilipídico e a capacidade funcional das células em responder à inflamação e ao sistema imunológico (Sordillo, 2018).

Assim, este estudo pretende contribuir para a melhor compreensão da suplementação de ácidos graxos de cadeia média, otimizando o desempenho produtivo e o metabolismo durante o período de transição. Paralelamente, busca-se compreender o comportamento e a imunomodulação das vias oxilipídicas em vacas em estresse calórico, considerando a infusão abomasal de ácidos graxos pró-inflamatórios e anti-inflamatórios. Portanto, a compreensão aprofundada das interações entre o período de transição, estresse calórico, inflamação e os efeitos da suplementação nutricional são essenciais para desenvolver estratégias eficazes que aprimorem a saúde e o desempenho das vacas leiteiras nesse período desafiador.

CAPÍTULO I. REVISÃO DE LITERATURA

Nesta revisão de literatura serão abordados temas correlacionados aos tópicos em estudo, visando fornecer uma base informativa que justifique e promova a análise da metodologia e resultados dos experimentos em questão.

2.1 Desafios fisiológicos na bovinocultura leiteira

Os desafios fisiológicos referem-se a condições que demandam adaptações nos processos normais do corpo para manter o equilíbrio interno, conhecido como homeostase. Essas condições são comuns em sistemas de produção de bovinos leiteiros, ocorrendo em várias fases da criação. Tais desafios podem abranger aspectos fisiológicos, metabólicos, nutricionais, ambientais e patológicos.

Atualmente, vacas leiteiras enfrentam maiores demandas nutricionais, ambientais e de manejo em comparação com décadas passadas (Bauman & Currie, 1980). Essas mudanças na bovinocultura leiteira são resultado de um intenso melhoramento genético para características produtivas nas últimas décadas, não necessariamente acompanhado de um melhoramento genético para características de saúde e de resiliência. Este aumento de produtividade e o aumento dos distúrbios metabólicos levantam preocupações globais sobre a saúde e longevidade desses animais. Isso tem impulsionado pesquisas focadas na prevenção e mitigação de doenças, ao invés do tratamento direto.

Entre diversos distúrbios metabólicos durante o ciclo de vida de uma vaca, aproximadamente 75% das doenças normalmente ocorrem no primeiro mês após o parto, durante o período de transição (LeBlanc et al., 2006). O período de transição tem sido associado a um período desafiador para o animal, compreendido pela interação do metabolismo energético e a imunossupressão, resultando em inflamação e estresse oxidativo (LeBlanc et al., 2006). Assim, estratégias nutricionais, que levem em consideração aspectos comportamentais e ambientais, surgem como fatores cruciais para melhorar a saúde e a longevidade desses animais.

O estresse calórico é um dos principais estressores ambientais, que tem ganhado atenção devido ao seu impacto no consumo de matéria seca (MS), no aumento da temperatura corporal e frequência respiratória (Thatcher, 1974; Tucker et al., 2007). Isso

resulta em maiores demandas energéticas para processos metabólicos e de manutenção, reduzindo consequentemente a produção de leite e a eficiência reprodutiva (St. Pierre et al., 2003). Embora essas mudanças sejam bem compreendidas, as alterações metabólicas e imunomoduladoras ainda estão sendo investigadas. Contudo, já está bem compreendido que os desafios fisiológicos e metabólicos enfrentados pelas vacas leiteiras sob estresse calórico têm impacto direto na produtividade e lucratividade dos rebanhos leiteiros (Grummer, 1993).

2.2 Período de transição

O período de transição compreende o período entre 21 dias antes do parto e 21 dias após o parto (Grummer, 1995, Drackley, 1999). Durante esse período, as vacas em transição passam por mudanças fisiológicas e metabólicas para se prepararem para o parto e início de lactação. Estas adaptações envolvem alterações no processo metabólico hepático, tecido adiposo, esquelético e endócrino (Head & Gulay, 2001).

Assim, a proximidade do parto leva a um rápido e exponencial crescimento fetal (NASEM, 2021), causando aumento da pressão nos órgãos do sistema digestivo e redução do espaço interno na cavidade abdominal (Forbes, 1968; Park et al., 2011), reduzindo a ingestão de matéria seca em até 30% (NRC, 2001; Barletta et al., 2017), mantendo o consumo de MS limitado, principalmente nas primeiras 4 semanas após o parto. Ainda nessa fase, ocorrem diversas alterações hormonais, incluindo aumento na concentração plasmática de estrógenos e corticosteroides (Chew et al., 1979). Assim, a concentração de insulina diminui, enquanto o hormônio do crescimento (GH) aumenta (Kunz et al., 1985). A glicose plasmática aumenta no dia do parto e diminui logo em seguida após este evento, devido ao aumento na utilização de glicose nos processos de homeostase fisiológica e produção de leite (Kunz et al., 1985). Este cenário propicia um aumento na resistência à insulina no pós-parto, resultando em maior saciedade (Abou-Rjeileh et al., 2022), juntamente com a ingestão de matéria seca limitada associada às altas demandas energéticas, decorrentes do desenvolvimento fetal, parto e síntese de colostro e produção de leite, o que acarreta em balanço energético negativo (Bell, 1995; Capuco et al., 1997; Brown & Allen, 2013; Agrawal et al., 2017).

Dessa forma, o período de transição tem sido considerado um período desafiador para as vacas leiteiras, que precisam passar por diversas alterações fisiológicas para retomar à homeostase. Essas mudanças abruptas acarretam em imunossupressão e estresse oxidativo, tornando as vacas leiteiras mais susceptíveis a doenças infecciosas e distúrbios metabólicos, como hipocalcemia, cetose, distocia no parto, prolapso de útero, retenção de placenta, mastite, metrite, deslocamento de abomaso e, em geral, baixa imunidade (Duffield et al., 1999; Kimura et al., 2006; Goff et al., 2008; Chapinal et al., 2011; Selfi et al., 2011; MCart et al., 2012; Sepulveda-Varas et al., 2015), resultando em baixa produção de leite e problemas reprodutivos (Jonsson et al., 1999; MCnally et al., 2014).

No entanto, alguns pesquisadores questionam a ideia de que vacas recém-paridas têm baixa imunidade. O estudo de Opgernorth et al. (2024) demonstrou que, após a administração intramamária de lipopolissacarídeo (LPS) em vacas em início e meio da lactação , todas as vacas desenvolveram febre, com intensidade maior nas vacas em lactação inicial. A haptoglobina aumentou após a aplicação de LPS, mas não houve diferença entre os grupos. As vacas em início de lactação tiveram um aumento mais significativo de citocinas pró-inflamatórias, embora isso não tenha afetado a produção de leite. As vacas em início de lactação exibem uma resposta imunológica à endotoxina muito mais intensa do que as vacas em lactação média, indicando que a hipótese de imunossupressão em vacas periparturientes requer mais estudos, pois as vacas mostram uma resposta imunológica dinâmica para restaurar a homeostase fisiológica (Opgernorth et al., 2024).

Contudo, a compreensão aprofundada das alterações fisiológicas e metabólicas busca aprimorar as estratégias nutricionais e de manejo, visando reduzir a exposição do animal ao balanço energético negativo e demais distúrbios metabólicos.

2.3 Adaptações fisiológicas associadas à mobilização de gordura durante o período de transição

O balanço energético negativo constitui uma adaptação fisiológica, desencadeando a intensa mobilização do tecido adiposo. Este processo resulta na liberação mais acentuada de ácidos graxos não esterificados (AGNE) na corrente

sanguínea, ocorrendo a um ritmo duas ou três vezes superior ao habitual, num intervalo de tempo relativamente rápido (Contreras & Sordillo, 2011). Esse mecanismo visa suprir a elevada demanda energética, sendo que as concentrações de AGNE apresentam uma correlação inversa com a ingestão de matéria seca (Overton & Waldron, 2004). Consequentemente, o fígado remove porções de AGNE da circulação sanguínea, metabolizando-os em corpos cetônicos ou triglicerídeos, ocasionando modificações na utilização de substratos no processo metabólico (Herdt, 2000).

Os AGNE presentes na corrente sanguínea podem ser utilizados por diversos tecidos corporais para produção de energia e preservar a glicose circulante (Pullen et al., 1989). O fígado metaboliza os AGNE em conformidade com as quantidades que chegam da corrente sanguínea. Sendo assim, a concentração plasmática de AGNE reflete a magnitude da mobilização do tecido adiposo (Grummer, 1993; Drackley, 1999).

A alta produção de corpos cetônicos pode resultar em cetose ou hipercetonemia, caracterizada por níveis de β-hidroxibutirato (BHB) superiores ou iguais a 1,2 mmol/L, aumentando a suscetibilidade a condições patológicas, tais como esteatose hepática, deslocamento de abomaso (Suthar et al., 2013) e redução na produção de leite e fertilidade (MCart et al., 2013). Estudos indicam que o período médio de persistência do balanço energético negativo pós-parto é de aproximadamente 45 dias, com um desvio padrão de 21 dias (Grummer & Rastani, 2004).

Os ácidos graxos não esterificados (AGNE) são absorvidos pelo fígado e são parcialmente ou totalmente oxidados (Bertics et al., 1992; Grum et al., 1994), gerando energia no processo de cetogênese. Este processo envolve a produção de glicose a partir de lipídios e ocorre nas mitocôndrias das células hepáticas.

Esta sobrecarga hepática resultante de uma cetogênese excessiva reduz a capacidade do fígado de realizar a gliconeogênese, aumentando a probabilidade de desenvolver distúrbios metabólicos, como lipidose hepática, inflamação e estresse oxidativo.

2.4 Estresse calórico em vacas leiteiras

O estresse calórico representa uma condição ambiental que ultrapassa a capacidade de regulação térmica do organismo, resultando em desconforto, disfunções

fisiológicas e impactos adversos na saúde, bem-estar e reprodução das vacas leiteiras (Mattachini et al., 2013; Das et al., 2016). Estudos meteorológicos indicam um aumento gradual na temperatura ambiente anualmente (Schleussner et al., 2016), intensificando a preocupação com o estresse térmico na pecuária. As vacas leiteiras são particularmente sensíveis às variações de temperatura devido à produção de calor proveniente das funções digestórias, atividade física, gastos metabólicos e produção de leite, enfrentando desafios na dissipação do calor corporal (Coppock, 1985; NRC, 2001).

O estresse térmico resulta da combinação de dois fatores principais: temperatura ambiental e umidade relativa do ar, sendo avaliado pelo Índice de Temperatura e Umidade (ITU; Berry et al., 1964; Morton et al., 2007). O ITU considerado adequado para vacas leiteiras é inferior a 68, com variações toleráveis na temperatura e umidade (Zimbelman et al., 2009). Classificações incluem ITU < 68 para animais sem estresse térmico, ITU entre 68 e 72 para estresse térmico moderado, ITU entre 72 e 78 para estresse térmico severo, e ITU > 78 para estresse térmico extremo (Habeeb et al., 2018). Exposição prolongada a ITU elevado pode resultar na morte de animais vulneráveis (Stull et al., 2008; Vitali et al., 2009). As temperaturas ideais para vacas lactantes situam-se entre -0,5 e 20°C, com uma temperatura crítica superior a 21°C (Berman et al., 1985; Johnson, 1988).

Efeitos associados, como aumento da temperatura corporal, taxa respiratória, redução do consumo de matéria seca e produção de leite, além de problemas reprodutivos, estão bem documentados (Thatcher, 1974; Cook et al., 2007; Tucker et al., 2007; Rhoads et al., 2009). Além disso, o estresse calórico geralmente eleva a temperatura retal, estabelecendo uma relação positiva. A cada aumento de 0,5°C acima da temperatura retal, uma vaca pode perder de 0,7 a 1,8 kg/leite/dia (Johnson et al., 1963; Zimbelman et al., 2009).

A redução na produção de leite está associada à diminuição do consumo de matéria seca, mas também à redistribuição de nutrientes e energia (Rhoads et al., 2009; Wheelock et al., 2010; Kvidera et al., 2017). Em condições de termoneutralidade, as vacas apresentam aumento na lipólise, resultando em maior concentração de ácidos graxos não esterificados (AGNE) no sangue, que são utilizados para a manutenção e produção de leite (Rhoads et al., 2009). No entanto, em situações de estresse térmico,

observa-se redução na concentração plasmática de AGNE (Wheelock et al., 2010). Essa alteração é influenciada por mudanças hormonais, como a elevação da liberação de insulina durante o estresse térmico. Com uma baixa concentração de AGNE, o organismo precisa recorrer à glicose para a manutenção, o que resulta em uma deficiência para a produção de leite (Rhoads et al., 2009). A interação entre a regulação da insulina e a glicose sanguínea é influenciada por estímulos do sistema imunológico (Dalmas, 2019).

Os neurônios, sendo sensíveis à temperatura, transmitem informações ao hipotálamo para regular estímulos relacionados à termorregulação (Curtis, 1983). Consequentemente, a somatotropina é impactada, desestabilizando as proteínas, carboidratos e lipídios no processo digestivo (Bauman e Currie, 1980; Collier et al., 2008; Wheelock et al., 2010). A diminuição na ingestão de nutrientes, a reduzida absorção devido à dilatação dos enterócitos, juntamente com desequilíbrios no ambiente ácido-base do rúmen e alterações hormonais, são fatores que também contribuem para a redução na produção de leite (McGuire et al., 1989; West, 2003).

O estresse calórico exerce impactos negativos sobre o sistema reprodutivo, reduzindo a fertilidade, taxa de concepção e aumentando a ocorrência de morte embrionária (West, 2003; Hansen, 2007; Oullet et al., 2021). Além disso, as elevadas temperaturas têm efeitos prejudiciais sobre o desenvolvimento embrionário (Hansen, 2007; Collier et al., 2008). Pesquisas indicam que embriões submetidos a estresse calórico podem sofrer efeitos negativos em seu crescimento e desenvolvimento, resultando em menor peso ao nascer, reduzida produção de leite e maior taxa de descarte da progênie até a fase adulta (Laporta et al., 2017; Oullet et al., 2021).

Assim, as alterações fisiológicas e comportamentais desencadeadas pelo estresse térmico têm impacto direto na saúde, bem-estar e desempenho produtivo das vacas leiteiras, e não devem ser subestimadas. Portanto, é crucial adotar estratégias que melhorem o conforto e minimizem os efeitos do estresse térmico, como a disponibilidade de sombra, ventilação, aspersão de água e acesso fácil à água fresca (West, 2003). Além disso, busca-se desenvolver estratégias nutricionais que auxiliem a mitigar os distúrbios metabólicos, acelerando o retorno à termoneutralidade, e compreender os mecanismos metabólicos provocados pelo estresse calórico.

2.5 Inflamação associada ao período de transição e estresse calórico

A resposta inflamatória é uma reação adaptativa do organismo diante de estímulos prejudiciais, como infecção e lesão tecidual, com o objetivo de restaurar a homeostase (Medzhitov, 2008). Enquanto a compreensão da inflamação aguda avançou consideravelmente, a inflamação crônica ou sistêmica ainda é menos compreendida e pode estar associada ao mau funcionamento do tecido, em vez dos causadores clássicos da inflamação, como infecção ou lesão. Embora os mecanismos da resposta inflamatória induzida por agentes infecciosos sejam mais conhecidos, a inflamação pode ser desencadeada por diversos fatores. A transição da inflamação aguda para a resolução envolve mudanças nos mediadores lipídicos pró-inflamatórios para anti-inflamatórios e na substituição de neutrófilos por macrófagos (Serhan & Savill, 2005; Serhan, 2007). Se a resposta aguda não conseguir eliminar o patógeno, a inflamação pode evoluir para um estado crônico (Kumar & Contran, 1994; Drayton et al., 2006).

Os sinais inflamatórios desempenham papéis duplos como indutores e mediadores da inflamação. Os indutores são os sinais que iniciam a resposta inflamatória, que pode incluir lipopolissacarídeos (LPS) e alérgenos. Eles ativam sensores específicos, como receptor toll like 4 (TLR-4), fator nuclear kappa-B (NF-kb) e imunoglobulina (IgE), desencadeando a produção de conjuntos específicos de mediadores, como fator tumoral de necrose alfa (TNF-α), interleucina 1 e 6 (IL-1, IL-6), prostaglandina E2 (PGE2) e aminas (Barton et al., 2008). Esses mediadores alteram o estado funcional dos tecidos e órgãos, os efetores da inflamação, para que possam se adaptar às novas condições. Assim, a inflamação comum consiste em indutores, sensores, mediadores e efetores, onde cada componente determina o tipo de resposta inflamatória.

Os indutores da inflamação podem ser exógenos ou endógenos. Os exógenos incluem padrões moleculares associados a patógenos (PAMPs) e fatores de virulência, enquanto os não microbianos englobam alérgenos, irritantes, corpos estranhos e compostos tóxicos (Medzhitov, 2008). Os PAMPs são reconhecidos por receptores de reconhecimento de padrões, enquanto os fatores de virulência são detectados indiretamente pelos danos teciduais (Medzhitov & Janeway, 1997). Alérgenos podem imitar a atividade de virulência dos parasitas e são detectados por mecanismos ainda não totalmente compreendidos (Sokol et al., 2008). Os corpos estranhos desencadeiam uma

resposta inflamatória quando são grandes demais para serem fagocitados ou causam danos à membrana fagossômica em macrófagos, sendo reconhecidos por sensores como o inflamassoma NALP3 (Sokol et al., 2008).

A inflamação aguda segue uma sequência definida, começando com uma fase de iniciação e fase de resolução. Na fase inicial, ocorre vasodilatação nos vasos sanguíneos da área afetada, levando a sinais de calor, inchaço e dor, controlados por peptídeos químicos, como citocinas e quimiocinas, e mediadores lipídicos, como prostaglandinas e leucotrienos (Majno & Joris, 2004). Essa permeabilidade vascular aumentada facilita a migração de leucócitos, principalmente neutrófilos, para o local da inflamação, onde neutrófilos-macrófagos fagocitam microrganismos invasores e iniciam o processo de reparo tecidual (Karp, 2010; Ward, 2010). À medida que os patógenos são eliminados e o tecido começa a se reparar, a inflamação entra em sua fase de resolução. Durante essa fase, mediadores anti-inflamatórios, como lipoxinas e resolvinas, ajudam a limitar a resposta inflamatória e promover a cicatrização (Spite et al., 2014). No entanto, se a presença contínua dos leucócitos não for devidamente controlada e eliminada, pode ocorrer danos teciduais colaterais e persistência da inflamação.

Assim, durante o estresse calórico, ocorre vasodilatação para facilitar a dissipação de calor e alcançar a termoneutralidade. Esse processo também afeta os enterócitos, aumentando sua vasodilatação. Bactérias gram-negativas presentes no intestino migram para a corrente sanguínea, levando consigo os lipopolissacarídeos (LPS) que compõem sua membrana estrutural. Esses LPS são detectados pelo LBP (Proteína Ligadora de Lipopolissacarídeo), ativando a via inflamatória conhecida como Receptor Toll Like 4/Fator Nuclear Kappa-B (TLR-4/NF-Kb; Neal et al., 2006; Lu et al., 2008). A ativação do TLR-4 desencadeia uma série de eventos intracelulares que resultam na produção e liberação de citoquinas inflamatórias, quimiocinas, moléculas de adesão e outros mediadores imunológicos (Grant & Stephens, 2015).

Além do LPS, o TLR-4 pode ser ativado por outros ligantes endógenos, como produtos de degradação celular, proteínas de choque térmico e ácidos graxos oxidados, desempenhando um papel na resposta inflamatória associada a danos teciduais e estresse oxidativo celular (Medzhitov, 2008). Essa resposta inflamatória é parte integrante da resposta imunológica inata e desempenha um papel crucial na eliminação

de patógenos. A transcrição e tradução do TNF-α podem ser influenciadas pela temperatura ambiental. Por exemplo, vacas que parem durante períodos de calor intenso no verão tendem a ter uma maior concentração de proteína TNF-α comparado a vacas que parem no inverno, sendo ainda mais pronunciado em vacas com maior perda de peso durante esse período (Zachut et al., 2020). Esses resultados sugerem uma possível resposta inflamatória mais intensa no tecido adiposo de vacas estressadas pelo calor e com maior grau de liberação de ácidos graxos (Zachut et al., 2020). Existem alguns mecanismos conhecidos para inibir ou bloquear a resposta inflamatória. Muitos mediadores pró-inflamatórios, como as prostaglandinas e citocinas, são bem estudados, e os tratamentos anti-inflamatórios frequentemente visam bloqueá-los ou antagonizá-los para controlar a inflamação excessiva (Spite et al., 2014).

Os ácidos graxos ômega-3, como ácido eicosapentaenoico (EPA) e o ácido docosahexaenoico (DHA), desempenham papel significativo na modulação da resposta inflamatória ao inibir a atividade da proteína de transcrição NF-kB. Esses ácidos graxos ativam os receptores acoplados à proteína G 120 (GPCR120), o que resulta na inibição da NF-kB e, por conseguinte, na redução da produção de citoquinas inflamatórias (Calder, 2013). Além disso, os ômega-3 têm a capacidade de inibir diretamente o estímulo do TLR-4, impedindo assim a ativação subsequente da NF-kB e a produção de citoquinas inflamatórias. Ademais, os ácidos graxos ômega-3 regulam a expressão gênica do PPAR-y, que também atua na inibição da NF-kB, contribuindo assim para a regulação da resposta inflamatória (Calder, 2013).

Vacas no período de transição enfrentam um aumento significativo no risco de distúrbios metabólicos e patologias devido às alterações hormonais que diminuem a imunidade (Zebeli et al., 2015). Após o parto, ocorre um aumento na produção de leite, porém muitas vezes as vacas não conseguem consumir nutrientes suficientes para atender a essa demanda, resultando em balanço energético negativo (Zebeli et al., 2015). Uma estratégia comum para lidar com isso é fornecer altas concentrações de concentrado na dieta, mas quando isso não é equilibrado com uma adequada proporção de forragem, pode ter efeitos adversos no rúmen (Zebeli et al., 2012). Estudos indicam que problemas de saúde no rúmen e no intestino podem levar a uma degradação incompleta, prejudicando os microorganismos ruminais, aumentando a taxa de

passagem, causando diarreia e reduzindo a absorção de nutrientes no intestino. Esses fatores contribuem para a inflamação sistêmica e aumentam o risco de distúrbios metabólicos, como cetose, hipocalcemia, laminite e deslocamento de abomaso (Plaizier et al., 2012; Zebeli & Metzler-Zebeli, 2012; Li et al., 2012; Metzler-Zebeli et al., 2013; Bradford et al., 2015).

Outro fator desencadeador de inflamação durante o período pré-parto é o balanço energético negativo, onde as vacas utilizam as reservas corporais de tecido adiposo como fonte de energia para a produção de leite, aumentando a probabilidade de desenvolver resistência à insulina nesse período crítico. A intensa mobilização lipídica, junto com a resistência à insulina, resultam em altos níveis de ácidos graxos não esterificados na corrente sanguínea. Esses ácidos graxos são metabolizados pelo fígado, liberando corpos cetônicos como o β-hidroxibutirato (BHB). Concentrações elevadas desses ácidos graxos podem desequilibrar o sistema imunológico, ampliar as respostas inflamatórias e sobrecarregar o sistema hepático (Contreras & Sordillo, 2011). Ainda não está claro se a redução no consumo é causada pela inflamação sistêmica ou se a diminuição no consumo leva ao balanço energético negativo e, consequentemente, à inflamação sistêmica no período de transição (Pascotini et al., 2020). Além disso, foi observado que após dois dias do início do período de secagem da vaca, os níveis circulantes de cortisol e ácidos graxos não esterificados aumentam (Putman et al., 2018). Esses resultados sugerem que as doenças clínicas que surgem após o parto estão frequentemente associadas ao período pré-parto (LeBlanc et al., 2010). Essa relação pode ser atribuída à mobilização de gordura devido à mudança para uma dieta de menor teor energético, à pressão intramamária resultante do processo de secagem e à involução mamária (Kushibiki et al., 2003; Sordillo & Mavangira, 2014).

As vacas enfrentam desafios de inflamação sistêmica, pois a inflamação desempenha um papel crucial na ativação e adaptação do sistema imunológico às mudanças fisiológicas. No entanto, a intensidade e a duração da resposta inflamatória podem variar entre os animais (Holland & Hamilton, 2013). O processo inflamatório libera mediadores inflamatórios, como citocinas, quimiocinas, moléculas de adesão e proteínas de fase aguda. Essas últimas aumentam suas concentrações no sangue em resposta à inflamação e são frequentemente usadas como biomarcadores desse processo (Kumar

& Cotran, 1994; Majno & Joris, 2004). Entre os marcadores mais comuns estão citocinas como o fator de necrose tumoral (TNF), interleucinas (IL) e proteínas de fase aguda, como haptoglobina, glicoproteína ácida, albumina, imunoglobulinas G e A, transferrina e ceruloplasmina (Zachut & Contreras, 2022).

O TNF-α é um poderoso mediador da inflamação, desencadeando alterações transcricionais mediadas por NF-kB e sinalização de quinases relacionadas ao sinal extracelular. A elevação dos níveis de TNF-α está associada ao aumento do número de macrófagos em vacas no pós-parto (Hotamisligil et al., 1995; Grant & Stephens, 2015). A proteína LBP, ao se ligar ao LPS de bactérias gram-negativas, estimula os macrófagos por meio da interação com o TLR-4 (Ceciliani et al., 2012). Baixas concentrações de LBP têm efeitos pró-inflamatórios, enquanto altas concentrações têm efeitos anti-inflamatórios (Lamping et al., 1998). Assim, os níveis de LBP aumentam após o parto em comparação com o período seco, especialmente em vacas com balanço energético negativo mais intenso em comparação com aquelas que perderam peso moderadamente (Zachut et al., 2018; Zachut et al., 2020).

As proteínas de fase aguda (APPs) são proteínas cujos níveis séricos se alteram em resposta aos processos inflamatórios, infecções, traumas ou outras condições que desencadeiam uma resposta de fase aguda no organismo. Elas desempenham papel crucial na regulação da resposta imunológica e na modulação da inflamação (Ceciliani et al., 2012). As APPs incluem uma variedade de proteínas, como haptoglobina, ceruloplasmina, proteína C reativa (PCR) e amilóide sérica A (SAA). Suas funções abrangem desde a modulação da coagulação sanguínea até o transporte de metais e a regulação do sistema imunológico, além do reparo tecidual. O tecido adiposo foi identificado como um local de síntese e possível secreção de proteínas de fase aguda (Ceciliani et al., 2012). A análise dos níveis de APPs pode ser utilizada como marcador para monitorar a presença e a gravidade de doenças inflamatórias e infecciosas, assim como a eficácia do tratamento.

As atividades biológicas da haptoglobina (HP) são diversas, incluindo a regulação das respostas imunes inatas nos glóbulos brancos, um efeito bacteriostático direto e uma atividade chaperona (Ceciliani et al., 2012). Assim, a função chaperona da haptoblobina está associada à sua capacidade de ligação com proteínas desnaturadas ou mal

formadas, as quais desestabilizam e causam danos celular (Ceciliani et al., 2012). Estudos proteômicos revelaram um aumento significativo na presença de haptoglobina no tecido adiposo de vacas com alta taxa de lipólise em comparação àquelas com baixa lipólise, sugerindo um possível aumento do estado inflamatório no tecido adiposo desses animais (Zachut et al., 2018). É relevante observar que esse aumento na concentração de haptoglobina no tecido adiposo pode ser parcialmente devido à sua liberação na corrente sanguínea, indicando uma possível correlação com a inflamação sistêmica nessas vacas. Portanto, a haptoglobina surge como um promissor marcador de inflamação no tecido adiposo bovino (Zachut & Contreras, 2022).

Além disso, a biossíntese de mediadores lipídicos pode desencadear resposta inflamatória no tecido adiposo. Esses mediadores incluem lipídios neutros, fosfolipídios (como ceramidas e esfingolipídios) e oxilipídeos (Sordillo, 2018; McFadden & Rico, 2019). Os oxilipídeos, derivados principalmente de ácidos graxos poli-insaturados (AGPI), como o ácido linoleico e o araquidônico, são produzidos durante a lipólise e podem modular rapidamente as respostas inflamatórias (Contreras et al., 2017). Entre os derivados do ácido linoleico, os ácidos hidroxioctadecadienóicos (HODEs) despertam interesse, pois são produzidos em grande quantidade durante o período periparturiente (Gartung et al., 2016; Contreras et al., 2020). Enquanto alguns HODEs, como o 13-HODE, promovem a polarização dos macrófagos para um estado anti-inflamatório e agem como ligantes do receptor nuclear PPAR-γ, reduzindo as respostas inflamatórias através de GPR13 e TLR-4, outros, como o 10-HODE e o 12-HODE, estão associados à desregulação da lipólise e ao estado inflamatório. A compreensão desses processos de oxidação de ácidos graxos é fundamental para elucidar a dinâmica da inflamação no tecido adiposo (Zachut & Contreras, 2022).

Em síntese, a interação entre o período de transição e o estresse térmico representa um desafio significativo para a saúde e o desempenho das vacas leiteiras. Durante esse período crítico, as alterações hormonais, metabólicas e a exposição ao calor excessivo, aumentam a suscetibilidade a distúrbios metabólicos e patológicos. Além disso, a influência da nutrição inadequada e do manejo inadequado durante esse período pode exacerbar a inflamação e seus efeitos adversos. Portanto, estratégias de manejo e nutrição direcionadas a minimizar o estresse térmico, promover a saúde

metabólica e fortalecer a resposta imune são fundamentais para garantir o bem-estar e a produtividade das vacas durante o período de transição, particularmente sob condições de estresse térmico.

2.6 Estresse oxidativo associado ao período de transição e estresse calórico

A partir da instabilidade de elétrons, surgem os radicais livres e, consequentemente, as espécies reativas de oxigênio (ERO), que incluem superóxido de ânions (O_2^-), peróxido de hidrogênio (H_2O_2) e radicais hidroxila livres (OH). Estes estão intimamente ligados ao metabolismo do oxigênio, exibindo alta reatividade com moléculas biológicas (Halliwell, 2007; Schieber & Chandel, 2014). Durante a peroxidação lipídica, os radicais livres capturam elétrons dos lipídeos das membranas celulares, introduzindo um oxigênio molecular nos ácidos graxos, resultando na deterioração da estrutura celular (Gitto et al., 2002; Sharma et al., 2011). Embora as ERO sejam naturalmente produzidas durante o metabolismo animal, um excesso de radicais livres e uma baixa disponibilidade de antioxidantes podem levar ao estresse oxidativo (Halliwell, 2007; Sordillo & Aitken, 2009). Este pode ser desencadeado por condições como inflamação, estresse, altas temperaturas (radiação ultravioleta) e toxinas ambientais.

Os antioxidantes atuam inibindo a oxidação, doando elétrons e mantendo a estabilidade celular, neutralizando as ERO. Enzimas antioxidantes como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), além de antioxidantes não enzimáticos como vitamina C, vitamina E e glutationa, desempenham papéis essenciais na prevenção do estresse oxidativo e na manutenção da saúde celular (Ifeanyi, 2018; Carmo de Carvalho & Martins et al., 2022).

O estresse oxidativo gerado pelas ERO pode resultar em dano oxidativo, incluindo danos ao DNA que aumentam o risco de câncer e outras doenças, danos a proteínas que contribuem para o envelhecimento e danos a lipídeos, resultando em peroxidação lipídica e inflamação celular (Sies, 1991; Haliwell & Whiteman, 2004). Esse processo pode desencadear inflamação crônica, pois o estresse oxidativo pode ativar vias inflamatórias, que por sua vez agravam ainda mais o estresse oxidativo, estabelecendo um ciclo prejudicial (Halliwell, 2006). Altos níveis de espécies reativas de oxigênio (ERO) não apenas desencadeiam estresse oxidativo, mas também prejudicam os sistemas de

reparo celular (Halliwell & Whiteman, 2004; Halliwell, 2006; 2007). No entanto, há evidências sugerindo que as ERO também podem contribuir para a eliminação de patógenos. Portanto, o impacto das ERO depende do grau de dano oxidativo presente (Halliwell, 2006).

Durante o período de transição das vacas leiteiras ocorre estresse fisiológico, devido à diferenciação celular do parênquima secretor, ao crescimento das glândulas mamárias e à síntese de leite, resultando em alta demanda por energia e oxigênio. Essa demanda aumentada de oxigênio desencadeia a produção de espécies reativas de oxigênio (Gitto et al., 2002; Contreras & Sordillo, 2011). No entanto, após o parto e durante a fase inicial da lactação, as vacas enfrentam um alto estresse oxidativo devido ao baixo consumo de matéria seca e de minerais essenciais como selênio, cobre e zinco, o que as torna mais suscetíveis a doenças e problemas metabólicos (Sordillo, 2016).

Durante esse desafio fisiológico, as vacas leiteiras em início de lactação aumentam suas taxas de lipólise no tecido adiposo, resultando na liberação de ácidos graxos livres (Zachut & Contreras, 2022). Esses ácidos graxos são então oxidados nas mitocôndrias através do processo de β-oxidação, que envolve uma série de reações enzimáticas. Durante esse processo, os ácidos graxos não esterificados (AGNE) são quebrados em unidades menores de acetil-CoA, que são subsequentemente oxidadas para produzir energia na forma de ATP no ciclo de Krebs (Lehninger, 2006). No entanto, a maior disponibilidade de AGNE para β-oxidação resulta em maior geração de ERO, que por sua vez ativa a lipase sensível a hormônios (Zhou et al., 2019).

Além disso, evidências sugerem que a β-oxidação é aumentada próximo ao parto devido à maior disponibilidade de ácidos graxos saturados e insaturados liberados pelas gotículas lipídicas, o que aumenta a atividade dos peroxissomos, organelas celulares responsáveis pela oxidação de ácidos graxos (Xie et al., 2011; Zachut & Contreras, 2022). Esse aumento na β-oxidação durante o periparto resulta em uma explosão oxidativa no tecido adiposo, levando à inflamação em ratos e bovinos (Kosteli et al., 2010; Contreras et al., 2015). Ainda, o estresse oxidativo também promove a peroxidação lipídica dos AGPI, que são utilizados na síntese de oxilipídeos pelas vias lipoxigenase (LOX), cicloxigenase (COX) e citocromo 450 (CYP450). Além disso, os radicais livres gerados

durante esse processo podem contribuir para a inflamação do tecido adiposo através da liberação de citocinas pró-inflamatórias (Lavrovsky et al., 2000; Yin et al., 2011).

Como já discutido, o estresse térmico é uma preocupação significativa para a saúde e o bem-estar das vacas leiteiras (Qu et al., 2015). Durante a lactação, as vacas apresentam uma produção metabólica de calor elevada devido à produção de leite e ao metabolismo ruminal, o que resulta em maior número de moléculas oxidativas (Tao et al., 2018; Li et al., 2021). O estresse térmico afeta diversas funções biológicas, incluindo a redução da ingestão de alimentos, o aumento da temperatura retal, frequência respiratória, secreção hormonal e do estresse oxidativo (da Costa et al., 2015; Kurokawa et al., 2016). Estudos mostram que vacas expostas a altas temperaturas apresentam balanço energético negativo menos acentuado, o que pode ser atribuído à redução no consumo de matéria seca e na produção de leite, resultando em um menor status antioxidante (Turk et al., 2015). Além disso, essas vacas tendem a ter níveis mais elevados de cortisol e citocinas inflamatórias (Webster et al., 2008; Ihsanullah et al., 2017).

O estresse oxidativo resultante do estresse térmico leva ao aumento das concentrações de radicais livres e espécies reativas de hidrogênio (Belhadj Slimen et al., 2016; Ganaie et al., 2021). Esses processos estão correlacionados com o aumento da frequência respiratória das vacas leiteiras, as quais elevam a respiração aeróbica celular (Schieber & Chandel, 2014; Kärkönen & Kuchitsu, 2015), aumentando a exposição dos pulmões a agentes oxidantes ambientais.

Além disso, o estresse térmico afeta diretamente as mitocôndrias, interferindo na fosforilação oxidativa e na síntese de ATP (Willis et al., 2000; Qian et al., 2004; White et al., 2012). As mitocôndrias, sensíveis ao calor, sofrem danos que impactam sua função respiratória, levando a menor captação de oxigênio e o aumento na formação de espécies reativas de oxigênio, contribuindo para o estresse oxidativo (Flanagan et al., 1998). Por fim, o estresse oxidativo desencadeado pelo estresse térmico pode gerar respostas inflamatórias que desregulam o metabolismo, resultando em apoptose celular, prejudicando a síntese de proteínas e afetando o desempenho produtivo dos animais (Guo et al., 2021).

Assim, estratégias nutricionais desempenham papel crucial na mitigação dos efeitos adversos dos desafios fisiológicos, como o período de transição e o estresse calórico. Em diversos estudos, os ácidos graxos de cadeia média, especialmente o ácido láurico (C12:0) presente no óleo de coco, emergem como poderosos antioxidantes. O óleo de coco, rico em propriedades bioativas, demonstrou aumentar a atividade de enzimas antioxidantes em ratos, reduzindo a peroxidação lipídica (Abujazia et al., 2012; Arunima & Rajamohan, 2013). Além disso, a ingestão desse óleo elevou significativamente a atividade de enzimas como a catalase, superóxido dismutase e glutationa peroxidase, mitigando o estresse oxidativo em comparação com outros óleos, devido à sua riqueza em polifenóis (Decker, 1997). Os polifenóis, presentes em abundância no coco, são conhecidos por suas propriedades antioxidantes e são estruturas químicas biologicamente ativas encontradas em frutas e vegetais. Entre esses compostos, os flavonoides se destacam, pois têm a capacidade de neutralizar radicais livres e/ou doar átomos de hidrogênio, inibindo reações em cadeia causadas por esses radicais (Decker, 1997).

Além disso, os ácidos graxos ômega-3 e ômega-6 também desempenham papel crucial na redução do estresse oxidativo devido às suas propriedades antioxidantes e anti-inflamatórias (Silvestre et al., 2011; Innes & Calder, 2018b; Santa et al., 2022). O ácido eicosapentaenoico (EPA) e ácido docosahexaenoico (DHA), componentes importantes de ômega-3, são precursores de oxilipídeos potentes que possuem grupos hidroxila OH, capazes de atuar como antioxidantes, neutralizando espécies reativas de oxigênio e modulando a resposta inflamatória (Chen et al., 2020). Portanto, a inclusão desses ácidos graxos na dieta pode contribuir para equilibrar o status oxidativo e inflamatório do organismo, oferecendo proteção contra o estresse oxidativo.

No entanto, são necessários mais estudos para compreender o estresse oxidativo em diferentes contextos fisiológicos e metabólicos. Avanços recentes na tecnologia ômica e nas análises de bioinformática têm permitido uma investigação mais aprofundada das vias e funções afetadas pelo estresse oxidativo (Zachut & Contreras, 2022).

3 Suplementação lipídica em vacas leiteiras

Durante um longo período, a suplementação lipídica era recomendada aos bovinos leiteiros com o propósito principal de oferecer uma fonte adicional de energia, seja para enriquecer a dieta em momentos desafiadores ou para aumentar o teor de gordura do leite, visando aprimorar o desempenho produtivo, eficiência reprodutiva ou status energético, baseado principalmente no teor lipídico (Palmquist & Jenkins, 1980). Atualmente se tem uma melhor compreensão das funções fisiológicas bioativas que os lipídeos exercem, além do seu papel como fonte de energia. O mais recente NRC de Bovinos Leiteiros (NASEM, 2021) traz uma nova abordagem para o estudo de lipídeos, explorando mais as funções individuais dos ácidos graxos (Almeida et al., 2023). Com o uso de novas tecnologias e melhor entendimento dos alimentos e suas funções, se tornou possível determinar o perfil de ácidos graxos de cada alimento inserido na dieta de vacas leiteiras (Daley et al., 2020).

Os suplementos lipídicos podem ser enriquecidos por ácidos graxos saturados, ácidos graxos insaturados ou mistos, de cadeia curta, média ou longa, fornecidos de forma livre ou protegidos em sabões de cálcio. Assim, possuem diversas fontes de ácidos graxos utilizados na dieta de vacas leiteiras, como gordura vegetal, gordura animal e óleos marinhos (os dois últimos proibidos sua suplementação em bovinos no Brasil; MAPA, 2004), e gorduras protegidas, com ácidos graxos puros ou mistos (Palmquist, 1993).

De acordo com a classificação de Daley et al. (2020), baseada nos teores de ácidos graxos, os alimentos se dividem em diversas categorias refletindo suas variações. Alimentos com baixo teor de ácidos graxos têm quantidades reduzidas de ácidos graxos saturados (AGS), monoinsaturados (AGMI) e poli-insaturados (AGPI

Os suplementos lipídicos destacam-se por incrementar o teor energético das dietas e atenuar os efeitos prejudiciais provenientes de dietas com alta proporção de carboidratos altamente fermentáveis. Além disso, esses suplementos têm o potencial de intensificar a produção de leite e aprimorar a qualidade dos produtos lácteos. Entretanto, seu uso pode resultar em efeitos variados, como a redução da digestibilidade de alguns componentes da dieta e interações complexas entre ácidos graxos devido às mudanças no ambiente ruminal (Doreau & Chilliard, 1997). Tais suplementos trazem respostas

produtivas diferentes, onde os ácidos graxos saturados apresentam respostas positivas quanto ao consumo de matéria seca, produção e energia do leite (Allen, 2000; Rico et al., 2020). Já os ácidos graxos insaturados resultam em redução do consumo de matéria seca, aumento da insulina plasmática, alteração da biohidrogenação ruminal e consequente redução dos teores de gordura do leite (Harvatine et al., 2009; Hristov et al., 2011; De Souza & Lock, 2019).

Dentre os ácidos graxos mais prevalentes e estudados, destacam-se o ácido palmítico (C16:0), ácido esteárico (C18:0), ácido oleico (C18:1), ácido linoleico (C18:2) e ácido linolênico (C18:3), geralmente suplementados em concentrações de 0,5 a 2% da matéria seca, dependendo das especificidades de cada estudo. O ácido palmítico, predominante no leite, tem sua suplementação associada ao aumento da produção de leite e de gordura do leite. Contudo, é reconhecido por induzir resistência à insulina, sugerindo precaução em sua suplementação periparto para evitar possíveis efeitos adversos, como a redução do peso das vacas durante o período de balanço energético negativo (De Souza & Lock, 2019). Por outro lado, a resistência à insulina pode aumentar a produção de leite (McFadden & Rico, 2019). Além disso, o estudo de De Souza & Lock (2019) observou que a suplementação de ácido palmítico, tanto no pré-parto quanto no pós-parto, resultou em maior produção e teor de gordura do leite, além da redução nos níveis circulantes de insulina.

O ácido esteárico, principal ácido graxo absorvido no intestino, pode ser incorporado tanto no leite quanto no tecido adiposo, apresentando efeito principalmente no status energético, e resultados variados relacionados à produção de leite e ao teor de gordura do leite (Piantoni et al., 2015; De Aguiar et al., 2022). Essa variabilidade é atribuída, em grande parte, à baixa digestibilidade do ácido esteárico, resultando em uma utilização menos eficiente pelo animal quando comparada a ácidos graxos como o palmítico (Piantoni et al., 2015; Boerman et al., 2017; Rico et al., 2017).

Em um estudo que investigou a digestibilidade de ácidos graxos a 1,5% da matéria seca, comparando uma dieta com inclusão de ácido palmítico e outra com ácido palmítico + esteárico, os resultados indicaram uma resposta superior na produção de leite para vacas que receberam apenas C16:0 em comparação com aquelas que receberam C16:0 + C18:0 em vacas de alta produção (Western et al., 2020). Além disso, no estudo de Rico

et al. (2020), que comparou os efeitos da infusão abomasal de ácido palmítico, ácido caprílico e cáprico, e ácido esteárico, foi observado que o ácido esteárico reduziu a eficiência na produção de leite, associada a uma menor absorção em comparação com o tratamento de ácido palmítico. O teor e produção de gordura no leite foram aumentados pelo ácido palmítico em relação ao ácido esteárico, sugerindo que o mecanismo subjacente ao aumento da secreção de gordura com o ácido palmítico não está relacionado à modulação da expressão de genes ligados à lipogênese, mas sim ao aumento da disponibilidade de substrato, conforme refletido no perfil de ácidos graxos do leite (Rico et al., 2020).

O ácido oleico desempenha papel crucial na manutenção da fluidez do leite, sendo incorporado tanto no leite quanto no tecido adiposo (Loften et al., 2014). Além de melhorar a digestibilidade total de lipídeos, tem o benefício adicional de reduzir a sensibilidade à insulina, resultando em efeitos favoráveis, especialmente no início da lactação (Prom et al., 2021; Abou-Rjeileh et al., 2023). Compreender esses efeitos em diferentes fases fisiológicas é fundamental para ajustar a relação entre os ácidos graxos palmítico e oleico.

No estudo conduzido por De Souza et al. (2019), que explorou diferentes proporções de ácido palmítico:oleico, como relações de 80:10, 73:17, 66:24 e 60:30, observou-se que a relação de 60:30 proporcionou os melhores resultados para a produção de leite em vacas de alta produção. Em outro experimento com as mesmas proporções, constatou-se que vacas tratadas com 80:10 apresentaram aumento na produção de leite, mas com diminuição no peso vivo em comparação ao grupo controle. No entanto, doses mais elevadas de ácido palmítico demonstraram maior produção de leite devido ao aumento da resistência à insulina, enquanto maiores doses de ácido oleico resultaram no aumento do peso corporal, indicando uma tendência ao acúmulo de tecido adiposo (De Souza et al., 2021). Por outro lado, o estudo de Sears et al. (2024) se concentrou nos efeitos específicos dos ácidos graxos palmítico, esteárico e oleico na digestibilidade da fibra. Eles observaram que o ácido palmítico aumentou a digestibilidade da fibra e influenciou a composição da comunidade bacteriana ruminal, favorecendo grupos bacterianos envolvidos na digestão de fibras.

Os ácidos graxos linoleico e linolênico, podem ser altamente digestíveis, porém sua inclusão pode comprometer a digestibilidade da fibra e causar diminuição na gordura

do leite (Lock & Garnsworthy, 2002; Hristov et al., 2005). O aumento na inclusão de ácido linoleico resulta em redução linear na produção de ácidos graxos provenientes da síntese *de novo* e de 16 carbonos (De Souza et al., 2018).

Além da suplementação de gordura aumentar a densidade energética das dietas, pode aumentar a absorção de nutrientes lipossolúveis (NRC, 2001). Recomenda-se manter a proporção de gordura total na dieta abaixo de 7% da MS, uma vez que exceder esse limite pode resultar em redução da ingestão de matéria seca, comprometimento da digestibilidade da fibra, diminuição do teor de gordura do leite e alteração da microbiota ruminal (NRC, 2001). Portanto, de forma mais conservadora, , sugere-se manter a gordura dietética total não superior a 6%, para prevenir a redução do consumo de matéria seca, preservar a digestão da fibra e manter uma fermentação ruminal saudável (Chilliard, 1993; Onetti & Grumer, 2004).

Especialmente no início da lactação, quando as vacas enfrentam balanço energético negativo, resultando em redução do consumo de matéria seca, a suplementação lipídica pode ser benéfica. Isso melhora a eficiência alimentar, permitindo maior produção de leite, saúde metabólica e eficiência reprodutiva (Onetti & Grumer, 2004). A temperatura também desempenha papel importante na resposta aos suplementos de gordura. A suplementação de gordura parece ser mais eficaz para aumentar o consumo e a ingestão de energia em vacas sob estresse térmico por calor. Isso se deve ao fato desses animais apresentarem uma menor digestão da gordura no rúmen, o que, consequentemente, reduz a produção metabólica de calor (West, 2003; Drackley et al., 2003). Dessa forma, o aporte energético proporcionado pela suplementação de gordura auxilia na obtenção de mais energia para a produção de leite durante períodos de estresse térmico.

Contudo, é crucial reconhecer que diferentes alimentos apresentam perfis variados de ácidos graxos, manifestando comportamentos metabólicos diversos. Esses comportamentos podem ser benéficos ou prejudiciais, dependendo dos objetivos e fases de criação dos animais. Portanto, uma compreensão aprofundada dos mecanismos, funções e potencial dos ácidos graxos em diferentes desafios fisiológicos é essencial para otimizar as estratégias nutricionais (Greco et al., 2015; De Souza & Lock, 2019).

3.1 Ácidos graxos de cadeia média

Os ácidos graxos de cadeia média (AGCM) são ácidos graxos saturados, contendo 6 a 14 átomos de carbono que normalmente estão ligados ao glicerol. Os AGCM são compostos por ácido capróico (C6:0), ácido caprílico (C8:0), ácido cáprico (C10:0), ácido laúrico (C12:0) e ácido mirístico (C14:0), predominantemente encontrados no óleo de coco, óleo de palma de dendê e laticínios (Dubois et al., 2007; Vyas et al., 2012). A principal fonte de AGCM é o óleo de coco, composto por 40 a 50% de C12:0, 9% de C8:0 e 6,5% de C10:0 (Hollmann et al., 2012).

Inicialmente, a suplementação de AGCM para ruminantes foi focada na mitigação da metanogênse, mostrando potencial para reduzir em até 50% a emissão de metano entérico e melhorar a utilização de nitrogênio (Dohme et al., 2001; Ajisaka et al., 2002; Machmuller et al., 2003; Soliva et al., 2003; Hristov & Jouany, 2005; Machmuller, 2006; Hristov et al., 2009). Essa redução ocorre pela dissociação dos prótons e ânions no citoplasma bacteriano, diminuindo o pH e inibindo enzimas citoplasmáticas, causando morte celular (Freese et al., 1973; Zentek et al., 2011). Além de seu impacto na metanogênese, os AGCM exibem propriedades antibacterianas e antivirais (Ababouch et al., 1992; Dawson et al., 2002; Hornung et al., 1994).

A dose de AGCM fornecida aos ruminantes pode causar resultados positivos e negativos, sendo que doses maiores que 1% na MS causam resultados produtivos e metabólicos mais expressivos (Machmuller, 2006). Porém, o aumento da dose de ACGM pode ocasionar redução na população de bactérias e protozoários ruminais, redução nas emissões de metano e nas digestibilidades de FDN e de MS (Dohme et al., 2000, Machmuller et al., 2001). Quando testadas doses abaixo de 1% na MS foram observados efeitos positivos na imunomodulação de neutrófilos, aumento da produção de leite e redução da incidência de cetose (Piepers e De Vliegher, 2013; Souza et al., 2015).

Portanto, os AGCM agem como redutores no metabolismo de protozoários ruminais, reduzindo a degradabilidade da fibra, e afetando o consumo de MS (Dohme et al., 2001; Hristov et al., 2004; Palmquist et al., 2006). Porém, em um trabalho de Faciola et al. (2005) foi relatado que a inclusão de ácido láurico na dieta total (TMR) não foi eficaz para redução da contagem de protozoários, diferentemente de quando infundido diretamente do rúmen.

Porém, ainda há dúvidas se os AGCM podem reduzir a ingestão de matéria seca. Dessa forma, quando AGCM foram infundidos no abomaso ou rúmen, não apresentaram nenhuma diferença quanto a ingestão de matéria seca (Kadegowda et al., 2008; Hristov et al., 2009; Vyas et al., 2012; Sun et al., 2013). Já quando os AGCM foram adicionados diretamente no concentrado, houve redução significativa na ingestão de MS (Drackley et al., 1992) e nenhuma diferença no trabalho de Dohme et al. (2004), levantando a questão que essas diferentes respostas podem estar ligadas com a quantidade do suplemento e a produção de leite do rebanho testado. Segundo Palmquist e Mattos (2006), quando a ingestão de matéria seca não foi afetada, a produção de leite foi mantida. Ainda, a suplementação de AGCM foi capaz de alterar as concentrações de grelina, o qual está relacionada à ingestão de MS (Fukomori et al., 2013), e também apresentou aumento nas concentrações de AGNE, colesterol total e BHB.

Estudos demonstram que a inclusão de AGCM isolados ou associados aos ácidos graxos de cadeia longa aumentam o teor de gordura e sólidos totais do leite (Kadegowda et al., 2008; Sun et al., 2013). Porém, quando houve inclusão de uma grande quantidade de AGCM de forma abrupta, reduziu os sólidos totais e a produção de leite (Hristov et al., 2011; Hollmann & Beede, 2012; Vyas et al., 2012). Ainda, Kadegowda et al. (2008) e Hristov et al. (2009) apontam que a suplementação de AGCM aumenta os teores de ácidos graxos monoinsaturados, poli-insaturados e ácidos linoleico conjugado *cis*-9, *trans*-11 no leite.

Ainda, a suplementação de 0,063% de AGCM na MS da dieta não afetou o desempenho produtivo e a digestibilidade de nutrientes comparado a animais não suplementados, mas aumentou o pH ruminal e reduziu a variação do pH ruminal diário, sugerindo que os AGCM podem reduzir as incidências de acidose ruminal subaguda (Burdick et al., 2022).

A suplementação de ácidos graxos de cadeia média, principalmente ácido láurico encontrado no óleo de coco, vêm apresentando resultados positivos como um imunoestimulador em desafios de estresse oxidativo e imunossupressão, possivelmente devido às modificações nas membranas celulares e mitocondriais provenientes das gorduras dietéticas (Rajaraman et al., 1997; Vigila & Baskaran, 2008; Lemieux et al., 2008; Leumieux et al., 2011). Piepers & Vliegher (2013) conduziram um estudo com a

suplementação oral de AGCM em novilhas e vacas algumas semanas antes do parto. Durante o início da lactação, foram observadas infecções intramamárias, contagem de células somáticas (CCS), apoptose celular, bem como a concentração de leucócitos e neutrófilos. Os resultados revelaram aumento significativo de apoptose celular entre os animais não suplementados, indicando um incremento nos neutrófilos, o que sugere redução na capacidade imunológica inata (Piepers & Vliegher, 2013). Estudos adicionais apontam que a suplementação de AGCM pode contribuir para a redução de infecções intramamárias e melhoria na resposta imunológica.

3.2 Ácidos graxos ômega-3 e ômega-6

Os ácidos graxos ômega-3 e ômega-6 são ácidos graxos poliinsaturados, contendo 18 a 22 carbonos na sua cadeia, com a última insaturação no terceiro carbono a partir do C ômega nos AG ômega-3 e no sexto carbono a partir do C ômega nos AG ômega-6 (Swern, 1982; Martin et al., 2005). Destacam-se o ácido eicosapentaenoico (C20:5 n-3, EPA) e o ácido docosahexaenoico (C22:6 n-3, DHA) como principais AG ômega-3, derivados do ácido alfa-linolênico (C18:3, ALA), e o ácido araquidônico (C20:4 n-6, AA) como o principal AG ômega-6, originado do ácido linoleico (C18:2; Innes & Calder, 2018a; Ishiara et al., 2019).

Os ácidos graxos ômega-3 e ômega-6 são considerados essenciais devido à incapacidade do organismo em sintetizá-los em quantidade suficiente, tornando necessária a suplementação dietética. Eles desempenham papéis cruciais como componentes estruturais de membranas e tecidos, além de modularem a resposta inflamatória (Scollan et al., 2006; Hadley et al., 2016; Innes & Calder, 2018b). Alimentos ricos em ômega-3 incluem o óleo de peixe, que é especialmente rico em EPA e DHA. O óleo e semente de linhaça, assim como as forragens frescas, são fontes de ácido alfa-linolênico. Por outro lado, óleos de soja e canola são fontes de ácido linoleico, e grãos de milho e sementes de girassol também são fontes de ômega-6 (Hodge et al., 1998; Ponnampalam et al., 2021). No entanto, é importante observar que no Brasil, o fornecimento de alimentos de origem animal, como o óleo de peixe, é proibido para ruminantes (MAPA, 2004).

Ambas as famílias ômega são essenciais e requerem uma suplementação equilibrada, uma vez que os ômega-3 ALA, EPA e DHA são reconhecidos por suas propriedades anti-inflamatórias, enquanto os ômega-6 LA e AA são associados à ação pró-inflamatória (Calder, 2010). Assim, a suplementação de ômega-3 pode trazer respostas positivas, reduzindo a inflamação e fortalecendo o sistema imunológico, através da modulação da expressão de genes relacionados, modulação do sistema endocanabinóide, vias oxilipídicas e via da cascata inflamatória TLR4/NF-kb (Silvestre et al., 2011; Calder, 2013; Greco et al., 2015; Kra et al., 2022). Os ácidos graxos ômega-3 desempenham boas respostas na inibição de citoquinas inflamatórias causadas pela inflamação induzida por LPS, justamente através da inativação do NF-kb, além de inibir outras citoquinas pró-inflamatórias; TNF-α, IL-1 e IL-6 (Calder, 2013; Liu et al., 2015).

Por outro lado, o ácido araquidônico, um ômega-6, é utilizado como substrato na via oxilipídica, gerando mediadores eicosanóides, como prostaglandinas, tromboxanos, leucotrienos e radicais livres de peróxidos, os quais regulam o início e a resolução em um processo inflamatório (Ponnampalam et al., 2021). De tal modo, a prostaglandina possui efeito pró-inflamatório, induzindo a produção de citoquinas IL-6, febre e dor (Innes & Calder, 2018a). Assim, pelo fato do ácido araquidônico ser precursor de potentes mediadores pró-inflamatórios, é comum indicá-lo exclusivamente pela sua ação pró-inflamatória, porém em um estudo em humanos saudáveis, o aumento da ingestão de ácido araquidônico ou ácido linoleico não aumentou marcadores inflamatórios (Raphael e Sordillo, 2013; Raphael et al., 2014). Além disso, o ácido araquidônico e o ácido linoleico desempenham papel importante na estrutura dos fosfolipídios da membrana celular (Raphael e Sordillo, 2013; Raphael et al., 2014). No entanto, é relevante notar que uma dieta rica em ácido araquidônico pode inibir os efeitos benéficos dos ômega-3 durante a resolução da inflamação (Innis, 2008).

Além das ações pró e anti-inflamatórias proporcionadas pelos ácidos graxos ômega-3 e ômega-6, eles podem ser aliados na modulação do perfil de ácidos graxos do leite e produção de leite. Vacas leiteiras demonstram habilidade na transferência de ácidos graxos ômega-3 para o leite, proporcionando benefícios significativos para o consumo humano ao enriquecer o leite com ácidos graxos anti-inflamatórios. Estudos indicam que os ácidos graxos ômega-3, especialmente o ALA, são transferidos de

maneira rápida e eficiente para o leite, enquanto o EPA e o DHA são prontamente incorporados à corrente sanguínea, participando ativamente em diversos processos metabólicos. Essas investigações evidenciam diferenças notáveis no transporte e na distribuição dos ácidos graxos poli-insaturados ômega-3 (Urrutia et al., 2023; Almeida et al., 2023).

O óleo de peixe, uma importante fonte de EPA e DHA, oferece benefícios como fonte de ômega-3, mas também pode ter desvantagens, como reduzir a ingestão de matéria seca (MS) e alterar a composição da gordura do leite quando fornecido em concentrações superiores a 1% na MS (Doreau e Chilliard, 1997; Keady et al., 2000; AbuGhazaleh et al., 2002). No entanto, quando fornecido em quantidades inferiores a 1% na MS, não foram observadas diferenças no consumo (Pirondini et al., 2015).

O impacto das fontes de ômega-3, como linhaça e óleo de peixe, na produção de leite apresenta resultados inconsistentes. Estudos que envolvem linhaça integral, extrato ou óleo encapsulado dessa semente demonstraram aumentos na produção de leite, variando de 2,7% a 6,4% (Petit et al., 2004; Moallem, 2009; Zachut et al., 2010a). No entanto, outros estudos não encontraram diferenças significativas na inclusão de linhaça em comparação com o grupo controle quanto à produção de leite (Gonthier et al., 2005; Petit et al., 2007; Neveu et al., 2013). Quanto ao óleo de peixe encapsulado, a inclusão de até 1% na MS aumentou a produção de leite, enquanto a produção diminuiu linearmente com a inclusão aumentada até 3% (Donovan et al., 2000), sem diferenças observadas quando a inclusão foi de 2% (Cant et al., 1997; AbuGhazaleh et al., 2002). Essas inclusões acima de 1% na MS podem reduzir o consumo, o que explica parcialmente a queda na produção de leite (Donovan et al., 2000).

Ainda, quando fornecido o óleo de peixe em concentrações superiores a 1% na MS, pode reduzir o teor de gordura do leite devido à biohidrogenação ruminal de ácidos graxos poli-insaturados, formando CLA *trans*-10, *cis*-12 e outros isômeros *trans* associados à redução da gordura do leite (Baumgard et al., 2001; Piperova et al., 2004; Lee e Jenkins, 2011). O fornecimento de outra fonte de ômega-3, como o óleo de linhaça ou linhaça não aquecida, também reduziu a gordura do leite (Mustafa et al., 2003; Moallem et al., 2013), enquanto a linhaça integral não mostrou diferença no teor de gordura do leite (Petit et al., 2007; Akraim et al., 2007).

Os ácidos graxos ômega-3 desempenham papel crucial na competência reprodutiva, especialmente no contexto do folículo ovariano, através da incorporação desses ácidos graxos na membrana celular e no ambiente intracelular (Zachut et al., 2010b; Moallem et al., 2013). Em estudo conduzido por Dirandeh et al. (2013), foram comparadas dietas à base de soja (fonte de ômega-6), linhaça (fonte de ômega-3) e óleo de palma (fonte de ácido graxo saturado) em vacas lactantes. Não foram observadas diferenças na produção de leite e no consumo de MS entre os grupos, mas a concentração de gordura no leite foi inferior no grupo que recebeu linhaça em comparação com os demais tratamentos. Embora os tratamentos não tenham influenciado a detecção de estro e a taxa de concepção, os animais que receberam fonte de ômega-3 apresentaram uma taxa de prenhez mais elevada aos 120 dias (Dirandeh et al., 2013).

Períodos de desafios fisiológicos podem desencadear um desequilíbrio de radicais livres, resultando em estresse oxidativo que afeta biomoléculas como proteínas, DNA e AGPI n-3 (DHA e EPA), que são particularmente suscetíveis devido à sua alta insaturação (Sevanian & Hochstein, 1985; Seki et al., 2022). Dietas ricas em gordura podem induzir estresse oxidativo no fígado, levando à diminuição de AGPI n-3 e à inibição da dessaturação enzimática (Araya et al., 2004; 2010; Valenzuela et al., 2015; Rincón-Cervera et al., 2016). Embora a influência do estresse oxidativo na atividade de alongamento de AGPI n-3 não tenha sido totalmente avaliada, estudos sugerem o papel do eixo Receptor Ativado por Proliferadores de Peroxissomo Gama e Fator de Crescimento de Fibroblastos 21 (PPAR-γ/FGF21) na dessaturase, o qual é considerado um componente essencial na modulação de processos metabólicos ligados ao metabolismo lipídico, homeostase energética e estresse oxidativo (Ortiz et al., 2020). Ainda, a relação reduzida entre EPA + DHA/alfa-linolênico indica a inibição das enzimas delta-5-dessaturase (Δ5D) e delta-6-dessaturase (Δ6D) no fígado de indivíduos com gordura hepática (Delarue et al., 2004; Araya et al., 2004). No entanto, o estresse oxidativo, que está negativamente relacionado à produção endógena de ácidos graxos ômega-3, pode ser revertido com a suplementação de antioxidantes (Rincón-Cervera et al., 2016; Valenzuela et al., 2017). Além disso, a suplementação exógena de ômega-3

pode ter efeitos antioxidantes, auxiliando na resposta ao estresse oxidativo (Lima et al., 2014; Santa et al., 2022).

É crucial ressaltar que a suplementação com ômega-3 e ômega-6 requer cuidados adicionais, pois tratam-se de ácidos graxos poli-insaturados que podem reduzir o teor de gordura do leite, levando à DGL, e são suscetíveis à oxidação, resultando em odor e sabor rancificados da gordura, o que pode reduzir o consumo dos animais. Portanto, é essencial destacar que a suplementação de ácidos graxos ômega está em constante desenvolvimento e possui potencial promissor tanto para a saúde animal quanto para a saúde humana (Almeida et al., 2023).

3.3 Metabolismo, digestão e absorção de ácidos graxos

Vacas consomem lipídeos na forma de triglicerídeos e galactolipídeos, a partir de forragens e concentrados. Embora essas fontes forneçam predominantemente ácidos graxos insaturados, como o ácido linolênico (C18:3) em forragens e ácido linoleico (C18:2) e ácido oleico (C18:1) em grãos, a gordura predominante no leite é composta por ácidos graxos saturados, como o ácido palmítico (C16:0) e o ácido esteárico (C18:0) (Jensen, 2002). Esse processo ocorre como uma defesa dos microrganismos ruminais, pois, ácidos graxos poli-insaturados e de cadeia média podem apresentar toxicidade às bactérias e protozoários do rúmen, relacionados à sua solubilidade em água e ao potencial de ruptura das membranas (Berchielli et al., 2011). Dessa forma, os microrganismos ruminais convertem ácidos graxos insaturados em saturados (Bauman et al., 1999; Jenkins et al., 2008).

Os lipídeos fornecidos pela dieta não são fermentados no rúmen, porém, sofrem dois eventos principais pela ação dos microrganismos ruminais: lipólise ou hidrólise microbiana e biohidrogenação. Durante a lipólise, os microrganismos hidrolisam os triglicerídeos e galactolipídeos, quebrando as ligações ésteres e liberando glicerol, ácidos graxos livres e galactose no caso dos galactolipídeos (Kozloski, 2011). Por sua vez, a biohidrogenação envolve enzimas isomerases e redutases atuando sobre ácidos graxos insaturados, alterando sua isomeria ou convertendo-os em ácidos graxos saturados por meio da ligação de moléculas de hidrogênio onde há duplas ligações nas cadeias de ácidos graxos (Jenkins et al. 2008). Desse modo, os principais ácidos graxos presentes

na dieta de ruminantes: C18:3, C18:2 e C18:1 são reduzidos principalmente a ácido esteárico (C18:0) no rúmen, e uma pequena porção de AG parcialmente hidrogenados conseguem escapar do processo de biohidrogenação na sua forma original (Jensen, 2002).

Aproximadamente 80 a 90% dos ácidos graxos livres chegam ao duodeno aderidos a partículas de alimentos presentes no ambiente (Look et al., 2006). No jejuno, os ácidos graxos se desprendem das partículas alimentares e sofrem a ação de sais biliares e enzimas pancreáticas, formando micelas (Moore & Christie, 1984). Assim, durante a absorção dos lipídeos pelos enterócitos, os ácidos graxos maiores de 10 carbonos são reesterificados com o glicerol, formando triglicerídeos. Triglicerídeos associados aos fosfolipídeos, colesterol livre e apolipoproteínas, formam quilomícrons e lipoproteínas de muito baixa densidade (VLDL) para serem liberados no sistema linfático e na corrente sanguínea (Kozlosky, 2011). Após a absorção, os ácidos graxos têm vários destinos e funções em diferentes órgãos do corpo. Eles atuam como precursores para a formação de outros ácidos graxos, são essenciais na produção de leite e na síntese da gordura do leite, funcionam como fonte primária de energia, contribuem para a estrutura das membranas celulares e a produção de hormônios, além de serem armazenados como reservas corporais (Hodson & Gunn, 2019).

Na glândula mamária, os ácidos graxos para a síntese de lipídios derivam de duas fontes distintas. A primeira é a síntese de novo, utilizando acetato e β-hidroxibutirato, originando ácidos graxos de cadeia curta e média, assim como parte dos ácidos graxos de 16 carbonos. A segunda fonte é composta por ácidos graxos pré-formados captados da corrente sanguínea, sendo principalmente ácidos graxos maiores que C16:0, absorvidos da dieta no intestino delgado e, em períodos de balanço energético negativo, mobilizados das reservas corporais até serem captados na glândula mamária (Bauman & Griinari, 2003; Mcdonald et al., 2010).

Em torno da metade dos ácidos graxos do leite em ruminantes têm origem na síntese de novo, com o acetato do rúmen sendo a principal fonte de carbono para essa síntese (Bauman & Davis, 1974). A via de síntese de novo na glândula mamária é predominantemente regulada pela expressão dos genes acetil CoA carboxilase (ACC) e ácido graxo sintase (FAS; Smith, 1994), enquanto a lipogênese proveniente da corrente

sanguínea envolve a expressão dos genes lipoproteína lipase (LPL), CD36, proteína ligadora de ácidos graxos (FABP), dessaturase de ácidos graxos (SCD), glicerol-3-fosfato aciltransferase (GPAT), diacilglicerol aciltransferase (DGAT), acilglicerol-3-fosfato aciltransferase (AGPAT), elemento regulatório de ligação a esterol 1 (SREBP1) e receptor ativado por proliferador de peroxissoma-gama (PPAR-γ; Griinari et al., 2000; Baumgard et al., 2002; Havartine E Bauman, 2006; Bernard et al., 2008; Hussein et al., 2013).

Na síntese de novo, a acetil-CoA é um precursor essencial, iniciando a lipogênese na glândula mamária de ruminantes (Bauman & Davis, 1974). Durante a lactação, a enzima acetil-CoA-carboxilase alfa (ACC-α) converte acetil-CoA em malonil-CoA, que, juntamente com a ácido graxo sintase (FAS), facilita o alongamento dos ácidos graxos até 16 carbonos na glândula mamária.

A absorção de ácidos graxos pré-formados envolve a ação da lipoproteína lipase (LPL), que quebra os triglicerídeos liberando ácidos graxos para serem absorvidos pela glândula mamária (Almeida et al., 2007), utilizando principalmente proteínas transportadoras de ácidos graxos (Doege & Stahl, 2006). Em seguida, a síntese de triglicerídeos ocorre por meio das enzimas glicerol-3-fosfato aciltransferase (GPAT) e diacilglicerol aciltransferase (DGAT; Bernard et al., 2008), resultando na formação de glóbulos de gordura secretados no leite.

3.4 Metabolismo de ácidos graxos de cadeia média

O processo metabólico dos ácidos graxos de cadeia média (AGCM) difere dos ácidos graxos de cadeia longa, pois o tamanho da cadeia de ácidos graxos pode impactar a rota de absorção no trato intestinal e a oxidação hepática (Hanczkowska, 2017). Quando os AGCM chegam ao intestino delgado, são absorvidos sob a ação da enzima lipase pancreática, esses podem ou não ser ligados a albumina e entram na corrente sanguínea de forma mais rápida do que ácidos graxos de cadeia longa, que são incorporados a quilomícrons e VLDL (Bach & Babayan, 1982; Jeukendrup et al., 1998). Porém, uma fração menor de AGCM ainda pode ser incorporada aos quilomícrons, juntamente aos ácidos graxos de cadeia longa (Wang et al., 2013). Após a absorção

pelos enterócitos, os AGCM são transportados até o fígado ou são solubilizados na fração aquosa do plasma (Berning et al., 1996; Jeukendrup et al., 1998; Hanczkowska, 2017).

No fígado, a enzima carnitina-palmitoil-transferase, controla a taxa de oxidação mitocondrial de ácidos graxos. As enzimas acetil-CoA e malonil-CoA são fisiologicamente inibidas, controlando ou bloqueando a entrada e oxidação de ácidos graxos na mitocôndria. Assim, a malonil-CoA inibe a carnitina-palmitil-transferase, aumentando a síntese de ácidos graxos no fígado. Porém, os ácidos graxos de cadeia média são relativamente independentes da carnitina, escapando desse mecanismo, que é quase que exclusivamente para ácidos graxos de cadeia longa (Colleone, 2002). Assim, quando os AGCM entram na matriz mitocondrial, são ativados por acil-Coa sintase, passando pela β-oxidação (Papamandjaris et al., 1998; Marten et al., 2006).

Contudo, os ácidos graxos de cadeia média possuem um peso molecular menor, resultando em uma absorção, digestão e metabolismo mais rápidos, se comparados aos ácidos graxos de cadeia longa (Spector, 1975; Bach & Babayan, 1982). A razão para isso é que os ácidos graxos com menos de 12 carbonos conseguem penetrar na mitocôndria sem necessitar de transportadores de membrana (Marten et al., 2006; Nelson & Cox, 2014). Assim, o metabolismo rápido dos AGCM resulta na economia líquida de glicose, buscando otimizar a utilização de ácidos graxos livres como fonte de energia (Kawaguchi et al. 2002; Rico et al. 2020).

3.5 Metabolismo de ácidos graxos ômega-3 e ômega-6

O metabolismo dos ácidos graxos ômega-3 e ômega-6 em vacas leiteiras difere dos demais ácidos graxos de cadeia longa. Suas fontes naturais na alimentação bovina, juntamente com a biohidrogenação ruminal, resultam em níveis relativamente baixos desses ácidos graxos ômega disponíveis para absorção (Dewhurst & Moloney, 2013). Como resultado, os ácidos graxos ômega-3 e 6 podem ser produzidos a partir de precursores metabólicos, como ácido linoleico (C18:2) e ácido linolênico (C18:3), por meio de um processo de alongamento e dessaturação durante a oxidação hepática (Videla et al., 2022).

Assim, a síntese de ômega-3 e 6 inicia-se com a disponibilidade de C18:2 e C18:3 no fígado. No retículo endoplasmático das células hepáticas ocorre a dessaturação e

alongamento dos ácidos graxos, através de enzimas elongases e dessaturases (Guillou et al., 2010). Na síntese de ômega-3, o ácido linolênico (C18:3 n-3) é transformado em ácido eicosapentaenoico (EPA, C20:5 n-3) por ações das enzimas delta-5-dessaturase (Δ5D), delta-6-dessaturase (Δ6D) e elongases 2 e 5. O EPA pode ser usado metabolicamente, mas uma parcela dele continua no ciclo de síntese de ômega-3, passando novamente pelas enzimas elongases 2 e 5 e Δ6D, resultando no ácido tetracosahexaenoico (C24:6 n-3). Posteriormente, ocorre um processo catabólico, no qual este ácido graxo é quebrado por meio da β-oxidação, formando o ácido docosahexaenoico (DHA; C22:6 n-3), que é então liberado na corrente sanguínea (Videla et al., 2022).

A síntese de ômega-6 segue um processo semelhante ao do ômega-3, mas com ácidos graxos derivados do ácido linoleico (C18:2 n-6). O C18:2 n-6 é processado por enzimas Δ5D, Δ6D e elongases 2 e 5, resultando no ácido araquidônico (C20:4 n-6). Este ácido graxo é parcialmente utilizado, e uma parte dele passa por etapas semelhantes de transformação pelas enzimas mencionadas anteriormente, exceto pela Δ5D, culminando no ácido tetracosapentaenoico (C24:5 n-6), que, por meio da β-oxidação, se converte em ácido docosapentaenoico (DPA; C22:5 n-6), sendo liberado na corrente sanguínea (Videla et al., 2022). Na síntese de ômega-3 e ômega-6, as enzimas elongases 2 e 5 trabalham em conjunto com outras quatro enzimas: β-cetoacil-CoA sintase, β-cetoacil-CoA redutase, β-hidroxiacil-CoA desidratase e trans-enoil-CoA redutase, acrescentando dois carbonos adicionais à cadeia (Cinti et al., 1992; Guillou et al., 2010). Ainda, os ácidos graxos ômega-3 e ômega-6 competem pelas enzimas elongases e dessaturases durante o processo de alongamento e adição de duplas ligações na cadeia (Aki et al., 1999; Leonard et al., 2004). Assim, não ocorre uma síntese simultânea e proporcional entre eles (Maniongul et al., 1993). A regulação da síntese e degradação de ácidos graxos no fígado é principalmente controlada pelos genes SRBP1 e PPAR-γ, cuja expressão é parcialmente influenciada pela insulina (Foretz et al., 1999; Hegarty et al., 2005).

A síntese de ácidos graxos ômega-3 e ômega-6 no fígado é um mecanismo adaptativo que objetiva a produção de energia e traz benefícios para a saúde do animal. No entanto, um excesso de β-oxidação pode acarretar problemas metabólicos a longo prazo, como estresse oxidativo (Videla et al., 2004).

3.6 Oxilipídeos

Os oxilipídeos, também conhecidos como oxilipinas ou eicosanoides, formam uma categoria de compostos lipídicos oxigenados que se originam a partir de ácidos graxos essenciais (Sordillo, 2018). Reconhecidos como potentes mediadores inflamatórios, esses compostos desempenham papéis significativos nos processos inflamatórios, imunológicos e na sinalização celular. Derivados da oxigenação de ácidos graxos poli-insaturados ômega-6 e ômega-3, os oxilipídeos são produzidos por meio de vias tanto enzimáticas quanto não enzimáticas (Sordillo, 2018). Os oxilipídeos provenientes de ácidos graxos ômega-3 estão relacionados a efeitos anti-inflamatórios, enquanto os derivados de ômega-6 exercem ação pró-inflamatória (Rafael & Sordillo, 2013; Innes & Calder, 2018a).

Dentre os oxilipídeos mais estudados derivados de ômega-6, estão as prostaglandinas, tromboxanos, leucotrienos e lipoxinas, todos derivados especificamente do ácido araquidônico e caracterizados por suas propriedades pró-inflamatórias (Zia et al., 1987; Atroshi et al., 1990). A produção da classe de oxilipídeos está condicionada à quantidade endógena do substrato do ácido graxo que são derivados, à própria via oxilipídica e ao estado fisiológico do animal. Esses compostos têm a capacidade de desempenhar funções distintas em diferentes estágios da inflamação, seja na fase inicial, resolução ou pós-resolução inflamatória (Raphael & Sordillo, 2013). Acredita-se que, quando atinge a produção de um determinado nível de oxilipídeos pró-inflamatórios, há a produção de outros tipos de oxilipídeos, como as lipoxinas, para facilitar o processo de resolução inflamatória (Mitchell et al., 2002).

Além disso, os oxilipídeos provenientes de ácidos graxos ômega-3, especialmente o ácido eicosapentaenoico (EPA) e o ácido docosahexaenoico (DHA), desempenham papel crucial na resolução da inflamação, devido ao seu potencial anti-inflamatório e à redução de mediadores pró-inflamatórios. As resolvinas e protectinas são categorias específicas desses oxilipídeos, exercendo papel essencial ao promover a resolução favorável de processos inflamatórios (Serhan et al., 2000). No entanto, a natureza exata da ação anti ou pró-inflamatória de cada oxilipídeo ainda requer uma compreensão mais aprofundada do papel individual de cada oxilipídeo (Sheppe et al., 2018). Dessa forma, não apenas a presença desses oxilipídeos e seus substratos influencia a regulação da

inflamação, mas também a incorporação relativa de ácidos graxos poliinsaturados específicos na camada fosfolipídica e o momento preciso da produção de determinados oxilipídeos durante o processo inflamatório (Raphael et al., 2014; Kuhn et al., 2017).

Assim, os oxilipídeos representam uma complexa rede de vias bioquímicas ativadas em resposta aos processos inflamatórios, estresse oxidativo ou outras desregulações fisiológicas (Mavangira & Sordillo, 2017; Kuhn et al., 2017). O aumento da produção de espécies reativas de oxigênio (ERO) durante disfunções metabólicas, pode gerar o estresse oxidativo, que a longo prazo associa-se às respostas inflamatórias (Sordillo & Aitken, 2009; Osório et al., 2014). Os ácidos graxos poliinsaturados presentes nos fosfolipídios da membrana são alvos primários para a modificação pelas ERO durante o estresse oxidativo. Esse cenário pode alterar o status redox, indicando um ambiente mais oxidativo. Portanto, a mudança no status redox celular influencia a oxidação dos ácidos graxos poliinsaturados, impactando diretamente na produção de oxilipídeos (Kuhn et al., 2015).

Kuhn et al. (2017) avaliaram o perfil de oxilipídeos no leite de vacas em diferentes fases de lactação. Assim, os ácidos graxos poliinsaturados e oxilipídeos foram mais baixos em vacas no início da lactação, comparado ao meio e ao fim da lactação. O oxilipídeo mais elevado no início da lactação foi o 20-hidroxieicosatetraenoico (HETE), frequentemente associado às doenças inflamatórias (Kuhn et al., 2017). Além disso, Putman et al. (2022) investigaram a associação dos oxilipídeos com doenças durante o período de transição, identificando alterações nas concentrações de alguns oxilipídeos em comparação com vacas saudáveis. Os perfis oxilipídicos incluem mediadores anti-inflamatórios e pró-inflamatórios, como PGD2, Prostaglandina F2 alfa (PGF2a), 9-HODE, 9-oxoODE, 13-oxoODE, 8-isoPGA2 e 12,13-DiHOME.

Vacas expostas a períodos prolongados de calor apresentaram modificações no perfil de ácidos graxos sanguíneos. Observou-se uma diminuição nos ácidos graxos monoinsaturados em animais sob estresse calórico, enquanto os ácidos graxos poliinsaturados aumentaram em comparação com vacas em condições de termoneutralidade (Mylostyvyi et al., 2021). Até o momento, não existem estudos específicos sobre as alterações no perfil oxilipídico em vacas submetidas à inflamação decorrente do estresse calórico.

Mais de 130 oxilipídeos foram identificados, impulsionados pelo avanço dos estudos em lipidômica e pela utilização de análises mais sensíveis com espectrômetros de massas inovadores (Wang et al., 2014). Entretanto, a atividade biológica individual de cada um ainda permanece pouco conhecida (Sordillo, 2018). O entendimento aprofundado dos oxilipídeos em vacas leiteiras é essencial para otimizar a saúde, o bem-estar e a produção de leite, especialmente diante de desafios fisiológicos como o período de transição e o estresse calórico.

3.7 Vias oxilipídicas: enzimática e não enzimática

A fase inicial da síntese de oxilipídeos envolve a liberação de ácidos graxos poliinsaturados dos fosfolipídeos de membrana por meio da ação da enzima fosfolipase (PLA2; Burke & Dennis, 2009). Essa enzima catalisa a hidrólise dos ésteres de ácidos graxos na posição sn-2 dos fosfolipídios, resultando na liberação de ácidos graxos livres, como ácido linoleico, ácido linolênico, ácido araquidônico, ácido docosapentaenoico e ácido eicosapentaenoico (Raphael & Sordillo, 2013). Esses ácidos graxos são então metabolizados por diferentes enzimas, levando à formação de diversos oxilipídeos. O produto inicial, obtido por meio da oxidação enzimática ou não enzimática dos ácidos graxos, passa por processos metabólicos adicionais, contribuindo para a complexidade da rede de oxilipídeos (Sordillo, 2018).

Os oxilipídeos podem originar-se tanto de processos enzimáticos quanto de não enzimáticos. Na abordagem enzimática, três classes principais de enzimas desempenham papéis distintos, sendo cada uma referida como uma via oxilipídica específica. As principais enzimas envolvidas são a ciclooxigenase (COX), a lipooxigenase (LOX) e o citocromo P450 (CYP450; Raphael et al., 2014). A fosfolipase-2 é responsável por quebrar os fosfolipídeos, liberando um ácido graxo livre, o qual pode seguir a via oxilipídica e ser metabolizado por uma das três enzimas, dependendo da disponibilidade e preferência específica do ácido graxo em questão (Arnold et al., 2010; Putman et al., 2022). Em geral, estas vias removem átomos de hidrogênio e inserem moléculas de oxigênio (Sordillo, 2018). Por outro lado, a via não enzimática é mais fortemente regulada por radicais livres que interagem diretamente com ácidos graxos

poliinsaturados presentes nos fosfolipídeos da membrana, originando os isoprostanos, uma categoria de oxilipídeos (Milne et al., 2015).

A via COX consiste em duas isoformas principais, COX-1 e COX-2, ambas catalisando a etapa inicial do ácido araquidônico, retirando um átomo de hidrogênio do AGPI e transferindo-o para sítios de COX que contenham tirosil (Wu et al., 2011). A COX-1 geralmente gera prostaglandinas, desempenhando funções homeostáticas, como regulação plaquetária e manutenção das funções fisiológicas normais (Zha et al., 2004; Sordillo, 2018). Em contraste, a via COX-2 está intimamente associada a respostas inflamatórias por meio da ativação do sistema NF-kB. Sua expressão aumenta em resposta a estímulos inflamatórios e dolorosos, levando ao aumento da produção de prostaglandina E2 (PGE2). No entanto, a afirmação de que apenas os oxilipídeos produzidos na via COX-2 são responsáveis pela propagação da resposta inflamatória não é mais respaldada pela literatura (Sordillo, 2018).

A via LOX engloba diversas isoformas, sendo as mais comuns a 5-LOX, 8-LOX, 12-LOX e 15-LOX, cada uma diferenciada pela habilidade de introduzir oxigênio em uma posição específica do ácido graxo (Kuhn & O'Donnell, 2006). Essas enzimas LOX são responsáveis pela produção de lipoxinas, leucotrienos e hidroxieicosatetraenóicos (HETE), desempenhando papéis essenciais na regulação inflamatória, imunológica e sinalização celular (Kuehl Jr & Egan, 1980; Natarajan & Nadler, 2004). No processo, as enzimas LOX utilizam Fe2+, formando o hidroxi ferroso, promovendo a extração de hidrogênio e a adição de oxigênio, resultando em um radical peroxi instável (Sordillo, 2018). Além disso, se sabe que 15-LOX demonstra preferência pelo ácido linoleico em sua via, em detrimento do ácido araquidônico, embora este último seja mais eficiente dentro da via (Soberman et al., 1985; Brash et al., 1997).

A CYP450 utiliza radicais de ferro no processo de epoxigenação e hidroxilação de AGPI, formando oxilipídeos diretamente ou utilizando algum oxilipídeo derivado da via COX, como PGE2 e prostaglandina (PGD2; Spector et al., 2004). A CYP450 gera epóxidos, hidroxiácidos e ésteres, desempenhando funções na regulação da pressão arterial, metabolismo de lipídios e hormônios, além de contribuir para a desintoxicação (Manikandan & Nagini, 2018). Contudo, é importante ressaltar que o ácido araquidônico é o principal e mais eficiente ácido graxo poliinsaturado utilizado nessas vias, embora os

demais ácidos graxos também sejam empregados. Ácidos graxos ômega-6 apresentam maior preferência pela via COX, enquanto os ômega-3 são metabolizados de forma mais eficiente nas vias LOX e CYP450 (Wada et al., 2007; Arnold et al., 2010).

A formação de oxilipídeos através da via não enzimática é impulsionada por radicais livres, espécies reativas de oxigênio (ERO) e espécies reativas de hidrogênio (Haliwell, 2007). A oxidação não enzimática demonstra preferência por ácidos graxos esterificados em fosfolipídeos, desencadeando o processo de autooxidação, especialmente em situações de estresse oxidativo (Sordillo & Aitken, 2009; Yin et al., 2011; Milne et al., 2015). Nesse contexto, a via não enzimática gera oxilipídeos conhecidos como isoprostanos, que são liberados dos fosfolipídeos da membrana pela ação da enzima fosfolipase A2, sendo, em sua maioria, excretados na urina (Sordillo, 2018). Os isoprostanos, devido à sua produção mais intensa em períodos de estresse oxidativo e inflamação, podem ser considerados pró-inflamatórios, evidenciados durante o período de transição, mastite ou processos inflamatórios (Halliwell, 2007; Sordillo, 2013; Mavangira et al., 2016). Assim, mais de 64 compostos isoprostanos foram identificados como derivados do ácido araquidônico, um ácido graxo pró-inflamatório (Kuehl Jr & Egan, 1980; Grantz et al., 2023). Contudo, existem isoprostanos derivados de ácidos graxos ômega-3, como ácido linolênico e DHA, que demonstram efeitos positivos na saúde humana (Joumard-Cubizolles et al., 2017). Apesar disso, os oxilipídeos isoprostanos derivados de ômega-3 apresentam concentrações geralmente mais baixas em comparação com os derivados de ômega-6 (Sordillo, 2018). Contudo, é relevante considerar que as vias enzimáticas COX, LOX e CYP450 também podem gerar peróxidos durante suas atividades, perturbando o equilíbrio oxidativo, e assim ativando a via não enzimática de forma adicional (Mavangira & Sordillo, 2018).

Como mencionado anteriormente, as alterações no estado redox celular exercem influência significativa sobre o nível e os produtos iniciais da oxigenação de ácidos graxos poliinsaturados, os quais são subsequentemente metabolizados para gerar mediadores lipídicos (Sordillo, 2018). Tomemos, por exemplo, a oxidação do ácido linoleico pela via 15-LOX, resultando na produção do oxilipídeo 13-hidroperoxoctadecaenóico (13-HPODE). Este composto pode ser convertido em 13-hidroxioctadecadienóico (13-HODE) por meio da ação de antioxidantes circulantes (Kuhn et al., 2015). Posteriormente, o 13-

HODE pode sofrer metabolização para formar 13-oxooctadecadienóico (13-oxoODE; Altamann et al., 2007). É relevante observar que o 13-HPODE é considerado um oxilipídeo pró-inflamatório, enquanto o 13-oxoODE é reconhecido por suas propriedades anti-inflamatórias. Uma questão crucial é que, em alguns casos, a produção de oxilipídeos anti-inflamatórios pode depender da prévia geração de oxilipídeos pró-inflamatórios, que atuam como sinalizadores de inflamação ou outras condições patológicas (Kuhn et al., 2015; Sordillo, 2018). Além disso, vale ressaltar que a produção de oxilipídeos pode variar conforme a patologia em questão e o tipo de célula afetada (Sordillo, 2018).

Contudo, uma compreensão mais aprofundada das origens dos oxilipídeos e do impacto que o ambiente pode exercer sobre os perfis desses mediadores lipídicos poderá abrir caminho para terapias mais direcionadas a doenças de natureza inflamatória. Ao decifrar o comportamento dos oxilipídeos e suas respectivas vias, torna-se viável modular e controlar os efeitos pró-inflamatórios por meio de intervenções nutricionais (Sordillo, 2016).

4 CONCLUSÃO

É importante desenvolver estratégias que minimizem os desafios metabólicos e fisiológicos enfrentados por vacas leiteiras, assegurando seu bem-estar e maximizando o desempenho produtivo. O período de transição representa uma fase crítica, tornando as vacas mais propensas a doenças metabólicas, enquanto que as mudanças climáticas estão se intensificando, e assim introduzindo desafios adicionais, como o aumento da temperatura ambiental, acarretando em períodos de maior exposição ao estresse calórico. A suplementação com ácidos graxos surge como uma abordagem eficiente, contudo há lacunas significativas no entendimento, especialmente em relação à utilização de ácidos graxos de cadeia média durante o período de transição. Além disso, o fornecimento de ácidos graxos ômega-3 e ômega-6 na modulação de mediadores lipídicos em animais sob estresse calórico carece de investigação, oferecendo a oportunidade para identificar biomarcadores associados aos processos inflamatórios ou estresse oxidativo.

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CAPÍTULO II. EFEITOS DA SUPLEMENTAÇÃO DE ÁCIDOS GRAXOS DE CADEIA MÉDIA EM VACAS LEITEIRAS NO PERÍODO DE TRANSIÇÃO

Hipótese

Considerando o contexto da suplementação de ácidos graxos de cadeia média (AGCM) em vacas leiteiras durante o período de transição, levantamos a hipótese de que a inclusão desses ácidos graxos em concentrações inferiores a 1% na MS da dieta pode representar uma estratégia eficaz para proporcionar suporte energético e imunológico aos animais. Acreditamos que a suplementação possa ocorrer não necessariamente impactando a produção de leite dessas vacas, mas trazendo algum benefício no metabolismo e na saúde dos animais suplementados.

Objetivo geral

Avaliar os efeitos da suplementação de AGCM em baixa dosagem no desempenho produtivo, metabólico e imunológico de vacas leiteiras no período de transição.

Objetivos específicos

- Avaliar o desempenho produtivo, incluindo a produção e composição do leite, bem como o perfil de ácidos graxos da gordura do leite, em vacas suplementadas com AGCM.
- Mensurar os impactos da suplementação de AGCM nos perfis sanguíneos e hepáticos pré e pós-parto.
- Analisar a expressão gênica relacionada a processos como inflamação, estresse oxidativo, microbiota, metabolismo de glicose, metabolismo de gorduras, receptores e fatores de transcrição em vacas suplementadas com AGCM.
- Avaliar a saúde geral de vacas submetidas à suplementação de AGCM.

EFFECTS OF MEDIUM-CHAIN FATTY ACID SUPPLEMENTATION IN DAIRY COWS IN THE TRANSITION PERIOD

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Abstract

Cows during the transition period are more susceptible to physiological and metabolic changes. Medium-chain fatty acids (MCFA; C6:0, C8:0, C10:0, and C12:0) have shown beneficial effects on the metabolism of lactating cows when supplemented at lower doses (< 1.0% of the dietary DM). This study aimed to evaluate the effects of MCFA supplementation on the production, metabolism, and immune system of periparturient dairy cows. One hundred sixty-eight Holsteins, 66 heifers (BW 662 ± 69 kg), and 102 cows (BW 718 ± 74 kg), were divided into two groups in a randomized complete block design for 35 days (21 days prepartum and 14 days postpartum). The treatments used were: T1: Control (no MCFA supplementation) and T2: 50 g of lipid supplement (0.065% MCFA in DM) based on coconut and palm oil, individually top-dressed once daily and mixed with corn meal. Milk production, milk composition and milk fatty acid profile, rumination, body weight, and body condition score (BCS) were measured, along with blood metabolites (total calcium, ionic calcium, glucose, GGT, AST, cholesterol, bilirubin, albumin, non-esterified fatty acids, and beta-hydroxybutyrate) on different days (-7, 0, 3, 7, and 14). An exploratory gene analysis (HPRT1, IL-6, SAA3, GPx3, CASP8, TLR4, MyD88, STAT1,

STAT5, LPK, ACACA, FASN, LPL, SCD, SREBF1) was also conducted. The model included treatment, time, parity, and their interactions as fixed effects, while block and animal (within treatment) were considered random effects. There was no significant effect of treatment on milk production, fat and protein contents, and total solids production ($P > 0.05$). However, there was a tendency towards increased protein production in multiparous MCFA-treated cows ($P = 0.08$). MCFA-treated cows showed a lower ($P = 0.03$) milk total solids content. There were also differences in milk fatty acids between groups, with a tendency to lower ($P < 0.10$) fatty acids in the MCFA-treated group, such as C11:0, C15:0, and C18:2 *cis*-9, *trans*-11. Blood metabolites did not differ ($P > 0.05$) between treatments. There were significant effects of treatment on the expression of PTX3, GPx1, and NFKB1 genes, with MCFA-treated cows showing different expression levels compared to the control group ($P < 0.05$). Therefore, the results indicate that supplementation with medium-chain fatty acids did not have a significant impact on milk production and composition, but influenced gene expression and the milk fatty acid profile. In conclusion, the results suggest that the dose of medium-chain fatty acids (MCFA) adopted in this trial may not have been sufficient to produce significant effects.

Keywords: caprylic acid, capric acid, lauric acid, lipid supplementation, periparturient cows.

1 Introduction

The transition period corresponds to a critical period for the health of dairy cows, characterized by a decrease in dry matter intake (DMI) and the establishment of a negative energy balance (Drackley, 1999; Barletta et al., 2017). This occurs due to the high energy demand resulting from exponential fetal growth and colostrum and milk synthesis (Brown & Allen, 2013). The lack of available energy leads to excessive adipose tissue mobilization, triggering inflammation and oxidative stress, often associated with immune dysfunction (Ling et al., 2018). As a result, the risk of health disorders during the immediate postpartum period is significantly elevated (Caixeta et al., 2017). Furthermore,

these metabolic disorders can lead to losses in production and reproduction, which have substantial economic implications (Ling et al., 2018).

During the critical period of early lactation, specific nutritional strategies are often implemented to benefit dairy cows and to mitigate health occurrences. One strategy involves supplementing medium-chain saturated fatty acids. Medium-chain fatty acids (MCFA), which include caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), and lauric acid (C12:0), have gained prominence due to their characteristics of faster absorption and metabolism, being a faster source of energy compared to long-chain fatty acids (Marten et al., 2006; Dubois et al., 2007). These MCFAs offer a series of benefits, such as antioxidant and anti-inflammatory actions, as well as improving cell mitochondrial function, which promotes greater energy production (Dawson et al., 2002; Nelson & Cox, 2014). It is important to highlight that, although MCFAs are not widely used in the dairy industry as lipid supplements, they are employed as food additives due to their potential beneficial health effects.

The inclusion of MCFA in the diet of dairy cows is not yet completely characterized and has shown varying results, depending on the source and dose administered to the animals. However, it is recognized that MCFAs have great potential to influence the ruminal environment, affecting pH and microbiota. This could result in a reduction in bacteria and protozoa, especially methanogenic bacteria, consequently leading to a decrease in methane emissions (Dohme et al., 2001; Machmuller et al., 2003). On the other hand, it was observed that MCFA supplementation may be associated with lower DMI, reduced neutral detergent fiber (NDF) digestibility, and eventually decreased milk production (Dohme et al., 2000; Machmuller et al., 2001; Hristov et al., 2004). These detrimental effects have a linear relationship as supplementation levels increase.

Therefore, our hypothesis is based on the idea that MCFA supplementation in concentrations lower than 1% dietary DM for transition dairy cows can contribute to the energy and immunological supply of these animals, without affecting milk production and composition. The objective of this study was to investigate the productive, metabolic, and immunological effects of low doses of MCFA supplementation during the transition period.

2 Material and Methods

2.1 Animals, experimental design and treatments

The study was approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Universidade Federal do Paraná, under protocol number 038/2021. The experiment was conducted on a commercial farm located at Paraná State, Southern Brazil. A total of 168 Holstein animals in the transition period were used, comprising 66 heifers (prepartum BW 662 ± 69 kg), and 102 cows (prepartum BW 718 ± 74 kg). Each animal was considered as an experimental unit and they were blocked in a randomized complete block design, based on similar body condition score (BCS), parity, and expected calving date. Within each block, animals were randomly assigned to one of two treatments.

The animals were subjected to two treatments: Control: basal diet + 150 g of ground corn (n=84) and MCFA: basal diet + 100 g of ground corn + 50 g of MCFA lipid supplement (n=84; Aromabiotic Cattle) for 35 days, including 21 days prepartum and 14 days postpartum. The lipid supplement contained 25% of MCFA (comprising 32% C8:0, 21% C10:0, and 47% C12:0) and 75% carrier ingredients (consisting of 63% ground corn, 11.5% silicon dioxide, and 0.5% flavoring component), as shown in Table 1. The basal diet provided to the animals is detailed in Table 1 and mainly consisted of corn silage, ground corn, soybean meal, vitamin/mineral supplement, and anionic salts (prepartum), formulated according to the nutritional requirements of dairy cows (NRC, 2001). The basal diet was provided in a total mixed ration (TMR), offered once a day in the morning for both treatments. The animals were restrained in the headlocks only at the time of MCFA or Control supplementation, receiving the treatments individually as top-dressing. Additionally, the animals had access to water ad libitum, and the diet allowed 10% of daily refusals.

2.2 Management, feeding, and rumination

Both groups, controls and MCFA-treated animals, were kept in the same free-stall barn and they were not physically separated, being divided only by cattle headlocks during supplementation. So, they had received uniform treatment in terms of management,

feeding, and weather conditions. During the prepartum period, the animals were divided into two groups, heifers and cows, but both remained in the same barn. All post-calving animals were kept together in a single group.

Feeding was scheduled to occur at 07h00 AM for the postpartum group and at 09h00 AM for the prepartum group. During feeding, the cattle headlocks were activated to contain the animals, so that when the feeding wagon passed by, the animals were already restrained in the stanchions. The lipid supplement plus corn meal for the treated-animals or only corn meal for the controls was then provided *top-dressed.* After 30 minutes, the total or partial consumption of the supplement was checked and recorded, ensuring that all the provided supplement was consumed, and the animals were released from the headlocks.

Throughout the experiment, group daily consumption was estimated using the following formula: the weight of TMR provided (in kg) minus the weight of leftovers (in kg), divided by the total number of animals in the group. Since the animals from both groups were mixed, it was not possible to estimate separated consumption by treatment.

However, ruminal activity was monitored through SmartBov earrings (SmartBow and Zoetis, AT), which were located on the cows' ears. These earrings recorded the rumination rate in minutes, based on ear movements, and after one hour, the rumination rate per hour was calculated using the Animal Pattern Recognition Intelligence (APRIL) system. This monitoring was recorded daily.

2.3 Feed collection and analysis

The TMR and individual forage samples included in the basal diet were collected in both the prepartum and postpartum groups every 2 weeks throughout the experimental period. These samples were collected by the same collector along the entire feedbunk and from the silos to avoid variability in sample composition. These feedstuffs were frozen at -20°C for subsequent nutritional analysis.

All processing and analyses of dry matter (DM), ash, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), ether extract (EE), and macrominerals were determined as follows. The samples were dried in an oven at 55°C for 72 hours and ground to pass through a 1mm diameter mesh screen using a stationary mill (Wiley Mill,

Thomas Scientific, Swedesboro, NJ). DM concentration was determined at 105°C for 24 hours, and ash content was assessed at 550°C for 8 hours. All analyses were conducted according to the AOAC methods (Official Methods of Analysis of AOAC International, 2016). The fractions of EE, NDF, and ADF were adapted for ANKOM 2000-type equipment. The CP fraction followed the Kjeldahl method. Calcium (Ca) was determined by titration, phosphorus (P) by colorimetry, and potassium (K) by photometry. The results are shown in Table 2.

2.4 Milk production and composition

Milking was performed three times daily at 04h00, 12h00, and 20h00h using a rotatory milking system. Milk production was individually measured from day 1 to day 100 of lactation (DIM). Specific milk meters from the farm's milking system (De Laval Inc.) were used, which quantified the total daily milk production per milking.

Throughout the experimental period, milk samples were collected every week using milk collectors connected to the milking system (De Laval Inc.). Two milk samples were obtained from each cow during each collection: one in a bottle containing bronopol for physical-chemical composition analysis and another in a bottle without bronopol for milk fatty acid profile analysis. All samples were stored at -20°C for subsequent analyses. Milk fat, total milk protein, milk lactose, and total milk solids content were measured by mid-infrared absorption spectrometry (Bentley Instruments, Minnesota, USA) and somatic cell count (SCC) was assessed by cytometry flow in the APCBRH laboratory, in Curitiba-PR.

Energy corrected milk (ECM) was estimated following NASEM guidelines (2021) using the formula: ECM = [(0.0929 x % fat) + (0.0547 x % protein) + (0.0395 x % lactose)] x kg of milk. Four percent fat-corrected milk (FCM) was estimated using the Gaines equation: FCM = (0.4 + 15 x % fat/100) x kg of milk. Somatic Cell Count (SCC) was transformed into a linear scale from 0 to 9 using the following equation: Linear SCC = -3.6438 + 1.4427 * Ln (SCC).

For the milk fatty acid profile analysis, the first step involved fat extraction and methylation following Rodriguez-Ruiz et al. (1998) procedures. Gas chromatography (Focus GC-Finnigan) was then used, employing a capillary column (100 m x 0.25 mm x 0.2 µm; CP-Sill 88). A 1 µL sample of esterified extract was injected into the

chromatograph, and the fatty acid content was identified by comparing retention times and the percentage of fatty acids obtained using Chromquest 4.1 software (Thermo Electron, Italy). Fatty acid peak identification was accomplished with the assistance of standards (Supelco TM Component FAME Mix, cat 18919) and the retention time of methyl esters.

2.5 Blood collection and analysis of blood metabolites

Blood samples were obtained through caudal coccygeal venipuncture using vacutainer tubes with anticoagulant on the following days of the experimental period: d-7, d0, d3, d7, and d14. After collection, the blood was subjected to centrifugation at 3000 rpm, at 10°C, for 30 minutes. The resulting plasma was stored at -20°C for further analysis. Plasma samples collected on different days were distributed for specific analyses as follows: total calcium (tCa), glucose, gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), cholesterol, bilirubin and albumin (d-7, d0, d3, d7, and d14), non-esterified fatty acids (NEFA, d-7, d0, and d7) and beta-hydroxybutyrate (BHB, d3, d7 and d14).

The samples were processed and analyzed on automated biochemical equipment (BS200 Mindray Starlab). The analysis of GGT, AST, bilirubin, and NEFA metabolites were conducted using the UV kinetic method, while cholesterol metabolites were evaluated by the colorimetric kinetic method. Glucose and BHB were measured using an enzymatic colorimetric method, and albumin was determined using the colorimetric method based on bromocresol green. The concentration of tCa was quantified using the colorimetric method with the Arsenazo III reagent.

During the experimental period, ketone bodies were also measured with specific BHB detection strips and glucometers, to indicate the prevalence of clinical or subclinical ketosis. These measurements were carried out on days 7 and 14 (FreeStyle Libre, Optium Neo Abbott). On the first day postpartum, blood was collected in a vacutainer tube with heparin for ionic calcium (iCa) analysis, approximately 20 hours after calving. Ionic calcium is the most relevant parameter to check possible events of clinical hypocalcemia (milk fever). These parameters were measured using EG7 cartridges and an iStat portable reading device (iStat, Sky wrappers Scientific).

2.6 Biological sample collection and gene expression

Blood samples were collected from the caudal coccygeal vein of each animal, using vacutainer tubes with EDTA on days d -7 and d +14 related to calving. The blood was processed by centrifugation at 3,500 rpm, 4ºC, for 15 minutes. The leukocyte cells were then isolated, transferred to 1.5 mL Eppendorf tubes, and stored in a -80ºC freezer for later analysis. A lysis solution (12 mM Tris-HCl pH 8.2; 0.32 M Sucrose and 5 mM EDTA and 1% Triton 100X) was used to eliminate the remaining red blood cells and white blood cells were used for gene expression analysis.

To extract RNA from white blood cells, the Pure Link RNA Mini Kit (Invitrogen, Carlsbad, CA) was used. The RNA was transferred to a silica membrane column and washed with pure RNA water to eliminate any impurities. RNA quality was assessed by the 260/280 ratio, which was approximately 2.0 (Nanodrop, ThermoScientific). DNase enzyme (Promega) was used to eliminate the DNA present, and then DNase was inactivated by 2mM EGTA at 65ºC for 10 min. Total RNA was reverse-transcribed using the enzyme GoScript Reverse Transcriptase (Promega), where the reaction was carried out at 42ºC for 60 min, followed by 15 min at 70ºC and cooling to 4ºC (Applied Biosystems). From this reaction, the cDNA was ready to be used in Polymerase Chain Reaction (PCR) analysis.

qRT-PCR reactions were prepared in a total volume of 20 µL, containing cDNA, oligonucleotide primers, and Syber Green PCR Master Mix (Life Technologies). Reactions were conducted in a StepOnePlus® Applied Biosystem thermal cycler (Life Technologies) with initial denaturation steps, denaturation cycles, primer annealing, and extension. Each sample was analyzed in duplicate, and the specificity of the reactions was confirmed by the dissociation curve, confirming the specificity of the reaction and the absence of formation of primer dimers or any other non-specific product. RT-PCR efficiency was performed for all primer pairs and the sizes of the fragments produced were analyzed by 1.5% agarose gel electrophoresis.

Four endogenous reference genes were selected (housekeeping; GAPDH, YWHAZ, HPRT1, and UBC) based on their efficiency and stability of expression between treatments, and 26 genes of interest involved with inflammation (IL-6, TNFa, IL-18, and NLRP3), oxidative stress (SOD1, CAT, GPX1, GPX3, and CASPASE8), microbial (PTX3

and SAA3), receptors (CD14 and TLR4), transcription factors (NF-kB1, MYD88, STAT1, STAT5, and FOXP3), glucose metabolism (LPK and G6PD), and fat metabolism (ACACA, FASN, LPL, SCD, and SREBF1; Table 3). Gene expression was quantified using the 2−ΔΔCT method (2008), which compares the expression of target genes to the geometric mean of reference genes. This allowed comparison of changes in gene expression between different treatments and samples.

To this end, it was calculated: ΔΔCt = (ΔCt treatment − ΔCt control). The proposed equation, by Schmittgen and Livak (2008), makes it possible to compare changes in the gene expression profile of each gene of interest about a calibrator (treatment: control) and between different samples. The number two at the base of the formula represents that the amount of genetic material in the reaction is doubled with each new cycle and the CT refers to the “threshold” cycle, which is the number of cycles necessary for the sample to reach the detection threshold in the phase exponential amplification.

2.7 Body weight and body condition score

Body weight and body condition score (BCS) were recorded at three moments during the experimental period, days -21, 0, and 14. Weight measurements were taken individually, using a bovine weight estimation tape (Bovitec ltda) on the thoracic perimeter. The BCS was assessed through visual observation of the median back vertebra, ilium bones, and tail insertion. The same observer consistently conducted this assessment using a scale of 1 to 5, in which 1 represented lean animals and 5 represented fat animals, with intervals of 0.25, as Wildman et al. (1982) described.

2.8 Disease detection and survival

The incidence of clinical diseases was monitored from the time of calving until 100 DIM, with all health conditions pre-defined before the start of the study. Cows were routinely subjected to daily examinations in the postpartum period, paying special attention to signs such as loss of appetite, reduced rumination, or decreased milk production. When such signs were observed, the diagnosis was made by the veterinarian responsible for the farm.

The health of the cows was thoroughly checked, including observation of the vulva to detect possible cases of retained placenta and metritis. Retained placenta was diagnosed when the placenta was not naturally expelled within 24 hours after calving. Metritis was characterized by the presence of reddish uterine secretion with an unpleasant odor, observed until d14 postpartum. The displacement of the abomasum was identified through auscultation and percussion close to the paralumbar fossae and was later confirmed and corrected surgically through omentopexy. As far as clinical mastitis is concerned, this was diagnosed before each milking, with all cows being thoroughly examined to check for the presence of abnormal milk in one or more quarters of the mammary gland.

Hyperketonemia, which refers to high levels of BHB in the blood, was assessed in two stages: initially, on days 7 and 14 after calving, using immediate reading strips, with values above or equal to 1.2 mmol/L being considered indicative of hyperketonemia. Then, on days 3, 7, and 14, BHB analysis as the gold standard was conducted, with values above or equal to 1.2 mmol/L indicating subclinical hyperketonemia and values above or equal to 2.9 mM/L indicating clinical hyperketonemia, while values below 1.2 mM/L were considered normal (Duffield, 2000).

To detect cows with hypocalcemia, both subclinical and clinical, blood calcium concentrations were assessed. The incidence of hypocalcemia was based on the detection of at least one sample with a serum Ca concentration of less than 1.10 or 2.0 mM/L, for clinical and subclinical hypocalcemia, respectively. On the first day after calving, ionic calcium (iCa) was assessed, with values below 1.10 mM/L indicating hypocalcemia and values above 1.10 mM/L indicating normocalcemia. On days 0, 3, 7, and 14 after calving, total Ca was analyzed, with cows that presented tCa less than 2.0 mM/L classified as hypocalcemic, and tCa greater than 2.1 mM/L as normocalcemic (McArt & Neves, 2020).

Morbidity was defined considering cows that had at least one of the following health conditions: dystocia, retained placenta, metritis, mastitis, displaced abomasum, hypocalcemia, hyperketonemia, and hoof problems that could potentially affect the animals' productive performance.

After the start of the experimental trial and the administration of the product, animals were excluded from the study only in cases where the farm decided to remove them from the herd due to low milk production, animal death, or health issues that required treatment with medications and resulted in significantly reduced feed intake, with the animal remaining in a hospitalized condition for more than 5 days.

2.9 Statistical analysis

Data were analyzed using the MIXED and PROC GLM procedures from SAS Academic (SAS Institute). Mixed models and repeated analyses over time were applied. The model included treatment, time, parity, and their interactions as fixed effects, while block and animal were considered random effects. Data normality was assessed using the Kolmogorov-Smirnov test. The means of each treatment were compared using the Tukey-Kramer test. Significant results were identified when P value < 0.05, while tendency were observed when P was between 0.05 and 0.10. The Restricted Maximum Likelihood Estimation (REML) method was chosen as the best statistical measure to fit the observed data. Simple Pearson correlations were calculated between the variables in each analysis, using the SAS CORR procedure. Outliers were excluded from statistical analysis. Additionally, 9 animals were removed from the study (4 MCFA and 5 Control) due to problems of low milk production, intercurrent mastitis and hoof problems affecting consumption.

3 Results

3.1 Intake and rumination

During the pre-calving period, cows recorded an average dry matter intake of 14 kg, while heifers had a lower average of 11 kg. However, after giving calving, an increase in consumption was observed. In the first 14 days postpartum, the average consumption of dry matter reached 19 kg/cow/day. It is important to highlight that these numbers were calculated based on pre- and postpartum groups, and were not individually measured by treatment. Despite this, the analyses indicated variations in rumination over the days and weeks (P < 0.001; Figure 1 A and B), but there were no differences between treatments (P = 0.40).

3.2 Production, composition, and milk fatty acid profile

Milk production until day 14, during the period in which the animals were receiving lipid supplementation, there was no effect of treatment (P = 0.61), nor was there an interaction between treatment and parity order (P = 0.47; Table 4). Furthermore, even on days 30, 60, and 100, after stopping medium-chain fatty acid (MCFA) supplementation, no residual effects on milk production were identified (P = 0.76, P = 0.88, and P = 0.99, respectively; Table 4).

Production and fat content were not affected by treatments (Table 4; P = 0.13 and P = 0.33). Likewise, protein production and content showed no differences for treatment (P = 0.67 and P = 0.79), although a tendency was observed in the interaction between treatment and parity for protein production (P = 0.08) and a significant effect for treatment x parity on protein content (P = 0.03; Table 4). Thus, second-calving cows in the control group showed a 9.59% increase in protein production compared to first-calving cows. Total solids production revealed a significant effect of treatment and parity (P = 0.03), with an increase of 4.79% in primiparous compared to secondiparous. Regarding total solids content, a treatment effect was observed, with a reduction in the MCFA group compared to the control group (P = 0.03). Lactose production and content were not influenced by treatment (P > 0.27).

Milk somatic cell count showed a tendency to treatment (P = 0.07), with an average of 1.48-fold higher in the group that received MCFA supplementation compared to the control group, according to the linear scale from 0 to 9 (Table 4). Energy-corrected milk and fat-corrected milk also demonstrated a tendency toward treatment, with a 10.78% and 12.10% reduction, respectively, in the MCFA group compared to the control (Table 4; P = 0.08 and P = 0.07).

Undecanoic fatty acid (C11:0) showed a tendency of 20% reduction in MCFA treatment compared with the control group (Table 5; P = 0.06). Furthermore, both pentadecanoic acid (C15:0) and vaccenic acid (C18:1 trans) were significantly reduced in the MCFA-treated group compared with the control, with a reduction of 12% (P = 0.04) and 11.26% (P = 0.03), respectively. Linolenic acid (C18:3 n-6) showed an increasing tendency in the MCFA-supplemented group compared with control (0.03 vs. 0.04; P = 0.07). Similarly, conjugated linoleic acid cis-9, trans-11 (C18:2 cis-9, trans-11) was

significantly reduced in the MCFA-treated group compared with the control (P = 0.02). When fatty acids were grouped into classes, odd-chain and branched-chain fatty acids, as well as polyunsaturated fatty acids, also showed reductions in the MCFA-treated group, with drops of 4.9% and 5%, respectively, compared with control (P = 0.06 and P = 0.07, respectively).

3.3 Blood metabolites

Blood metabolites came from plasma, and are detailed in Table 6. The GGT showed a tendency for treatment, reducing by 3.47% in MCFA compared to Control (P = 0.09; Table 6). Glucose showed a tendency for treatment and day, being higher in the MCFA-treated group compared to Control on days -10 and 3, at 3.19% and 3.44%, respectively (P = 0.05; Figure 2D). Total calcium showed a tendency for interaction between treatment and day, being reduced by 3.32% on day 0 in the MCFA treatment compared to the Control (P = 0.08; Figure 2H). The AST, bilirubin, albumin, cholesterol, NEFA, BHB, and ionic calcium had no treatment effect (P > 0.05; Table 6).

3.4 Gene expression in leukocyte cells

Twenty-nine genes were analyzed, and 14 were expressed: the 3 housekeeping genes (GAPDH YWHAZ, UBC) and 10 target genes (TNFα, PTx3, SOD1, CAT, GPx1, IL18, NLRP3, CD14, NFKB1, FOXP3, G6PD). A significant effect of treatment on the PTX3 and GPx1 genes was observed, with cows treated with MCFA showing a higher expression of these genes compared to cows in the control group (P < 0.05; Figure 3A). The NFKB1 gene, cows treated with MCFA showed a lower expression compared to the control group (P < 0.05; Figure 3A). For the GPx1 gene, an interaction between treatment and parity was also identified, with multiparous dams treated with MCFA showing an increase in GPx1 expression compared to Control (P < 0.05; Figure 3B). Still, only some animals expressed the TNFα and NLRP3 genes, with no difference between treatments (P > 0.05). The following genes were not expressed in the samples analyzed: HPRT1, IL-6, SAA3, GPx3, CASP8, TLR4, MyD88, STAT1, STAT5, LPK, ACACA, FASN, LPL, SCD and SREBF1.

3.5 Body weight and body condition score

The body weight and body condition score of the cows showed no differences between treatments during the pre-partum, parturition and post-partum periods ($P > 0.05$; Table 7). It was observed that the Control group had an average reduction of 21 kg from pre-partum to post-partum, while the MCFA group recorded an average reduction of 14 kg during the same period (Table 7).

3.6 Incidence of diseases and metabolic disorders

During the experimental period, a total of 209 incidences of diseases or metabolic disorders were recorded, detailed in Table 8. These incidences were observed in both Control and MCFA group. The disorders reported were: ketosis (54.76% vs. 53.57%), hypocalcemia (28.57% vs. 34.52%), retained placenta (2.38% vs. 3.57%), metritis (4.76% vs. 3.57%), dystocic calving (17.85% vs. 15.47%), abomasal displacement (3.57% vs. 4.76%), mastitis (2.38% vs. 5.95%) and hoof problems (8.33% vs. 4.76%), for the control and MCFA groups, respectively.

Furthermore, throughout the experiment, 9 animals were discarded, 5 cows from the Control group and 4 from the MCFA group. These discards occurred due to low milk production, intercurrent mastitis, hoof problems, and calving complications that affected locomotion and supplement consumption, in addition to the need to administer medications. It is important to highlight that these discards were not related to the treatments themselves.

4 Discussion

The exact consumption of dry matter per animal or treated group was not measured. However, consumption was estimated based on the total amount of natural matter supplied, subtracting leftovers from the group. During the supplementation period, the animals were monitored to ensure that they completely consumed the supplement provided as top-dress, therefore, it is assumed that the cows consumed all of the product offered.

Rumination varied over the days. In the first three days, there was a slight decrease in rumination, possibly due to adaptation to the supplementation and acceptance of the product, but then it returned to normal. As calving approached, there was a drop in rumination peaks, which is natural, as dairy cows tend to reduce their consumption in the days before calving and for a few days after calving, due to the smaller rumen space caused by fetal growth (NRC, 2001; Barletta et al., 2017). However, there was no difference between treatments over the days, indicating that rumination followed a natural pattern without the influence of treatments.

To maintain a good rumination rate, averaging 50 to 80 food bolus movements in 6 to 10 hours per day, cows need a stable and diverse community of rumen microorganisms for greater feed and rumination efficiency (Shabat et al., 2016; Beauchemin, 2018). Supplementation with 0.063% dry matter of medium-chain fatty acids (MCFA) in lactating cows reduced daily fluctuations in rumen pH, creating a more stable environment for rumen microorganisms. However, this did not affect rumen microbial communities or the production of volatile fatty acids in the rumen, and there was no significant impact on the rumen microbiota (Burdick et al., 2022). Therefore, as MCFAs did not impact the ruminal microbiota, there was also no difference in rumination rate in this study, suggesting that the bacterial community was well adapted to MCFAs.

Furthermore, fiber digestibility strongly influences rumination in dairy cows (Nørgaard et al., 2011). Studies show that MCFAs increase the efficiency of fiber digestibility compared to long-chain fatty acids (Machmüller, 2006). However, research has also demonstrated that including more than 1.3% MCFA in dry matter reduced fiber digestibility (Dohme et al., 2001; Hollmann et al., 2012; Faciola & Broderick, 2013), while inclusion of less than 1%, such as 0.063% or 0.96%, did not alter fiber digestibility (Hristov et al., 2009; Burdick et al., 2022). Furthermore, MCFAs tend to reduce food consumption (Hollmann et al., 2012; Faciola & Broderick, 2013), which was only observed at the beginning of this study but was not confirmed by statistical analysis. Thus, as in this study, the inclusion was less than 1% of dry matter, there was no difference in rumination.

Milk productionand fat and lactose content also showed no treatment effect. Milk production may be related to the consumption and rumination of animals, which did not differ from each other. However, there are inconsistent results in the literature on the

effects of MCFAs on milk fat. Cows that were supplemented with a mix of C8:0 and C10:0 had increased milk fat compared to cows that received C16:0 or flaxseed-derived PUFA (Van Zijderveld et al., 2011), but no results were observed for milk fat when compared to non-supplemented animals (Fukumori et al., 2013; Sugino et al., 2014). Some studies suggest that C12:0 is the MCFA that most influences food consumption, and production of milk and its derivatives, being a potent inhibitor of rumen microorganisms (Dohme et al., 2001). Thus, in our study, we included 48.98% of C12:0 in the lipid supplement.

Milk production and protein content were influenced by treatment and parity, with higher production in second-calving cows compared to first-calving cows in the control group. Total solids also showed differences for treatment and parity, being higher in the control group and first-calving cows compared to second-calving cows. This suggests that these effects are related to the milk production of these cows. Second-parous cows produced more milk and, consequently, more protein, while primiparous cows produced less milk, resulting in a higher concentration of total solids.

Still, protein and total solids levels were lower in the MCFA-treated group, as was energy- and fat-corrected milk production. The reason for these results is unclear. A study by Rico et al. (2020) observed a tendency toward reduced milk fat production and fat-corrected milk with MCFA supplementation compared to C16:0, but the results are not conclusive. Furthermore, the high value of total solids found in this study can be attributed to the collection of milk within a few days after calving, resulting in transitional milk that is more consistent and richer in fat.

Supplementation with MCFA slightly altered the fatty acid profile of milk, although the results are inconclusive. A reduction in the levels of pentadecanoic acid (C15:0) and odd-chain and branched-chain fatty acids, which are considered markers of rumen fermentation and microbial activity in the rumen (Buitenhuis et al., 2019), was observed. Furthermore, we expected that supplementation would increase the levels of medium-chain fatty acids, such as C8:0, C10:0, and C12:0 in milk, but the amount supplemented may have been insufficient to achieve this effect.

Furthermore, the influence of rumen bacteria on the formation of milk fatty acids is most significant in odd-chain fatty acids, such as C15:0 and C17:0, and in 18-carbon polyunsaturated fatty acids (Månsson, 2018). Buitenhuis et al. (2019) demonstrated that

variations in the composition of the ruminal microbiome significantly affect the content of odd-chain fatty acids and C18 polyunsaturated fatty acids, with a lesser influence on short- and medium-chain fatty acids present in milk. Indicating lower microbial activity in the treated group compared to the Control group.

The study observed a tendency towards reduced GGT levels in blood plasma in the group treated with MCFA. GGT is an enzyme used as a clinical marker to assess liver health and function. Low GGT values suggest that cows in the transition period do not face serious liver problems (Bossaert et al., 2012). Glucose also showed a trend for treatment and days analyzed, being higher in the MCFA-treated group compared to the control on days -10 and 3. During the transition period, the liver function of cows undergoes major changes, including greater release of bile acids into the bloodstream (Wang et al., 2023). Bile acid accumulation during late pregnancy may influence glucose homeostasis. Cholic, glycolic and taurocholic acid are the bile acids most present in the blood of dairy cows (Washizu et al., 1991). The study by Wang et al. (2023) observed a reduction in bile acids 14 days postpartum with MCFA supplementation, suggesting that supplementation promotes the metabolism of these acids, improves liver function, and increases insulin resistance in dairy cows (Roopashree et al., 2021).

Furthermore, Kawaguchi et al. (2002) showed that the activation of MCFA generates a net economy of glucose in rat hepatocytes, through an increase in the activity of AMPK1, an important enzyme in glycolysis. The study by Rico et al. (2020) also observed an increase in hepatic AMPK1 expression in groups treated with MCFA and C18:0, suggesting a glucose saving in cells.

As for total calcium, there was a tendency for an interaction between treatment and days analyzed, with reduced levels on day 0 in the group treated with MCFA. Although this interaction occurred, both groups demonstrated hypocalcemia. Day 0, or day of calving, is marked by a high demand for calcium for the production of colostrum and milk, resulting in hypocalcemia at the beginning of lactation. Caixeta et al. (2017) indicate that cows with serum calcium concentrations below 2.15 mmol/L in the first three days after calving are classified as having subclinical hypocalcemia and are more prone to disease and less likely to become pregnant compared to normocalcemic cows.

The levels of AST, bilirubin, albumin, cholesterol, NEFA, BHB and ionic calcium were not influenced by the treatment. However, we expected that MCFA supplementation could decrease the plasma levels of NEFA and BHB due to the rapid absorption of MCFA by the liver, which would efficiently regulate the metabolism of cows during the transition period (Marten et al., 2006; Nelson & Cox, 2014; Wang et al., 2023).

The MCFA treatment had a significant effect on the expression of the PTX3 and GPx1 genes, with treated cows showing higher levels of these genes compared to the control group. Pentraxin 3 (PTX3) is a protein involved in the inflammatory response, contributing to the innate immune response by recognizing and eliminating pathogens through the immune system. This suggests that healthy cows have a lower expression of PTX3, since this protein has a high affinity with the TNF-stimulated gene 6 (TSG-6), facilitating the recognition of pathogens by macrophages and dendritic cells (Genís et al., 2018). The study by Genís et al. (2018) on metritis in cows with different parity orders revealed an increase in the expression of the PTX3 gene in the endometrial tissue of healthy cows, indicating that PTX3 can promote the recruitment and activation of macrophages and dendritic cells, amplifying the feedback effect on the expression of PTX3 (Mansouri-Attia et al., 2013). This suggests that, even with a low dose of MCFA in the present study, the treated group may have shown greater immune activation compared to the control group.

Glutathione peroxidase 1 (GPX1) is an antioxidant enzyme that protects cells from oxidative stress by catalyzing the reduction of hydrogen peroxides and hyperperoxidized fatty acids, contributing to the maintenance of redox balance and preventing cellular damage caused by reactive oxygen species (Adeniran et al., 2022). In the study by Adeniran et al. (2022), the antioxidant rate of LPS-induced bovine metritis during postpartum was assessed through GPX1 expression. They observed a significant increase in mRNA concentration in the LPS-treated group compared to the control. Thus, greater expression of this gene attenuates LPS-mediated responses, reducing oxidative stress. Furthermore, GPX1 and GPX4 have been documented to participate in diverse reactions, including antioxidant activities, inhibition of inflammatory cytokines, and elimination of peroxynitrite in the inflammatory phase of the wound healing process (Hariharan & Dharmaraj, 2020). Therefore, a higher concentration of GPX1, as found in

this study in cows supplemented with MCFA, suggests that the immune function to prevent oxidative damage is stronger (Hosnedlova et al., 2017).

Furthermore, an interaction between MCFA treatment and parity in the GPx1 gene was identified, with treated multiparous cows showing higher GPx1 expression compared to the control group at d14 postpartum. A study with multiparous women showed that GPX1 mRNA abundance was highest 35 days before parturition, decreased during parturition, and increased again in early lactation (Aitken et al., 2009). This increase in GPX1 activity in early lactation may be a protective response against the oxidative damage that occurs during abundant milk production and secretion, where GPX1 mRNA expression is sensitive to changes in the accumulation of reactive oxygen species (ROS), making it an effective indicator of oxidative stress (Aitken et al., 2009). The reason for the more pronounced expression of GPX1 in multiparous cows than in primiparous and secondiparous cows in our study is not entirely clear, but older cows tend to have a more efficient immune system.

In our study, cows treated with MCFA showed lower expression of the nuclear factor kappa β1 (NF-κB1) gene compared to cows in the control group. NF-κB1 is related to the regulation of inflammatory and immunological responses, being activated by processes such as inflammation, infections, injuries, or oxidative stress (Zhao et al., 2017). The study by Sun et al. (2021) on ketotic cows showed that the expression of NF-κB1, NLRP3 inflammasome, and caspase 9, as well as a greater abundance of pro-inflammatory cytokines mRNA (TNFA, IL6, and IL1B) in the mammary gland, was present in cows with ketosis. Reactive oxygen species can activate NF-κB1, leading to increased production of pro-inflammatory cytokines (Nakajima and Kitamura, 2013; Calder, 2013). Changes in the redox environment also affect the regulation of the NLRP3 inflammasome, resulting in the activation of caspase 1 (Rubartelli, 2012). Similarly, LPS inflammation activates NF-κB1, inducing the production of pro-inflammatory cytokines (Calder, 2013). These findings highlight that metabolic stress can cause systemic inflammation in early lactation dairy cows. However, our results indicate that cows supplemented with MCFA were not sufficiently inflamed to activate the NF-κB1 system.

Incidences of metabolic disorders or diseases in animals have been observed, although the absence of statistical analysis limits conclusions. Numerically, the group

treated with medium-chain fatty acids showed a lower incidence of ketosis, even though the blood BHB concentration did not show differences between the groups. On the other hand, the incidence of hypocalcemia was higher in the MCFA-treated group compared to the control group, which is consistent with the lower total calcium levels observed on day 0, indicating hypocalcemia. Furthermore, there was a higher incidence of mastitis in the group treated with MCFA compared to the control group, consistent with the findings of higher somatic cell counts in the milk of this group. These results are inconclusive but suggest that MCFA treatment did not provide expected immunopathological support to the treated animals.

Thus, the study showed that supplementation with medium-chain fatty acids (MCFA) did not significantly alter rumination, milk production, or milk composition in treated cows. Furthermore, although greater expression of the PTX3 and GPx1 genes was observed in cows treated with MCFA, the effects on immune function and disease incidence were not conclusive. Although the results indicate that MCFA supplementation may have some impacts on cow health, more research is needed to clarify these effects and determine the practical implications for dairy production.

5 Conclusion

The inclusion of 0.065% MCFA in the diet of cows during the transition period did not significantly impact production performance but caused subtle changes in milk composition, milk fatty acid profile, blood metabolites, and expression of genes related to inflammation and oxidative stress. A discrete effect was observed on the PTX3, GPX1, and NF-κB1 genes, suggesting a positive modulation of immunity in cows treated with MCFA. However, these changes do not appear to significantly influence production, health, or metabolic disorders. We believe that the very low dose used in this study may have limited the scope of the desired effects. More research is needed to investigate the potential of MCFA on the health and production of cows in the transition period.

6 Study limitations and future perspectives

The study was conducted on a commercial farm, and because of that, it was not possible to measure the individual dry matter intake of the animals based on the

management adopted on the farm. It is important to note that dry matter intake is significantly influenced by MCFA supplementation, due to changes in the rumen microbiota that can affect intake and digestibility. Furthermore, the collection of milk samples for physicochemical composition analyzes was limited for the same reasons.

The dose of MCFA we used was relatively low, which may explain the lack of significant results in terms of productive variables. It is important to highlight that there is a gap in knowledge regarding the ideal dose of MCFA supplementation, and the results of previous studies vary. Therefore, it would be beneficial to conduct additional research that explores different doses, including a dose-response analysis, to better understand the metabolic and productive effects of MCFAs.

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8 Tables and figures

Table 1: Composition of the diet formulated for pre- and postpartum cows.

	Prepartum		Postpartum	
Ingredients, % DM	**Control**	**MCFA**	**Control**	**MCFA**
Corn silage	77.44	77.44	34.53	34.53
Oat haylage	-	-	1.22	1.22
Ryegrass haylage	-	-	3.05	3.05
Wheat straw	4.95	4.95	-	-
Ground corn	-	-	21.25	21.25
Soybean meal	14.59	14.59	13.54	13.54
Soybean hulls	-	-	8.16	8.16
Cottonseed	-	-	7.56	7.56
Barley	-	-	7.71	7.71
Water	-	-	0.001	0.001
Prepartum premix[1]	2.04	2.04	-	-
Lactation premix[2]	-	-	2.44	2.44
Ground corn carrier[3]/g	0.150	0.100	0.150	0.100
Lipid supplement[4]/g	-	0.050	-	0.050

[1]Prepartum premix composition: Ca (min) 106.00 g/kg, Ca (max) 130.00 g/kg, P 30.00 g/kg, S 90.00 g/kg, Mg 20.00 g/kg, Na 31.00 g/kg, Cl 130.00 g/kg, Co 12.00 g/kg, Cu 600.00 mg/kg, Cr 30.00 mg/kg, Fe 600.00 mg/kg, I 60.00 mg/kg, Mn 1600.00 mg/kg, Se 16.00 mg/kg, Zn 2400.00 mg/kg, Vitamin A 480000.00 IU/kg, Vitamin D3 200000.00 IU/kg, Vitamin E 12000.00 IU/kg, Biotin 80.00 mg/kg, Saccharomyces cerevisiae 1.5x10^9 CFU/kg, Monensin 500.00 mg/kg, F 300.00 mg/kg. [2]Lactation premix composition: Biotin 64 mg/kg, Ca (min) 140 g/kg, Ca (max) 110 g/kg, Co 11 mg/kg, Cu 400 mg/kg, Cr 16 mg/kg, S 4000 mg/kg, P 16 g/kg, I 16 mg/kg, Mg 56 g/kg, Mn 1120 mg/kg, Methionine 14 mg/kg, Monensin sodium 500 mg/kg, K 320 mg/kg, Se 14 mg/kg, Na 115 g/kg, Vitamin A 160 IU/kg, Vitamin D3 57.6 IU/kg, Vitamin E 960 IU/kg, Zn 2.24 mg/kg. [3]Corn used as a diluent of the lipid supplement: Control 150g and MCFA 100g. [4]Lipid supplement composition: 20.4% capric acid, 30.6% caprylic acid, 48.9% lauric acid, and carrier ingredients 63% extruded corn, 11.5% silicon dioxide, and 0.5% flavoring additive. A total of 12.25 g of pure MCFA was provided per animal per day.

Table 2: Chemical analysis of the nutrition composition of forages and TMR samples.

	Prepartum		Postpartum	
Nutritional composition, % DM	**Control**	**MCFA**	**Control**	**MCFA**
DM	51.00	50.93	49.30	49.26
NDF	34.40	34.47	34.08	34.12
ADF	18.38	18.44	17.70	17.73
Lignin	2.59	2.60	2.80	2.81
CP	11.99	12.00	15.56	15.57
TDN	70.94	70.88	68.75	68.72
EE	3.01	3.01	4.54	4.55
Net Energy	1.63	1.63	1.51	1.51
Digestible Energy	3.13	3.13	3.06	3.06
NFC	48.48	48.39	43.29	43.23
Starch	26.50	26.36	27.51	27.44
Ash	3.30	3.31	4.09	4.10
Ca	2.33	2.34	3.63	3.64
P	0.76	0.76	0.64	0.64
K	0.69	0.69	8.51	8.53

DM: Dry matte; NDF: neutral detergent fiber; ADF: fiber in acid detergent; CP: crude protein; TDN: Total digestible nitrogen; EE: ether extract; NFC: Non-fiber carbohydrate.

Table 3: Genes analyzed, primer sequence, and GenBank identification code.

Gene	Direction	Primer sequence (5' to 3')	GenBank code	Fragment (pb)[1]
Housekeeping				
GAPDH	Forward	GGTGATGCTGGTGCTGAG-3'	AJ431207	181
	Reverse	TGACAATCTTGAGGGTGTTG		
YWHAZ	Forward	AGGCTGAGCGATATGATGAC	NM_174814	140
	Reverse	GACCCTCCAAGATGACCTAC		
HPRT1	Forward	TGCTGAGGATTTGGAGAAGG	NM_001034035	154
	Reverse	CAACAGGTCGGCAAAGAACT		
UBC	Forward	ATGCAGATCTTTGTGAAGAC	NM_001206307	189
	Reverse	CTTCTGGATGTTGTAGTC		
Cytokines				
IL-6	Forward	TGCTGGTCTTCTGGAGTATC	NM_173923	153
	Reverse	GTGGCTGGAGTGGTTATTAG		
TNF-α	Forward	TCTTCTCAAGCCTCAAGTAACAAGC	EU276079	103
	Reverse	CCATGAGGGCATTGGCATAC		
Antimicrobials				
PTX3	Forward	TATGCCATGGTGCTTTCAGA	NM_001076259	182
	Reverse	CCAATGAACAATGGACAACAA		

SAA3	Forward	CTCAAGGAAGCTGGTCAAGG	NM_181016	240
	Reverse	CTTCGAATCCTCCCGTACCT		
Oxidative stress				
SOD1	Forward	TGTTGCCATCGTGGATATTGTAG	NM_174615	102
	Reverse	CCCAAGTCATCTGGTTTTTCATG		
CAT	Forward	GCTCCAAATTACTACCCCAATAGC	NM_001035386	104
	Reverse	GCACTGTTGAAGCGCTGTACA		
GPX1	Forward	GCAAGGTGCTGCTCATTGAG	NM_174076	82
	Reverse	CGCTGCAGGTCATTCATCTG		
GPx3	Forward	CTAGCCACCCTCAAGTATGTTCG	XM_005683183	76
	Reverse	TCACATCGCCTTTCTCAAACAGT		
CASPASE 8	Forward	GTGGAGATGGAGAAGAGGA	NM_001045970	193
	Reverse	CTGGAAGCGATTGTGACA		
Inflammasome				
IL-18	Forward	ACTGTTCAGATAATGCACCCCAG	NM_174091	100
	Reverse	TTCTTACACTGCACAGAGATGGTTAC		
NLRP3	Forward	CTTTCTGGACTCTGACCGGG	NM_001102219	312
	Reverse	CTCCCATTCTGGCTCTTCCC		
Receptors				
CD14	Forward	GTAAATGACCTGACTCTGGACGG	NM_174008	195
	Reverse	ATTCCTCTTCCCTCTCTTCCC		

TLR4	Forward	GACCCTTGCGTACAGGTTGT	NM_174198	103
	Reverse	GGTCCAGCATCTTGGTTGAT		
Transcription factors				
NFKB1	Forward	CTCAAAGCAGCAGGAGCAGA	NM_001076409	102
	Reverse	CGGTACGACCCCTTCATCC		
MyD88	Forward	ACTATCGGCTGAAGTTGTGC	NM_001014382	139
	Reverse	TCCTTGCTTTGCAGGTATTC		
STAT1	Forward	TATCCAGAGCACTGTAATGT	NM_001077900	285
	Reverse	GTGGCATTCAACAACTCTAT		
STAT5	Forward	CGCAGCTCCAGAACACGTAC	NM_001012673	187
	Reverse	ACCAGTCGCAGTTCCTCAAA		
FOXP3	Forward	AAGAGCCCAGGGACAACTTTC	NM_001045933	74
	Reverse	GGGTTCAAGGAGGAAGAGGAA		
Glucose metabolism				
LPK	Forward	AGACTCAACTTCTCCCACGG	NM_001076176	286
	Reverse	TGACTCGGACGATATTGGGG		
G6PD	Forward	CAACCAGCTGTCCAACCACAT	NM_001244135	100
	Reverse	CACCATGAGGTTCTGGACCAT		
Fat metabolism				
ACACA	Forward	TGGTCTGGCCTTACACATGA	NM_174224	112
	Reverse	TGCTGGAGAGGCTACAGTGA		

FASN	Forward	CTGAGTCGGAGAACCTGGAG	NM_001012669	156
	Reverse	CGAAGAAGGAAGCGTCAAAC		
LPL	Forward	GAGCCAAAAGAAGCAGCAAG	NM_001075120	182
	Reverse	AGGCAGGGTAAAAGGGATGT		
SCD	Forward	ACAATTCCCGACGTGGCTT	NM_173959	254
	Reverse	GGCATAACGGAATAAGGTGGC		
SREBF1	Forward	ACCGCTCTTCCATCAATGAC	NM_001113302	120
	Reverse	GCTGAAGGAAGCGGATGTAG		

[1]DNA base pair fragment. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta, HPRT1: Hypoxanthine phosphoribosyltransferase 1, UBC: Ubiquitin C, IL-6: Interleukin 6, TNF-α: Tumor necrosis factor alpha, PTX3: Pentraxin 3, SAA3: Serum amyloid A3, SOD1: Superoxide dismutase 1, CAT: Catalase, GPX1: Glutathione peroxidase 1, GPx3: Glutathione peroxidase 3, CASPASE 8: Caspase 8, IL-18: Interleukin 18, NLRP3: NLR family pyrin domain containing 3, CD14: Cluster of differentiation 14, TLR4: Toll-like receptor 4, NFKB1: Nuclear factor kappa B subunit 1, MyD88: Myeloid differentiation primary response 88, STAT1: Signal transducer and activator of transcription 1, STAT5: Signal transducer and activator of transcription 5, FOXP3: Forkhead box P3, LPK: Liver pyruvate kinase, G6PD: Glucose-6-phosphate dehydrogenase, ACACA: Acetyl-CoA carboxylase alpha, FASN: Fatty acid synthase, LPL: Lipoprotein lipase, SCD: Stearoyl-CoA desaturase, SREBF1: Sterol regulatory element-binding transcription factor 1.

Table 4: Milk production and milk composition of cows supplemented with MCFA.

				P value	
	Control[1]	**MCFA[2]**	**SEM**	**Treatment**	**Treat. x Parity**
Milk yield, kg/d					
d14	31.79	32.26	0.65	0.61	0.47
d30	37.11	37.40	0.67	0.76	0.44
d60	40.76	40.91	0.71	0.88	0.97
d100	43.07	43.06	0.72	0.99	0.94
Milk composition[3]					
Fat, kg/d	1.79	1.58	0.09	0.13	0.33
Fat, %	5.23	5.00	0.18	0.33	0.99
Protein, kg/d	1.20	1.27	0.05	0.67	0.08
Protein, %	3.54	3.50	0.04	0.79	0.03
Lactose, kg/d	1.56	1.55	0.06	0.86	0.26
Lactose, %	4.60	4.64	0.02	0.27	0.36
Total solids, kg/d	4.93	4.78	0.22	0.03	0.40
Total solids, %	14.51	14.36	0.20	0.96	0.03
SCC	2.02	3.01	0.23	0.07	0.51
ECM	41.72	37.23	1.80	0.08	0.12
FCM, 4%	42.93	37.74	1.90	0.07	0.17

[1]Control group received 150 g/d corn meal. [2]Treatment group received 50 g/d MCFA + 100 g/d corn meal (Aromabiotic Cattle, Agrifirm). [3]Milk composition (g/kg) was calculated based on milk production on d14. *P* value was considered significant when $P < 0.05$.

Table 5: Fatty acids profile of milk from cows supplemented with MCFA.

Fatty acid, g/100	Control[1]	MCFA[2]	SEM	*P* value
C4:0	3.22	3.25	0.19	0.77
C6:0	1.96	1.93	0.1	0.74
C8:0	1.14	1.11	0.06	0.58
C10:0	2.58	2.45	0.56	0.49
C10:1	0.15	0.14	0.003	0.80
C11:0	0.05	0.04	0.005	0.06
C12:0	0.02	0.02	0.002	0.44
C12:1	0.03	0.03	0.002	0.59
C13:0	0.09	0.07	0.001	0.72
C14:0	9.35	9.22	3.57	0.78
C14:1 *cis*-9	0.54	0.49	0.02	0.18
C15:0	0.73	0.64	0.02	0.04
C16:0	28.58	28.42	11.75	0.86
C16:1 *cis*-9	1.68	1.59	0.15	0.42
C17:0	0.66	0.63	0.007	0.18
C17:1	0.27	0.26	0.006	0.51
C18:0	13.35	14.18	4.8	0.14
C18:1 *trans*	2.22	1.97	0.18	0.03
C18:1 *cis*-9	24.12	24.58	17.38	0.67
C18:1 *cis*-11	0.88	0.84	0.03	0.38
C18:1 *cis*-12	0.34	0.34	0.004	0.70
C18:1 *cis*-13	0.11	0.11	0.001	0.65
C18:1 *trans*-16	0.27	0.27	0.003	0.62
C18:1 *cis*-15	0.1	0.1	0.004	0.69
C18:2 *cis*-9, *cis*-12	2.38	2.28	0.09	0.17
C20:0	0.1	0.11	0.003	0.25
C18:3 n-6	0.03	0.04	0.002	0.07
C18:3 n-3	0.22	0.21	0.001	0.17
C20:1	0.01	0.02	0.008	0.47
C18:2 *cis*-9, *trans*-11	0.25	0.22	0.003	0.02
C18:2 *trans*-10, *cis*-12	-	-	-	-
C22:0	0.08	0.08	0.001	0.89
C20:4 n-6	0.21	0.21	0.004	0.88
C20:5 n-3	0.01	0.01	0.003	0.96
FA class, %				
De novo FA[3]	21.71	21.2	15.23	0.62
Mixed FA[3]	30.25	30.02	12	0.79
Preformed FA[3]	44.84	45.68	33.53	0.57

Odd-chain and branched-chain FA[4]	3.02	2.87	0.09	0.06
Satured FA[4]	65.87	65.87	23.98	0.93
Monounsaturated FA[4]	30.75	30.75	22.98	0.99
Polyunsaturated FA[4]	3.20	3.04	0.12	0.07
Unsaturated FA[4]	33.95	33.79	23.76	0.9

[1]Control group received 150 g/d corn meal. [2]Treatment group received 50 g/d MCFA + 100 g/d corn meal (Aromabiotic Cattle, Agrifirm). *P* value was considered significant when $P < 0.05$. [3]FA 16 C from extraction from plasma, and 16 C FA originate from both sources (mixed). [4]Sum of all odd- and branched-chain fatty acids; Saturated fatty acids; Monounsaturated fatty acids; Polyunsaturated fatty acids; Unsaturated fatty acids.

Table 6: Metabolites in blood plasma from cows supplemented with MCFA.

				P value		
Metabolites	**Control[1]**	**MCFA[2]**	**SEM**	**Treat**	**Treat x Day**	**Treat x Parity[3]**
GGT, U/L[4]	27.89	26.92	0.26	0.09	0.29	0.23
AST/TGO, U/L[5]	78.88	77.24	1.37	0.39	0.37	0.85
Bilirrubin, mg/dL	0.16	0.15	0.02	0.97	0.44	0.54
Albumin, g/dL	3.34	3.33	0.01	0.82	0.47	0.35
Cholesterol, mg/dL	79.02	79.8	1.4	0.68	0.22	0.43
Glucose, mg/dL	64.60	64.9	0.6	0.69	0.05	0.16
NEFA, mg/dL[6]	1.16	1.09	0.01	0.95	0.95	0.92
BHB, mmol/L[7]	1.06	1.08	0.02	0.62	0.19	0.40
Total Ca, mg/dL	7.75	7.74	0.05	0.96	0.08	0.52
Ionic Ca, mmol/dL	1.08	1.09	0.01	0.43	-	0.25

[1]Control group received 150 g/d corn meal. [2]Treatment group received 50 g/d MCFA + 100 g/d corn meal (Aromabiotic Cattle, Agrifirm). [3]Treatment *P* value x cow parity. [4]GGT: Gamma-glutamyl transferase. [5]AST/TGO: Aspartate aminotransferase/ glutamic-oxalacetic transaminase. [6]NEFA: Nonesterified fatty acids. [7]BHB: beta-hydroxybutyrate. *P* value was considered significant when $P < 0.05$.

Table 7: Body weight and body condition score of cows that participated in the study.

	Treatment			
	Control[1]	**MCFA[2]**	**SEM**	***P* value**
BW, kg[3]				
Prepartum	696.19	697.47	6.07	0.58
Calving	704.99	708.39	6.19	0.62
Postpartum	675.06	683.41	6.22	0.23
Difference	21.13	14.06	-	-
BCS, 1-5[4]				
Prepartum	3.10	3.10	0.02	0.85
Calving	2.96	2.97	0.02	0.68
Postpartum	2.97	2.96	0.02	0.73

[1]Control group received 150 g/d corn meal. [2]Treatment group received 50 g/d MCFA + 100 g/d corn meal (Aromabiotic Cattle, Agrifirm). [3]Body weight. [4]Body condition score. *Pre-calving BW was collected on d -21, calving on d0, and post-partum on d14. *P* value was considered significant when $P < 0.05$.

Table 8: Incidence of diseases during the experimental period.

Incidence, %[1]	Control[2]	MCFA[3]
Subclinical ketosis	54.76 (n = 46)	53.57 (n = 45)
Subclinical hypocalcemia	28.57 (n = 24)	34.52 (n = 29)
Retained placenta	2.38 (n = 2)	3.57 (n = 3)
Metritis	4.76 (n = 4)	3.57 (n = 3)
Dystocia	17.85 (n = 15)	15.47 (n = 13)
Displaced abomasum	3.57 (n = 3)	4.76 (n = 4)
Clinical mastitis	2.38 (n = 2)	5.95 (n = 5)
Hoof problems	8.33 (n = 7)	4.76 (n = 4)

[1]Incidence of diseases or metabolic problems affected in cows during the transition period. [2]Control group received 150 g/d corn meal. [3]Treatment group received 50 g/d MCFA + 100 g/d corn meal (Aromabiotic Cattle, Agrifirm).

Figures

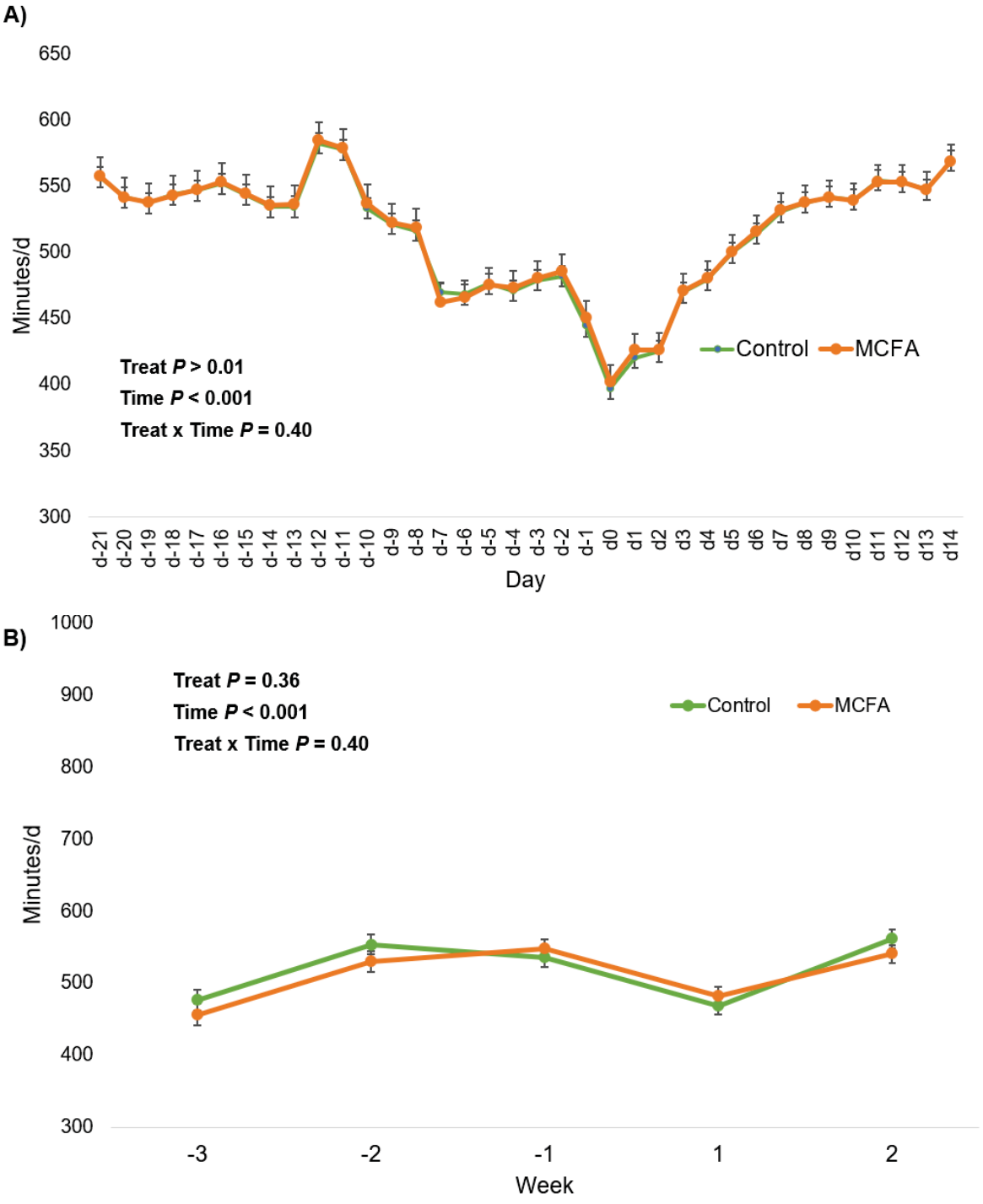
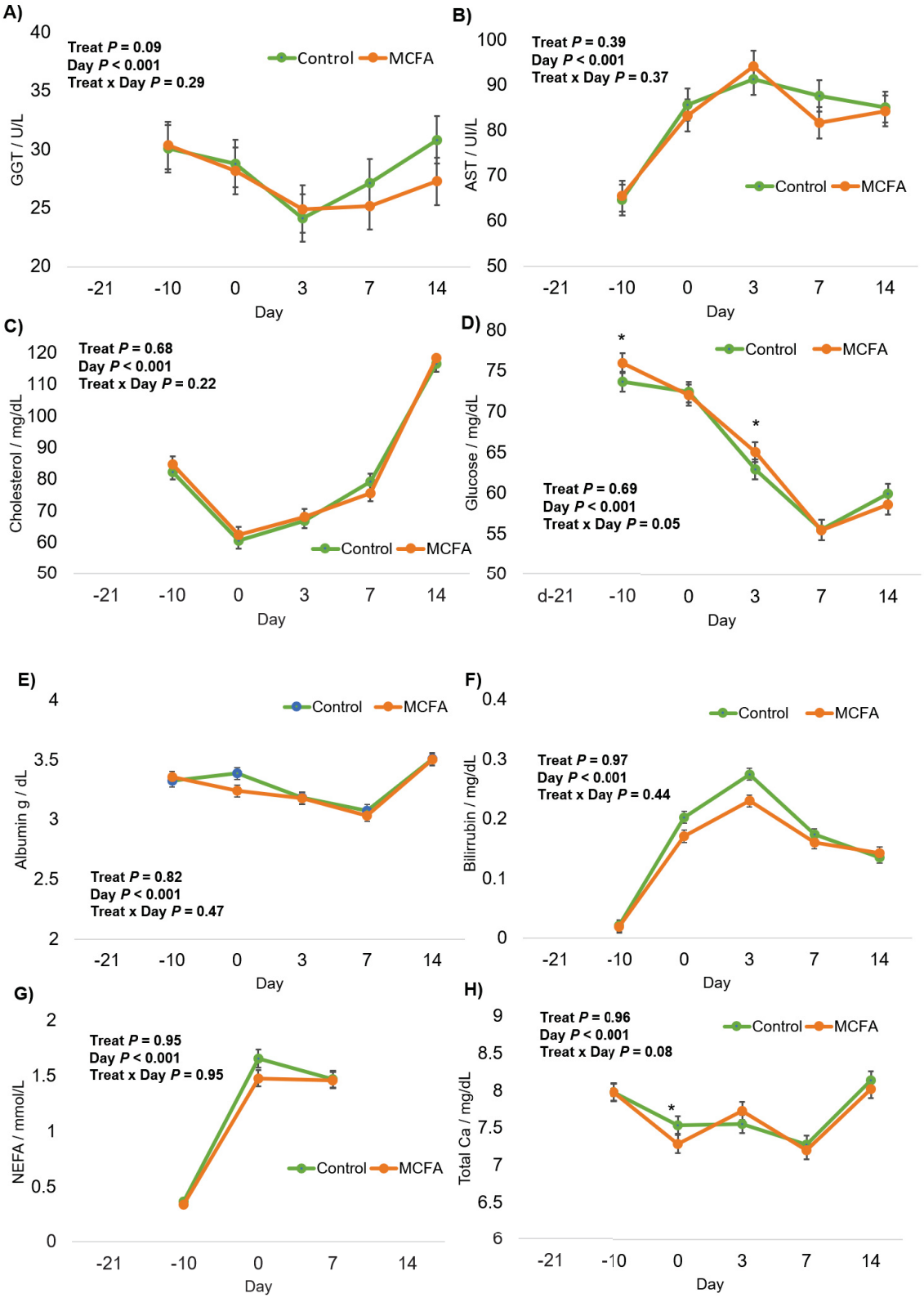


Figure 1: Rumination of cows in the transition period during the experimental period. Control group received 150 g/d corn meal. Treatment group received 50 g/d MCFA + 100 g/d corn meal (Aromabiotic Cattle, Agrifirm). A) Rumination measured from day -21 to 14. B) Rumination measured from week -3 to 2. *P* value was considered significant when $P < 0.05$.


A)
Treat P = 0.09
Day P < 0.001
Treat x Day P = 0.29
GGT / U/L
Control
MCFA
Day
B)
Treat P = 0.39
Day P < 0.001
Treat x Day P = 0.37
AST / UI/L
C)
Treat P = 0.68
Day P < 0.001
Treat x Day P = 0.22
Cholesterol / mg/dL
D)
Treat P = 0.69
Day P < 0.001
Treat x Day P = 0.05
Glucose / mg/dL
E)
Treat P = 0.82
Day P < 0.001
Treat x Day P = 0.47
Albumin g / dL
F)
Treat P = 0.97
Day P < 0.001
Treat x Day P = 0.44
Bilirrubin / mg/dL
G)
Treat P = 0.95
Day P < 0.001
Treat x Day P = 0.95
NEFA / mmol/L
H)
Treat P = 0.96
Day P < 0.001
Treat x Day P = 0.08
Total Ca / mg/dL

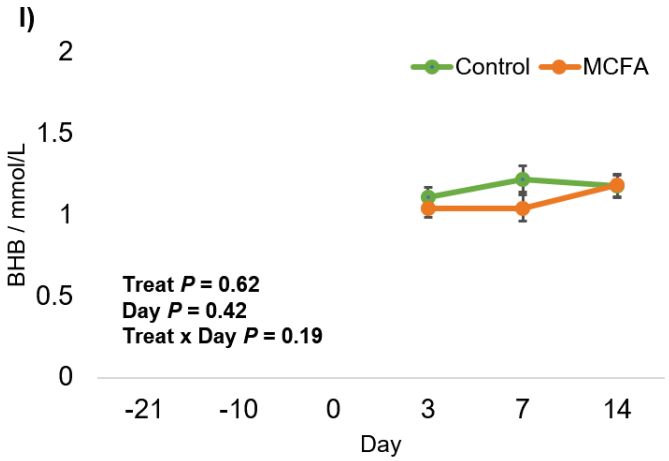


Figure 2: Blood metabolites expressed per day.
Control group received 150 g/d corn meal. Treatment group received 50 g/d MCFA + 100 g/d corn meal (Aromabiotic Cattle, Agrifirm). A) GGT: Gamma-glutamyl transferase. B) AST/TGO: Aspartate aminotransferase/ glutamic-oxalacetic transaminase. C) Cholesterol. D) Glucose. E) Albumin. F) Bilirrubin. G) NEFA: Nonesterified fatty acids. H) Total calcium. I) BHB: beta-hydroxybutyrate in plasma. Measured at different times *P* value was considered significant when $P < 0.05$. The * symbol demonstrates the treatment x day interaction.

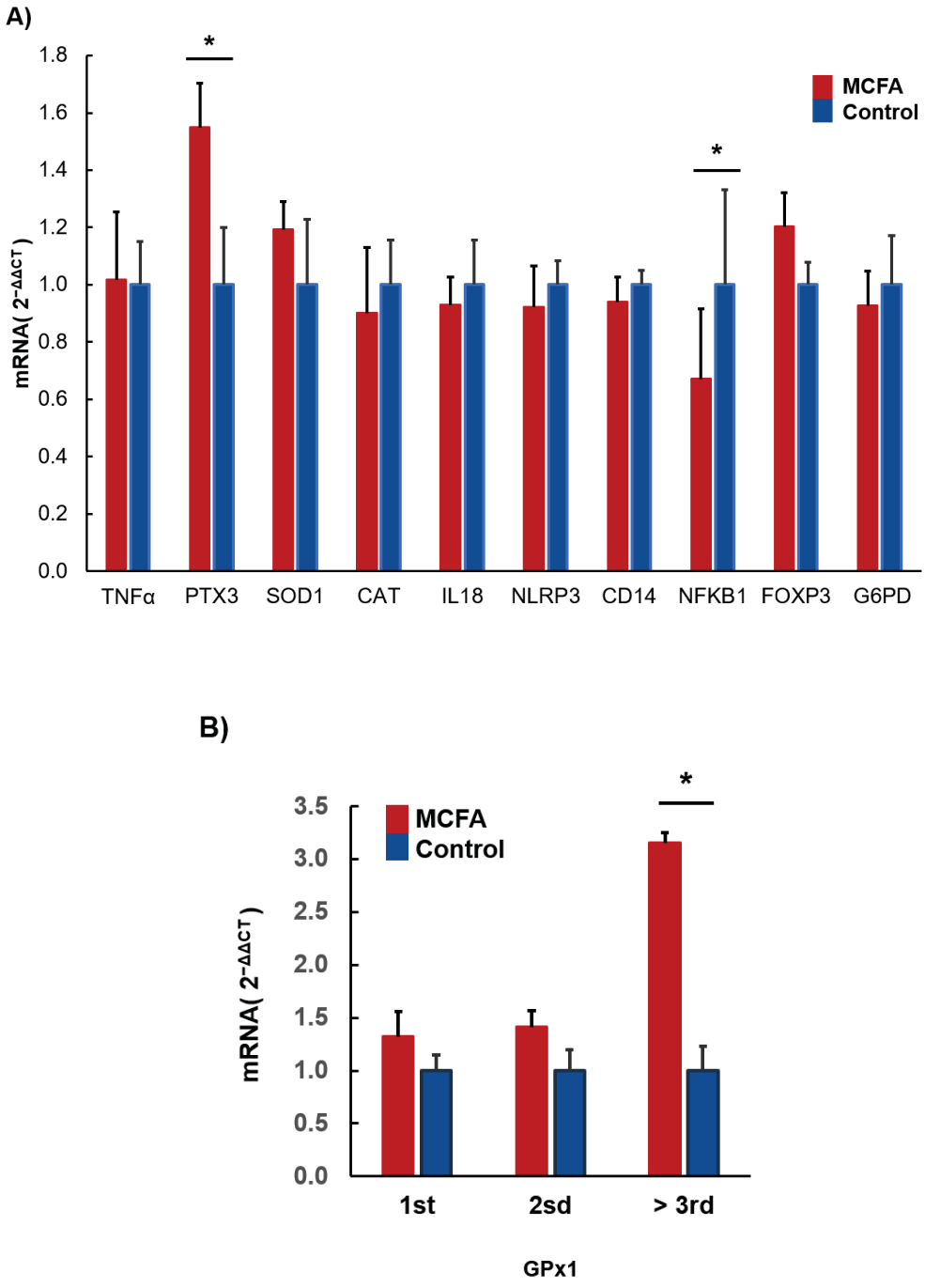


Figure 3: Gene expression of mRNA in leukocyte cells.
The genes TNFα, PTx3, SOD1, CAT, GPx1, IL18, NLRP3, CD14, NFKB1, FOXP3, G6PD in leukocyte cells of Holstein cows subjected to medium chain fatty acid (MCFA) or Control. Gene expression was quantified using the 2−ΔΔCT method, gene expression results are presented as Mean and SEM. In the columns subscripted by the symbol *, the means differ significantly $P < 0.05$. A) Effect of the treatment. B) Effect of the treatment/parity interaction.

CAPÍTULO III. INFUSÃO ABOMASAL DE ÁCIDOS GRAXOS ÔMEGA-3 E ÔMEGA-6 E SEU IMPACTO NOS MEDIADORES LIPÍDICOS PLASMÁTICOS EM VACAS LEITEIRAS SOB ESTRESSE CALÓRICO

Hipótese

Nossa hipótese é que a infusão abomasal de ácidos graxos ômega-3 terá um efeito mitigador da inflamação e dos sintomas clínicos em vacas submetidas a estresse térmico, por meio da produção aumentada de oxilipídeos com propriedades anti-inflamatórias. Por outro lado, a infusão de ácidos graxos ômega-6 pode acentuar a resposta inflamatória associada ao desafio fisiológico do estresse térmico, devido as suas características pró-inflamatórias.

Objetivo geral

Avaliar os efeitos da infusão abomasal de ácidos graxos ômega-3 e ômega-6 sobre a resposta de biomarcadores inflamatórios em vacas leiteiras submetidas ao estresse calórico.

Objetivos específicos

- Avaliar o perfil oxilipídico em vacas leiteiras expostas ao estresse calórico;
- Compreender as vias oxilipídicas na resposta inflamatória induzida pelo estresse calórico;
- Avaliar o potencial agravante da inclusão de ômega-6 nos efeitos negativos do estresse calórico;
- Avaliar a capacidade do ômega-3 em atenuar os efeitos deletérios do estresse calórico;
- Identificar mediadores lipídicos como biomarcadores potenciais para respostas inflamatórias induzidas pelo estresse térmico em estudos subsequentes.

ABOMASAL INFUSION OF OMEGA-3 AND OMEGA-6 FATTY ACIDS AND THEIR IMPACT ON PLASMA LIPID MEDIATORS IN DAIRY COWS UNDER HEAT STRESS

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Abstract

Heat stress in dairy cows has the potential to trigger inflammatory responses, which can be influenced by specific oxylipids derived from dietary lipids. Diets rich in omega-6 fatty acids are potent pro-inflammatory agents, and when combined with heat stress, they can exacerbate inflammation. Conversely, omega-3 fatty acids have anti-inflammatory properties and can be used as a therapeutic nutritional strategy during physiological challenges. This study aimed to assess the effects of abomasal infusion of n-6 and n-3 fatty acids and investigate inflammation in heat-stressed dairy cows using plasma oxylipids as biomarkers. Twelve lactating Holstein cows (38.5 ± 9.8 kg of milk/day; 85 ± 33 DIM) were randomly allocated to treatment groups in a replicated incomplete Latin square design with two 10-day periods. The treatments included: 1) Thermoneutral pair feeding + corn oil (TNPF/n6; Max THI=64), 2) Heat stress + corn oil (HS/n6; containing 55% linoleic acid; Max THI=84), and 3) Heat stress + fish oil (HS/n3; containing 8.3% EPA, 19% DHA; Max THI=84). The oils (159 g/d) were infused into the abomasum in two boluses. Blood samples were collected 4 hours after abomasal infusion on days 0, 5, and 10 for lipidomic analysis by LC-MS/MS. Data were analyzed using a mixed model, with cow and period considered random effects, and treatment, time, and their interactions as fixed effects. The fatty acids EPA, DHA, DPA, and AA, along with the oxylipids from the lipoxygenase pathway 5-HETE, 5-oxoETE, 15-HETE, 15-oxoETE, 17-HDoHe, as well as the CYP 450 oxylipids: 19,20-EpDPE, 19,20-DiHDPA, were increased in HS/n3 compared to HS/n6. Conversely, 9-oxoODE was reduced in HS/n6 compared to TNPF/n6. HS/n3

treatment led to elevated concentrations of anti-inflammatory oxylipids compared to HS/n6 ($P < 0.005$). There was no effect of treatments on the cyclooxygenase enzymatic pathway and non-enzymatic pathway. Twenty components exhibited positive correlations with milk production and composition, skin temperature, rectal temperature, inflammatory markers, and oxylipids. DHA and EPA demonstrated a negative correlation with rectal temperature, skin temperature, LBP, milk production, and milk fat. During heat stress, substrate availability, synthesis pathway, and the duration of animal exposure to stress indicate the potential for exogenous modulation of lipid mediators by omega-3. However, the optimal dose remains unknown.

Keywords: cyclooxygenase, cytochrome P450, fatty acids, inflammation, lipoxygenase, omega therapy.

1 Introduction

Heat stress (HS) in dairy cows is a subject that has been of concern to producers and scientists, as the air temperature tends to increase by up to 2°C annually, causing discomfort and production losses to dairy cattle (Herbut et al., 2018). HS reduces dry matter intake (DMI), milk production, and health impacts including reproductive delays, oxidative stress, and inflammation (Yadav et al., 2016). Furthermore, prolonged exposure to heat can cause tissue damage, triggering an inflammatory response exacerbated by the production of cytokines, reactive oxygen species (ROS), and prostaglandins (Bargath et al., 2019). Furthermore, HS also affects intestinal permeability, allowing gram-negative bacteria to enter the bloodstream, triggering a bacterial infection response, and activating the immune system (Hu et al., 2022; Chandler et al., 2023). Lipopolysaccharide (LPS) present in gram negative bacterial walls plays a crucial role in inducing inflammation by acting on the signaling pathway known as Toll-Like Receptor/NF-KB (TLR-4/NF-KB), resulting in the release of more cytokines and adhesion molecules (Calder, 2013; Chirivi et al., 2022).

The oils most commonly used for formulating diets for dairy cows are soybean oil and corn oil, both sources of omega-6 (n-6) fatty acids. Studies have shown that n-6-type polyunsaturated fatty acids (PUFA) are precursors of potent pro-inflammatory mediators,

including arachidonic acid (AA) and other lipid mediators (Innes and Calder, 2018). Excessive n-6 PUFA intake, when combined with low levels of omega-3 (n-3) PUFA intake, has been associated with elevated levels of inflammatory biomarkers such as thromboxane, leukotriene, interleukin-1 and 6 (IL-1 and IL-6), as well as C-reactive protein, leading to increased inflammation and chronic disease (Mariamenatu and Abdu, 2021).

Conversely, a higher dietary intake of PUFA n-3 appears to be beneficial to health, due to its anti-inflammatory potential (Kra et al., 2021). The evidence for this is that a higher ratio of PUFA n-3 to n-6 reduced cancer cell proliferation in patients with colorectal cancer, breast cancer, and inflammatory, autoimmune, and cardiovascular diseases (Simopoulos, 2002). Furthermore, diets enriched with fish oil and flaxseed for postpartum dairy cows have also demonstrated beneficial health outcomes, reducing plasma levels of AA and its potential pro-inflammatory mediators (Kra et al., 2021; Kra et al., 2022).

The source of n-3 and n-6 PUFA supplied to the animals directly influences the pathways and production of oxylipids (Van Winters, 2023). Oxylipids, which are oxidized products of PUFAs, have their biological action largely determined by the substrate from which they are generated and play crucial roles in the regulation of inflammation, being able to act as inflammatory or pro-inflammatory mediators (Sordillo, 2018; Mavangira and Sordillo, 2018). In general, omega-6 derivatives tend to be pro-inflammatory, while n-3 derivatives are often associated with anti-inflammatory properties (Oliveira et al., 2021). It is important to point out that the action of oxylipids is intrinsically related to how they are formed, which can occur both non-enzymatically, often mediated by free radicals and with a preference for fatty acids esterified in phospholipids, and enzymatically, involving enzymes such as cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450, being more compatible with hydrolyzed free PUFAs (Putman et al., 2022). Recent studies, such as the one conducted by Kuhn et al. (2017) identified high concentrations of oxylipids in early lactation dairy cows, a period associated with inflammation and health risks. Complementarily, in another study that evaluated cows in the dry period until subsequent lactation, changes in the oxylipid profile were observed in cows that developed some disease (Putman et al., 2022). These findings highlight the relevance of studying oxylipids, demonstrating their potent inflammatory biomarkers in plasma.

Although inflammation is a natural process in the body's defense response, its excessive or chronic occurrence can result in tissue damage, pathologies, and diseases (Bradford et al., 2015). Thus, we hypothesize that abomasal infusion of n-3 will have a reducing effect on inflammation and clinical symptoms in cows under HS, through the production of anti-inflammatory oxylipids, while n-6 may accentuate the inflammation associated with the physiological challenge of HS. The objective of this study was to evaluate the effects of abomasal infusion of n-3 and n-6 fatty acids and to investigate the plasma concentration of oxylipids and other lipid mediators in cows exposed to HS conditions.

2 Material and Methods

2.1 Animals, experimental design and treatments

The management practices used with the animals in this experiment were approved by the CRSAD animal care committee (2019-BL-386), based on the Canadian Council on Animal Care standards for the use of Farm Animals (1993).

Twelve multiparous Holstein dairy cows (38.5 ± 9.8 kg of milk/day; 84.5 ± 32.8 DIM) were used in an incomplete Latin square design replicated with two periods of 10 days. The animals were housed in a tie-stall system divided by two controlled climate chambers. Cows were randomly allocated to one of three different treatments: 1) thermoneutrality in pair feeding + corn oil (TNPF; 159 g/d corn oil; 55% 18:2 n-6; Maximum temperature and humidity index (THI) = 64), 2) Heat stress + corn oil (HS/n6; 159 g/d corn oil; THI = 84) and 3) Heat stress + fish oil (HS/n3; 159 g /d of fish oil; 8.3% 20:5 n-3, 19% 22:6 n-3; THI = 84). Corn oil was provided as a source of omega 6 fatty acids and fish oil as a source of omega 3. Thus, the dose of oils used was divided twice a day, at 8:00 and 14:30 hours, and infused into the abomasum through specific boluses. Cows in thermoneutrality were fed in pairs to eliminate the difference in the effects of DMI compared with cows in heat stress. The diets were formulated to meet the nutritional requirements of lactating cows, by the NASEM (2021). The detailed composition of the diet ingredients is shown in Table 1. Thus, animals from all treatments received water and food ad libitum.

2.2 Feeding

The cows were fed individually with a total mixed ration (TMR), for two meals a day at 09h00 and 14h00. Consequently, all leftover feed was quantified and documented before the morning meal. Samples of both TMR and forage were gathered every week and subsequently subjected to a drying process in a forced air oven set at 55°C for 96 hours. These dried samples were then finely ground to a particle size of 1 mm using a Wiley mill from Thomas Scientific. The nutritional analysis of these food samples adhered to the methodology outlined in Ruiz-Gonzalez et al. (2023). Adjustments to the dietary ingredient ratios were made every week, guided by the dry matter (DM) concentrations found in the diet components.

Typical dairy cow diets of corn silage, corn grain, and soybeans are generally rich in omega-6 fatty acids. The choice of corn oil as a comparison with fish oil was made due to its high content of 18:2 n-6, which allowed contrasting the fatty acid profile under conditions of equal heat stress (HS-n3 vs HS- n6), while n-6 was also provided in TNPF-n6 to determine the impact of THI within the same oil type.

2.3 Temperature and hyperthermia indicators

Cows were exposed to heat stress, with temperature and humidity index (THI) calculated according to Schüller et al. (2014). The THI ranged from 72.0 to 84.0 in a controlled temperature environment (29 to 39°C) and uncontrolled relative humidity (20 to 50%; Edgetech Instrument Inc.). In contrast, the TNPF group experienced thermoneutral conditions with a constant temperature of 20°C and relative humidity ranging from 55 to 64% (THI = 61.0 to 64.0).

The daily cyclic pattern of HS was kept constant throughout the experiment. During the experimental periods, cows were transferred from one chamber to another according to the treatment sequence. They underwent a 7-day acclimatization period under thermoneutral conditions (THI = 61.0 to 64.0) with light and 12-hour darkness.

Heat stress indicators were evaluated, such as respiratory rate, skin temperature, and rectal temperature. Respiratory rate was determined by visual observation of flank movements at 08:00 and 17:00 h on days 0, 2, 5, 7, and 10 of the experiment. Skin and rectal temperatures were measured daily at 08:00, 14:00, and 17:00 using an infrared

temperature gun (MiniTemp MT6, Raytek Corp.) and a portable rectal thermometer for cattle (AG-102 angular, AG-Medix). The variations and regulation of the temperature chamber and measurements of heat stress indicators are described in detail in the article by Ruiz González et al. (2023). It is important to highlight that in this study, we are analyzing the measurements recorded on days 0, 5, and 10.

2.4 Blood sampling and analysis of lipid mediators

Blood samples were collected from the coccygeal vein before feeding and 4 hours after feeding, on days 0, 5, and 10 of each experimental period. These samples were placed in vacutainer tubes containing EDTA, to avoid lipid peroxidation, a mixture consisting of methanol, ethanol, water, butylated hydroxytoluene, EDTA, triphenylphosphine and indomethacin was added to each sample (Mavangira et al., 2015). Subsequently, the samples were centrifuged at 1,500 rpm at 4°C for 20 minutes, resulting in plasma that was stored at -80°C, awaiting further analysis using liquid chromatography coupled to tandem mass spectrometry (LS-MS/MS).

Plasma processing followed the protocol described by Mavangira et al. (2015). Briefly, plasma was thawed on ice, diluted with a 4% formic acid solution, and mixed with reducing antioxidants to prevent degradation of preformed oxylipids and lipid peroxidation, as mentioned by O'Donnell et al. (2009). Then, internal standards containing 0.25 µM 5-Hydroxyeicosatetraenoic acid (5(S)-HETE-d_8), 0.25 µM 15-5-Hydroxyeicosatetraenoic acid (15(S)-HETE-d_8), 0.5 µM 8(9)-epoxyeicosatrienoic acid (8(9)-EET-d_{11}), 0.5 µM prostaglandin E2 (PGE2-d_9), and 0.25 µM 8,9-Dihydroxyeicosatrienoic acid (8,9-DHET-d_{11}) to samples. Lipid extraction in the solid phase occurred by loading the samples into columns and applying nitrogen; the columns were washed with 3 mL of 5% methanol. Sample elution was performed with 2.5 mL of acetonitrile, followed by the removal of volatile solvents using a SpeedVac Savant (Thermo Fisher Scientific). The final residues were reconstituted in methanol and stored in specific chromatography bottles at -80°C, as described by Putman et al. (2022).

The quantification of oxylipids was performed with a standard curve established through internal standards analyzed by LC-MS/MS on the Xevo-TQ-S system. Liquid chromatography used an Ascentis Express HPLC C18 column at 50°C with a mobile

phase A of water with 0.1% acetic acid and a mobile phase B of acetonitrile, with a specific linear gradient. Data were analyzed with linear curves using standards in five-fold dilutions, ranging from 0.01 to 100 nM. IsoP quantification was performed with a Waters at 50°C. The mobile phases were composed of acetic acid, acetonitrile and methanol. The elution gradient was specific for each mobile phase. Detection was done in negative ion mode via electrospray ionization. Data were analyzed with the Waters MassLynx software, version 4.1, following previous protocols (Mavangira et al., 2015; Putman et al., 2022). The method allowed the detection of 31 compounds, including fatty acids and enzymatic and non-enzymatic oxylipids in this study (Table 1).

2.5 Other measures

The production and metabolic results were previously documented by Ruiz-González et al. (unpublished), providing more comprehensive details. A database with measurements of respiratory rate (RR), rectal temperature, skin temperature, dry matter intake (DMI), milk production, milk composition, bovine lipopolysaccharide (LBP), non-esterified fatty acids (NEFA), glucose and three insulin resistance assessment indexes: quantitative insulin sensitivity check index (QUIKI), revised quantitative insulin sensitivity check index (RQUIKI), and homeostasis model assessment of insulin resistance (HOMA-IR) were correlated with the oxylipid profile identified in the present study using the MetaboAnalyst software.

However, abomasal infusions of omega-3 and omega-6 have been conducted to prevent the harmful effects of PUFA on rumen bacteria, including the toxic effects of PUFA on these bacteria and milk fatty depression (MFD; Maia et al., 2007; 2010). Furthermore, the level of stress adopted in this study was similar to the study by Ruiz-González et al. (2023). This study used a protocol that simulates diurnal variation in THI and can induce a full range of heat stress effects, including production losses and increased inflammation. The 10-day protocol duration was chosen based on the observation of Ruiz-González et al. (unpublished), who reported steady-state conditions in heat stress responses after 7 days of induction. A similar heat stress phenotype was expected to be achieved here.

2.6 Statistical analysis

Oxylipids were analyzed using the MIXED procedure from JMP: Statistical Software (Statistical DiscoveryTM, from SAS). Data were analyzed as repeated measures over time, considering sequence, period, and cow as a random effect, and day 0 (covariate), treatment, time, and their interactions as fixed effects. Thus, the analysis considered time as a repeated variable and the cow per treatment as the subject. Day 0 of each experimental period was considered the covariate for the remaining days: 5 and 10. Preplanned contrasts compared the effect of TNPF/n6 vs. HS/n6 and HS/n6 vs. HS/n3, with 12 observations for each variable and period. The data were also normalized and the mean was calculated using the Tukey test. The results were considered significant with $P < 0.05$ for treatment effect and time, and a tendency between $P > 0.05$ and 0.10. For interactions, $P < 0.05$ was considered significant, and $P > 0.05$ and < 0.15 for interactions: treatment x time. The preplanned contrasts were considered significant at $P < 0.05$.

Oxylipids were associated with measurements from the database mentioned above, published by Ruiz-González et al. (unpublished), using the MetaboAnalyst 5.0 platform of the R package (Xia et al., 2009). Data from MetaboAnalyst 5.0 were subjected to normalization using the sum method, generalized log-transformed, and Pareto-scaled. For statistical analysis, a combined approach was used, involving multivariate analysis, analysis of variance (ANOVA), partial least squares discriminant (PLS-DA), and Pearson's correlation coefficient. Additionally, for visualization, heatmaps were generated that highlight the magnitude of changes in relative abundance using a color gradient, as well as the correlation of 25 principal components with the variable of interest. Initially, a two-factor metabolomic analysis (Treatment and Time) was conducted but revealed no significant interactions. As a result, a separate one-factor analysis considering only the Treatment or Time factor was performed, with the significance statement established based on the false discovery rate (FDR) at P values less than 0.05.

3 Results

Thirty-one lipid compounds were identified, including fatty acids and oxylipids from the 3 oxylipid pathways: LOX, COX, and CPY450. Of the compounds identified, 20 were

affected by treatments, and 11 were not significant. The results of all identified compounds are presented in detail in Table 2. The production and metabolic variables associated with the results of lipid mediators are in the article by Ruiz-González et al. (unpublished).

3.1 Complementary results

Summary of production and metabolism results from the study by Ruiz-González et al. (unpublished; $P < 0.001$), which were used in the multivariate analyses of the current study. Maximum rectal temperature was recorded at 5h00, with an increase of 2.7°C in cows subjected to HS/n6 compared to TNPF. However, HS/n3 reduced rectal temperature by 0.9°C compared to cows in the HS/n6 group. Respiratory rate increased 3.5-fold in the HS/n6 group compared to TNPF but decreased by 16% in the HS/n3 group. Dry matter intake decreased progressively in all groups before stabilizing on day 5, with a 36% reduction.

Milk production also decreased progressively in all treatments, being 17% lower in the HS/n6 group compared to TNPF. However, HS/n3 increased milk production by 9% compared to HS/n6. Milk fat and protein yields were 25% and 30% lower in the HS/n6 group compared to TNPF, respectively, but there were no differences between HS/n6 and HS/n3. Lactose production decreased by 20% in the HS/n6 group compared to TNPF, while HS/n3 increased lactose production by 12% compared to HS/n6.

Pre- and postprandial insulin plasma concentrations increased in the HS/n6 group compared to TNPF, while decreased in the HS/n3 group compared to HS/n6. Pre-prandial NEFA plasma concentration was significantly higher in TNPF but decreased in the HS/n3 group compared to HS/n6 on the same days. There was a significant increase in plasma LBP concentrations in the HS/n6 group compared to TNPF, but a reduction in the HS/n3 group compared to HS/n6. Insulin sensitivity indices QUIKI pre- and postprandial, RQUIKI, and HOMA-IR were significantly affected by the different treatments. Cows subjected to HS showed decreased insulin sensitivity and increased HOMA-IR indices compared to TNPF. The HS/n3 tended to attenuate these adverse effects of heat stress, resulting in improvements in insulin sensitivity indices and a decrease in HOMA-IR indices compared to the HS/n6 group.

3.2 Fatty acids

The fatty acids identified in the blood plasma, both linolenic acid and linoleic acid did not show significant differences between treatments (P > 0.22) but exhibited variation over time (*P* = 0.001; Figure 1A and B).

The omega-3 fatty acids eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA) showed differences between treatments (*P* < 0.05), time (*P* < 0.03), and the interaction between treatment and time (*P* < 0.09; Figure 1C, D and E). EPA concentrations showed a gradual increase over the days in HS/n3 compared with HS/n6, 3.04 times on day 5 and 7.14 times on day 10. The DHA followed a similar pattern, with a 4.29-fold increase on day 5 and an 8.48-fold increase on day 10 in the HS/n3 treatment compared with HS/n6. The DPA revealed differences only on day 10, with a concentration 2.35-fold higher in the HS/n3 treatment compared with HS/n6. Arachidonic acid showed a 1.98-fold increase in the HS/n3 treatment compared with HS/n6 on day 10 (Figure 1F).

3.3 Oxylipids

There was a treatment x time interaction for oxylipids from the LOX pathway, 5-Hydroxyeicosatetraenoic acid (5-HETE) and 5-Oxoeicosatetraenoic acid (5-oxoETE) on day 10. Notably, the HS/n3 treatment showed concentrations 2.46-fold higher than HS/n6 for 5-HETE (*P* = 0.05; Figure 2A), while for 5-oxoETE, this relationship was 3.12-fold higher (*P* = 0.03; Figure 2B).

Furthermore, 9-Hydroxyoctadecadienoic acid (9-HODE) showed an effect for treatment (*P* = 0.03) and time (*P* < 0.001). It was observed that animals subjected to TNPF/n6 and HS/n6 without differences, indicating that omega-6 increased its concentration similarly. However, compared with HS/n6, HS/n3 had a 1.18-fold lower concentration (Figure 2C). A similar behavior was observed for 9-Oxooctadecadienoic acid (9-oxoODE), derived from 9-HODE, except for the fact that the latter showed significance for treatment, time, and the interaction between them (Figure 2D). Notably, on day 10, 9-oxoODE concentration reduced by 1.97-fold in HS/n6 compared with TNPF/n6 (*P* = 0.03), without effect for HS/n6 vs. HS/n3.

The lipid mediator 13-Hydroxyoctadecadienoic acid (13-HODE) showed a difference for treatment (P = 0.03) and day (P = 0.01). The TNPF/n6 did not differ from the HS/n6, however, the HS/n6 was 1.38-fold higher than the HS/n3 (Figure 2E). Furthermore, on day 10, a change of 1.23-fold lower was observed in HS/n6 compared with TNPF/n6 (Figure 2E). The 13-Oxooctadecadienoic acid (13-oxoODE) showed a tendency for treatment (P = 0.07), with a difference between TNPF/n6 and HS/n3, a comparison that is not relevant to this study (Figure 2F).

Furthermore, 15-Hydroxyeicosatetraenoic acid (15-HETE) demonstrated an effect for treatment and time on days 5 and 10. On day 5, HS/n3 treatment was 1.80-fold greater than HS/n6 (P = 0.003), while on day 10 this relationship was 3.18-fold higher (P = 0.003; Figure 2G). Its oxylipid derivative, 5-Oxoeicosatetraenoic acid (5-oxoETE; Figure 2H), showed an effect for treatment only on day 10, with a 3.65-fold higher concentration in HS/n3 compared with HS/n6. However, in Table 2, it was observed that the relationship between these oxylipids, substrates, and derivatives was not significant: 5-HETE:5-oxoETE (P = 0.64), 9-HODE:9oxoODE (P = 0.23), 13-HODE:13oxoODE (P = 0.66), and 15-HETE:15-oxoETE (P = 1.47).

Finally, the lipid mediator 17-Hydroxydocosahexaenoic acid (17-HDoHe) showed differences for treatment (P < 0.001), time (P = 0.002), and the interaction between treatment and time (P < 0.001; Figure 3A). These effects were most prominent on days 5 and 10. On day 5, HS/n3 treatment was 3.52-fold greater (P < 0.001), and on day 10 it was 4.42-fold greater (P < 0.001) compared with HS/n6.

Lipid mediators derived from the CYP450 enzymatic pathway, 19,20-Epoxydocosapentaenoic acid (19,20-EpDPE) and 19,20-Dihydroxydocosapentaenoic acid (19,20-DiHDPA), also demonstrated differences for treatment (P < 0.001), time (P < 0.05) and the interaction between treatment and time (P < 0.003, Figure 3). Both compounds showed an increase in concentration in the HS/n3 treatment group compared with HS/n6 on both days 5 and 10. On day 5, a 5.43-fold increase was observed for 19,20-EpDPE and 3.72-fold for 19,20-DiHDPA (Figure 3B and C, P = 0.003, P = 0.001), while on day 10 these increases were 6.32 and 9.14-fold, respectively, when compared with the HS/n6 group (Figure 3B and C, P = 0.003, P = 0.001).

3.4 Multivariate analysis

Using the MetaboAnalyst software, an analysis of the association between productive and metabolic variables of lipid mediators was carried out, using a two-factor approach. No interactions were observed between treatments and time. Thus, the single-factor analysis made associations for treatment individually (Figure 4).

Through the application of PCA and PLS-DA analysis, the differentiation between the 3 treatment groups is evident, with a greater distinction between TNPF/n6 and HS/n3. However, the HS/n6 treatment shows overlap with both treatments (Figure 4A). The VIP (Projection Importance Value) indicates that EPA, DHA, and the lipid mediators 17-HDoHe and 19,20-DiHDPA play a role in the discrepancies observed in HS/n3, and are less expressed in HS/n6, except for 17-HDoHE which is less expressed in TNF/n6. Finally, TNPF/n6 has a greater influence on linoleic and linolenic fatty acids, pre-prandial nonesterified fatty acids (NEFA), milk production, and some oxylipids, such as 9-oxoODE, 9,10-Epoxyoctadecaenoic acid (9,10-EpOME), 13-HODE, 13-oxoODE, 9-HODE, 12,13-Epoxyoctadeenoic (12,13-EpOME) and Thromboxane B2 (TXB2; Figure 4B). The other observed variables remained neutral.

Heatmap clustering analysis reveals the 25 most significant variables for heat variation in a dataset consisting of 93 samples, of which 47 were selected by Metaboanalyst software (Figure 5A). In Figure 5B, the means of the most expressed samples in each treatment for a specific variable are presented. Thus, it is observed that the group exposed to heat stress with abomasal infusion of omega-3 (HS/n3) exhibited high concentrations of 17,18-Dihydroxyeicosatetraenoic acid (17,18-DiHETE), 17-HDoHE, 19,20-DiHDPA, EPA, and DHA, together with a more moderate response in respiratory rate (Figure 5A and B). Conversely, the expression of insulin, RQUICK, pre- and postprandial HOMA-IR, and respiratory frequency was more pronounced when cows were subjected to heat stress with omega-6 infusion (HS/n6; Figure 5A and B). Total solids, milk fat and protein, linoleic acid, 9,10-EpOME, 13-oxoODE, 12,13-EpOME, 13-HODE, and 9-HODE showed milder concentrations for HS/n6, but higher than for HS/ n3 (Figure 5A and B). These variables were also notably expressed in animals in TNPF/n6, along with pre and postprandial QUICK, linolenic acid, and 9-oxoODE (Figure 5A and B).

The DHA, EPA, LA, and AA were adopted as correlation elements. The EPA and DHA showed positive correlations with components such as DHA, EPA, 19,20-DiHDPA, 17-HDoHE, 19,20-EpDPE, and 17,18-DiHETE (Figure 6A and B). Furthermore, we observed positive correlations with approximately 20 components, associated with milk production, milk composition, skin temperature, rectal temperature, inflammatory markers, and oxylipids, more detailed in Figure 6A and B. Linoleic acid (LA) showed positive correlations with milk production, protein, fat and total milk solids, pre-prandial NEFA, pre- and post-prandial QUIKI, linolenic acid, arachidonic acid and DPA, in addition to oxylipids from the LOX and CYP450 enzymatic pathways (Figure 6C). The LA showed a negative correlation with DHA, EPA, respiratory frequency, and some oxylipids from the CYP450 pathway 19,20-DIHDPA, 15-oxoETE, and 17-HDoHE (Figure 6C). Arachidonic acid (AA) had a positive correlation with DPA, ALA, EPA, and oxylipids from the enzymatic and non-enzymatic pathways (Figure 6D). Furthermore, AA demonstrated a negative correlation with metabolic factors related to insulin resistance, RQUICK and HOMA-IR, pre and postprandial insulin, LBP, skin temperature, and respiratory rate (Figure 6D).

4 Discussion

In this study, we intended to investigate whether the infusion of omega-6 fatty acids could intensify the inflammatory response caused by heat stress, due to their pro-inflammatory characteristics. Furthermore, we wanted to determine whether omega-3 fatty acids could mitigate inflammation and adverse effects in heat stress vacations by increasing the production of oxylipids with anti-inflammatory properties. As a result, we observed a positive modulation of lipid mediators depending on the different treatments and days of analysis.

The plasma concentration of essential fatty acids plays a crucial role since these fatty acids serve as precursors for the synthesis of other lipid compounds and are well-known for their anti-inflammatory properties (Moallem et al., 2018). Omega-3 fatty acids offer health benefits and have anti-inflammatory properties, while excess omega-6 fatty acids may contribute to the development of chronic and autoimmune diseases (James et al., 2000). These polyunsaturated fatty acids are absorbed by the intestine or metabolized in the liver, transported through the bloodstream, and distributed throughout tissues,

which can be found as phospholipids, cholesterol esters, and triglycerides, or can be metabolized into bioactive species, such as oxylipids (Astarita et al., 2014).

Thus, the concentrations of linolenic and linoleic acid exhibited variations over time, with greater emphasis on the tenth day. This observation suggests a possible relationship with the progressive increase in heat stress throughout the experiment, which may deregulate metabolism and contribute to a concomitant increase in inflammation. Furthermore, it is plausible that there is an accumulation of fatty acids in the bloodstream due to the supplementation carried out during the study.

The concentrations of EPA and DHA fatty acids were higher in the HS/n3 group on days 5 and 10, while DPA and AA showed an increase only on day 10. These results corroborate previous studies that demonstrated that several polyunsaturated fatty acids (PUFAs), including linoleic acid, AA, EPA, and DHA, are important substrates for the synthesis of oxylipids (Raphael & Sordillo, 2013).

The availability of fatty acids and their uptake by the body are crucial for the production of other long-chain fatty acids and specific oxylipids. For example, reduction of linolenic acid in humans resulted in a decrease in plasma oxylipids derived from this fatty acid. While linolenic acid contributes to the linoleic acid content in the lipid membrane, it can also be utilized in de novo AA synthesis (Ramsden et al., 2012). Similarly, linolenic acid can be converted into EPA and DHA. However, in dairy cows, dietary supplementation of polyunsaturated fatty acids is more challenging due to the isomerization and biohydrogenation of these fatty acids by microorganisms in the rumen, which results in the conversion of polyunsaturated fatty acids to saturated fatty acids (Glasser et al., 2008; Jenkins et al., 2008; Ryman et al., 2017). Therefore, AA plays a fundamental role in metabolism, fueling pro-inflammatory pathways, while EPA, DHA, and DPA are essential for the synthesis of resolvins, which act to reduce the production of pro-inflammatory cytokines, such as TNF-α and IL-1 (Spite et al., 2014). Resolvins are important in resolving inflammation as they help reduce the production of pro-inflammatory cytokines and promote the removal of inflammatory cells from the site of inflammation (Spite et al., 2014).

The initial products of lipid peroxidation include hydroxyeicosatetraenoics (HETE) and hydroxyoctadecadienoics (HODE). The reduced oxylipids can then serve as

substrates for dehydrogenase metabolism, producing oxoeicosatetraenoics (oxoETE), dihydroxyeicosapentaenoics (DiHETE), or oxooctadecadienoics (oxoODE) (Sordillo et al., 2005; Ramsden et al., 2012).

Higher concentrations of 5-HETE and 15-HETE and their derivatives 5-oxoETE and 15-oxoETE were observed in the HS/n3 group on day 10. These oxylipids are classified as pro-inflammatory oxylipids, originating from arachidonic acid through the lipoxygenase (LOX). These results are in line with the higher concentrations of AA in the HS/n3 group, indicating a similar behavior for their HETE derivatives. Thus, the results suggest that omega-3 infusion under heat stress increased pro-inflammatory oxylipids, compared with the group with omega-6 infusion under heat stress. Arachidonic acid is metabolized by enzymes and non-enzymatic reactions to generate hydroxyeicosatetraenoic acids (HETEs; Ciampi et al., 2022). In a study involving healthy cows during the transition period, an increase in the plasma concentration of oxylipids, especially 5-HETE and 20-HETE, was observed (Contreras et al., 2017). This occurs because 5-HETE regulates the activity of macrophages, increasing their phagocytosis capacity in cattle (Sordillo et al., 2008). 15-HETE is a powerful inducer of the expression of adhesion molecules in endothelial cells, thus regulating the migration of neutrophils and mononuclear cells into tissues (Sordillo et al., 2008). Therefore, HS/n3 treatment appears to confer a greater defense response against pathogens.

The concentration of 9-HODE was highest on day 10, especially in the TNPF/n6 group, which was also observed for its derivative 9-oxoODE. Furthermore, a similar pattern was observed for 13-HODE and 13-oxoODE. These are products derived from linoleic acid through the lipoxygenase (LOX) pathway. Although linoleic acid is associated with pro-inflammatory effects, these specific oxylipids have anti-inflammatory properties. There are two possible explanations for the fact that 9-HODE, 13-HODE, and their derivatives 9-oxoODE and 13-oxoODE are more concentrated in the TNPF/n6 group: 1) Animals in thermoneutral conditions may not present prominent inflammatory signs, resulting in less need to produce pro-inflammatory oxylipids; or 2) There may be better utilization of the omega-6 fatty acid from the abomasal infusion by animals under thermoneutral conditions.

The 9-HODE is known to be utilized during oxidative stress, which can result in reduced plasma concentrations (Sordillo, 2018). In a study investigating oxylipids in early lactation cows, it was observed that 13-HODE and 13-oxoODE levels were reduced compared with later stages of lactation (Raphael et al., 2014). As early lactation is characterized by metabolic dysregulation and inflammation, this decrease is in line with our findings, where heat-stressed cows also showed a reduction in these oxylipids. However, in other studies, such as the evaluation of oxylipids in periparturient cows and those with *Streptococcus uberis* or *Escherichia coli* mastitis in vitro, 9-HODE and 13-HODE were increased, which may lead to the upregulation of pro-inflammatory oxilypids (Contreras et al., 2012; Mavangira et al., 2015; Ryman et al., 2015). The 13-HODE is considered the most abundant plasma oxylipid in early lactation dairy cows and is correlated with IL-12, which is a pro-inflammatory interleukin that plays a key role in pathogen defense (Raphael et al., 2014). The oxidation of linoleic acid through the LOX pathway results in the formation of the initial product 13-hydroperoxyoctadecadienoic acid (13-HPODE), which has pro-inflammatory properties and can be converted into 13-HODE, and later into 13-oxoODE, which are anti-inflammatory (Kuhn et al., 2015). Therefore, metabolism must have the ability to convert 13-HPODE to 13-HODE during inflammatory processes (Ryman et al., 2016). Furthermore, 13-HODE is one of the oxylipids that has already demonstrated the ability to be a potent marker for conditions of inflammation and oxidative stress (Ciampi et al., 2022).

Furthermore, 17-HDoHE demonstrated a more prominent concentration in the HS/n3 group on days 5 and 10. This lipid mediator is derived from DHA via the LOX pathway and is known for its potent anti-inflammatory properties. Both EPA and DHA follow the same behavior pattern as their derivative 17-HDoHE in this study. Therefore, this result suggests that the abomasal infusion of omega-3 stimulated the production of the anti-inflammatory oxylipid 17-HDoHE, also indicating that the greater bioavailability of these omega-3 fatty acids favored the production of anti-inflammatory oxylipids (Astarita et al., 2014). Ogawa et al. (2020) investigated cornea healing in mice, it was observed that 17-HDoHE was the most effective lipid mediator in promoting the recruitment of eosinophils during the healing process. Importantly, 17-HDoHE is recognized as a pro-resolving mediator of inflammation, as it stimulates both the antibody-mediated immune

response and phagocytosis by macrophages (Chiu et al., 2012; Ramon et al., 2014). Furthermore, evidence indicates that 17-HDoHE can reduce obesity-associated inflammation and suppress the production of pro-inflammatory cytokines (Neuhofer et al., 2013; Ogawa et al., 2020). Therefore, the observed increase in this lipid mediator in the group treated with omega-3 suggests that these fatty acids assist in the immune response against pathogens and promote an effective anti-inflammatory response.

The oxylipids 19,20-EpDPE and 19,20-DiHDPA showed higher concentrations in the HS/n3 group on days 5 and 10. These compounds are derived from EPA and DHA, where 19,20-EpDPE is produced before, and after 19, 20-DiHDPA via the cytochrome P450 (CYP450) pathway, these are known for their anti-inflammatory properties, a pattern similar to that observed for 17-HDoHE. Previous studies have described omega-3-derived metabolites via the CYP450 pathway for their anti-inflammatory, analgesic, platelet aggregation inhibition, and muscle relaxation properties in humans and rats (VanRollins, 1995; Morin et al., 2009; 2010; Morisseau et al., 2010).

The PLS-DA and VIP data analyses aim to highlight and identify the characteristics that contribute most to variation between groups (Astarita et al., 2014). In the present study, we observed a clear differentiation between the three treatment groups, with a more pronounced distinction between TNPF/n6 and HS/n3. However, the HS/n6 treatment showed an overlap with both treatments. The fatty acids EPA, DHA, 17-HDoHE, and 19,20-DiHDPA were more important in the HS/n3 group compared with the HS/n6, suggesting that omega-3-derived anti-inflammatory oxylipids are more readily produced when there is a greater bioavailability of these fatty acids, mitigating the harmful effects of heat stress. On the other hand, the TNPF/n6 group demonstrated a greater influence of linoleic and linolenic fatty acids, as well as NEFA, MY, 9-oxoODE, 9,10-EpOME, 13-HODE, 13-oxoODE, 9-HODE, 12, 13-EpOME, and TXB2.

Linoleic and linolenic fatty acids are the main substrates used for the metabolism and production of other long-chain fatty acids and lipid mediators. It is believed that animals in thermoneutrality use these fatty acids more efficiently, resulting in higher concentrations compared with animals under heat stress, which metabolize these fatty acids more quickly due to increased metabolic demands. Thus, during heat stress, animals direct a considerable portion of energy to regulate body temperature, which leads

to the rapid oxidation of omega-3 fatty acids as an effective way of generating energy to meet energy demands.

It has been described that inflammation induced by heat stress increases adipose tissue lipogenesis and reduces lipolysis, explaining the higher concentration of NEFA in the thermoneutrality group compared with the heat stress group (Wheelock et al., 2010). Animals in thermoneutrality direct their energy towards milk production, while stressed animals prioritize thermoregulation and other metabolic demands, which results in a reduction in milk production (Rhoads et al., 2009). However, the understanding of the lipid mediators most expressed in the HS/n3 group remains incomplete, despite the observed modulation, which includes four anti-inflammatory oxylipids derived from linoleic acid and three oxylipids derived from arachidonic acid, with anti-inflammatory and pro-inflammatory properties.

We observed higher concentrations of 17,18-DiHETE, 17-HDoHE, 19,20-DiHDPA, EPA, and DHA, along with a moderate respiratory rate response in the HS/n3 group in the heat map analysis. This higher concentration of EPA and DHA in the oxylipid pathway indicates a greater availability of these fatty acids for the production of lipid mediators. EPA is converted into DHA, and through the CYP450 pathway, produces 19,20-EpDPE and, subsequently, 19,20-DiHDPA (Astarita et al., 2014). Simultaneously, EPA acts as a substrate for the production of 17,18-EpETE and then 17,18-DiHETE. 17-HDoHE is generated from DHA in the LOX pathway (Astarita et al., 2014). Such fatty acids and lipid mediators derived from omega-3 have anti-inflammatory properties, justifying their greater expression in the HS/n3 group.

Biological indicators, such as pre- and post-prandial insulin, R-QUICK, and HOMA-IR, in addition to respiratory frequency, showed greater expression in the group treated with HS/n6. These markers, such as insulin, R-QUICK, and HOMA-IR, play a crucial role in assessing insulin resistance in individuals, and insulin sensitivity may be correlated with systemic inflammation (Holland et al., 2011). During heat stress, a high release of circulating insulin is observed, aiming to promote the increased uptake of glucose by cells for use as an energy source, resulting in low concentrations of NEFA and glucose (Rhoads et al., 2009; Gantner et al., 2017). The regulation of insulin and blood glucose is influenced

by the complex interaction with stimuli from the immune system, which can be hyperstimulated during heat stress (Dalmas, 2019).

In the heat map analysis, it was highlighted that total solids, proteins, and fats in milk showed higher expression in response to the changes induced by the HS/n6 treatment, although their concentrations were lower compared with the HS/n3 and TNPF/n6 treatments. (Ruiz-González et al., unpublished). This finding suggests that heat stress exacerbated by the presence of omega-6 leads animals to direct more energy to thermoregulation, resulting in an insufficient allocation of energy for the production of milk components.

The lipid mediators that excelled in the HS/n6 treatment included 9,10-EpOME and 12,13-EpOME, derived from the CYP450 pathway, and 9-HODE, 13-HODE, and 13-oxoODE from the LOX pathway. These derive from linoleic acid and demonstrate anti-inflammatory properties, although some, such as 9,10-EpOME and 12,13-EpOME, are not yet fully characterized. On the other hand, the pre-and postprandial markers QUICK, linolenic acid, and 9-oxoODE were more expressed in the TNPF/n6 group. QUICK also serves as an indicator of insulin sensitivity, and although it is more common to observe insulin sensitivity in heat-stressed animals, it may also suggest an inflammatory process resulting from the abomasal infusion of omega-6 fatty acids. Furthermore, 9-oxoODE is an anti-inflammatory oxylipid derived from the pro-inflammatory 9-HpODE and 9-HODE, suggesting a beneficial response from the body in mitigating inflammation, as 9-oxoODE is already in the anti-inflammatory form.

EPA and DHA were associated with 25 different components, revealing a positive correlation between them (EPA and DHA) and lipid mediators such as 17-HDoHE, 17,18-DiHETE, and 19,20-DIHDPA, all derived from these fatty acids. Increases in the bioavailability of these omega-3 fatty acids also resulted in correlated increases in their anti-inflammatory derivatives. 19,20-EpDPE showed a positive correlation only with DHA.

Additionally, markers of insulin sensitivity and inflammation (such as LBP) were negatively correlated with EPA and DHA. As discussed earlier, thermal stress triggers a systemic inflammatory response that increases insulin sensitivity and LBP levels in the bloodstream. In this context, evidence suggests that omega-3 fatty acid infusion may attenuate inflammatory effects by modulating the immune system (Ruiz-González et al.,

unpublished). Furthermore, EPA and DHA showed a negative correlation with skin and rectal temperature, highlighting their ability to mitigate the effects of thermal stress. By reducing endotoxemia caused by LBP, these fatty acids can also lower body temperature.

This is especially relevant considering that TLR4 receptor activation by the immune system can trigger a febrile response, exacerbating hyperthermia in dairy cows already under thermal stress (Blatteis, 2006; Waldron et al., 2006). Milk production and components showed a negative correlation with EPA and DHA. This relationship can be attributed to the fact that these polyunsaturated fatty acids, derived from fish oil, are known to reduce milk fat by producing intermediate fatty acids in the rumen, such as trans-10, cis-12 conjugated linoleic acid (CLA), thereby disrupting milk fat synthesis regulation pathways (Baumgard et al., 2001). Additionally, lipid mediators 13-HODE correlated negatively with EPA and DHA, while 9-HODE, 13-oxoODE, and 14,15-DHET correlated negatively only with DHA. These are oxylipids derived from linoleic acid and arachidonic acid, associated with pro-inflammatory action (Spite et al., 2014).

Linoleic acid and arachidonic acid showed positive correlation with linolenic acid and DPA, whereas only arachidonic acid correlated with EPA. Additionally, they exhibited positive correlation with 9-oxoODE, 9,10-EpOME, 12,13-EpOME, 11,12-DHET, and 14,15-DHET, which are either anti-inflammatory or poorly characterized lipid mediators. Only linoleic acid positively correlated with 13-HODE and 13-oxoODE, which are anti-inflammatory mediators. These lipid mediators use linoleic acid and arachidonic acid as substrates in LOX and CYP450 enzymatic pathways, explaining their positive correlation (Astarita et al., 2014). The same pattern is observed in the positive correlation of arachidonic acid with 5-HETE, 5-oxoETE, 5-iso-prostaglandin (5-iPF2α-VI), 8-iso-prostaglandin A2 (8-iso-PGA2), and 8,12-iso-isoprostane-F2α-VI (8,12-iso-IPF2α-VI), which are lipid mediators that utilize arachidonic acid as a precursor of pro-inflammatory mediators in LOX and non-enzymatic pathways. Linoleic acid also positively correlated with NEFA concentration and QUIKI, as well as with milk production, fat, protein, and total milk solids. We believe this result may be attributed to the higher concentration of linoleic acid in the TNPF/n6 and HS/n6 treatments, which were more productive. The observed negative correlation between linoleic acid and respiratory rate, 19,20-DiHDPA, 17-HDoHE, 15-oxoETE, and QUIKI, as well as the negative correlation between arachidonic

acid and insulin resistance biomarkers, LBP, skin temperature, and respiratory rate, is not clear.

In summary, this study offers valuable insights into the interaction between omega-3 and omega-6 fatty acids, inflammatory response, and heat stress in dairy cows. The results indicate that omega-6 infusion during heat stress can intensify the inflammatory response, while omega-3 appears to have a mitigating effect, promoting the production of oxylipids with anti-inflammatory properties. However, the levels of these markers can vary depending on the intensity of the stress, the exposure time, and the individual adaptation of the animals to heat stress (Gupta et al., 2013). The optimal dose of omega for cows in these conditions, as well as in other scenarios where inflammation is a concern for the animal, has not yet been determined. This finding highlights the importance of considering the lipid composition of the diet in situations of heat stress, not only for the health of the animals but also for the quality of the dairy product. Furthermore, it highlights the continued need for research to better understand the underlying mechanisms and develop more effective management strategies to minimize the negative impacts of heat stress on dairy production.

5 Conclusion

Cows subjected to heat stress underwent an inflammatory response, and plasma oxylipid concentrations may largely depend on the available substrate. However, oxylipid metabolism was also impacted by heat stress itself. Our results indicate that omega-3 infusion partially alleviated hyperthermia and promoted the production of anti-inflammatory oxylipids, exerting an immunomodulatory effect on these compounds. This effect was more pronounced on day 10 when the animals were exposed to environmental stress for a longer time. The LOX enzymatic pathway was particularly significant, probably due to systemic inflammation and oxidative imbalance resulting from heat stress. However, abomasal infusion of fish oil-derived omega-3 was able to mitigate clinical signs of inflammation in cows, while omega-6 infusion appeared to aggravate the inflammatory response. The different patterns observed between fatty acids and associated metabolites highlight the complexity of lipid mediators about metabolic factors. Strategies to modulate oxylipid biosynthesis may include approaches that do not exacerbate inflammation or that

actively promote the resolution of inflammation through endogenous pathways. Therefore, it is suggested that the controlled introduction of fatty acids into the diet of dairy cows can promote health benefits. However, more research is needed in this area to better understand the modulation in different types and levels of inflammation.

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7 Table and figures

Table 1: Lipid mediators detected in this study.

Lipids[1]	Complete name	Precursor[2]	Omega[3]	Inflammation[4]
Enzymatic pathway[5]				
EPA	Eicosapentanoic acid	-	n-3	Anti-inflammatory
DHA	Docosahexaenoic acid	-	n-3	Anti-inflammatory
DPA	Docosapentanoic acid	-	n-3	Anti-inflammatory
ALA	Linolenic acid	-	n-3	Anti-inflammatory
LA	Linoleic acid	-	n-6	Proinflammatory
AA	Arachidonic acid	-	n-6	Proinflammatory
LOX				
5-HETE	5-Hydroxyeicosatetraenoic acid	AA	n-6	Proinflammatory
5-oxoETE	5-Oxoeicosatetraenoic acid	AA	n-6	Proinflammatory
9-HODE	9-Hydroxyoctadecadienoic acid	LA	n-6	Anti-inflammatory
9-oxoODE	9-Oxooctadecadienoic acid	LA	n-6	Anti-inflammatory
11-HETE	11-5-Hydroxyeicosatetraenoic acid	AA	n-6	Proinflammatory
13-HODE	13-Hydroxyoctadecadienoic acid	LA	n-6	Anti-inflammatory
13-oxoODE	13-Oxooctadecadienoic acid	LA	n-6	Anti-inflammatory
15-HETE	15-5-Hydroxyeicosatetraenoic acid	AA	n-6	Anti-inflammatory
15-oxoETE	15-Oxoeicosatetraenoic acid	AA	n-6	Anti-inflammatory
17-HDoHE	17-Hydroxydocosahexaenoic acid	DHA	n-3	Anti-inflammatory
COX				
TXB2	Thromboxane B2	AA	n-6	Proinflammatory
CYP450				
8,9-DHET	8,9-Dihydroxyeicosatrienoic acid	AA	n-6	Poorly characterized
9,10-DiHOME	9,10-Dihydroxyoctadecenoic acid	LA	n-6	Proinflammatory
9,10-EpOME	9,10-Epoxyoctadecenoic acid	LA	n-6	Poorly characterized
11,12-DHET	11,12-Dihydroxyeicosatrienoic acid	AA	n-6	Poorly characterized

12,13-DiHOME	12,13-Dihydroxyoctadecenoic acid	LA	n-6	Proinflammatory
12,13-EpOME	12,13-Epoxyoctadecenoic acid	LA	n-6	Poorly characterized
14,15-DHET	14,15-Dihydroxyeicosatrienoic acid	AA	n-6	Poorly characterized
17,18-DiHETE	17,18-Dihydroxyeicosatetraenoic acid	EPA	n-3	Anti-inflammatory
19,20-DiHDPA	19,20-Dihydroxydocosapentaenoic acid	DPA/DHA	n-3	Anti-inflammatory
19,20-EpDPE	19,20-Epoxydocosapentaenoic acid	DPA/DHA	n-3	Anti-inflammatory
20-HETE	20-5-Hydroxyeicosatetraenoic acid	EPA	n-3	Anti-inflammatory
Non-enzymatic[6]				
8-iso-PGA2	8-iso-Prostaglandin A2	AA	n-6	Poorly characterized
8,12-iso-iPF2alpha-VI	8,12-iso-Isoprostane-F2α-VI	AA	n-6	Poorly characterized
5-iPF2alpha-VI	5-iso Prostaglandin F2α-VI	AA	n-6	Poorly characterized

[1]Lipid mediators detected in the study with LC-MS/MS analysis. [2]Fatty acid used as a by-product to form the final product. [3]Omega complex of the precursor of lipid mediator [4]Inflammatory action, indicating which lipid mediators are predominantly pro-inflammatory or anti-inflammatory (Putman et al., 2022). [5]Lipid medi detected in the enzymatic oxidation pathway: Lipoxygenase (LOX), Cyclooxygenase (COX), and Cytochrome 450 (CYP450). [6]Lipid mediators detected in the enzymatic oxygenation pathway.

Table 2: Fatty acids and oxylipids quantified in blood plasma in dairy cows under heat stress and supplemented with omega-3 and omega-6.

Metabolites/nmol/mL	Treatment x Day[1]						*P* value[6]			Contrasts[7]	
	Day[3]	TNPF/n6	HS/n6	HS/n3	Day mean[4]	SEM[5]	Treat	Day	Treat x Day	TNPF x HS/n6	HS/n6 x HS/n3
Enzymatic pathway											
EPA	0	2.70	4.21	1.96	2.96	1.50	<0.0001	0.001	<0.0001		
EPA	5	2.80	4.09	12.46	6.45						*
EPA	10	3.56	3.19	22.80	9.85						*
EPA	**Treat mean[2]**	3.02	3.83	12.40							
DHA	0	2.39	3.41	2.25	2.02	2.03	<0.0001	0.003	<0.0001		
DHA	5	3.32	3.76	16.15	7.74						*
DHA	10	4.73	3.39	28.76	12.29						*
DHA	**Treat mean**	3.48	3.52	15.05							
DPA	0	2.29	4.26	2.49	3.01	1.58	0.05	0.01	0.09		
DPA	5	3.80	6.12	7.62	5.85						
DPA	10	5.14	5.13	12.06	7.45						*
DPA	**Treat mean**	3.74	5.17	7.39							
ALA	0	20.00	29.93	20.78	23.57	7.19	0.62	0.02	0.31		
ALA	5	33.08	35.53	26.77	31.79						
ALA	10	55.75	31.90	39.59	42.42						
ALA	**Treat mean**	36.28	32.45	29.05							
LA	0	56.56	82.68	59.02	66.09	14.87	0.22	0.001	0.31		
LA	5	116.75	128.86	82.88	109.49						
LA	10	168.00	116.32	109.88	131.40						
LA	**Treat mean**	113.77	109.29	83.93							
AA	0	17.47	24.72	17.06	19.75	5.85	0.07	0.01	0.09		

AA	5	22.57	30.53	35.09	29.40						
AA	10	26.87	25.78	50.94	34.53						*
AA	**Treat mean**	22.30	27.01	34.36							
LOX[8]											
5-HETE	0	172.10	219.98	147.01	179.70	51.85	0.12	0.21	0.05		
5-HETE	5	147.10	160.72	245.93	184.58						
5-HETE	10	211.16	147.06	361.65	239.95						*
5-HETE	**Treat mean**	176.78	175.92	251.53							
5-oxoETE	0	7.95	13.08	7.43	9.49	3.29	0.15	0.33	0.03		
5-oxoETE	5	7.46	9.16	14.27	10.30						
5-oxoETE	10	12.50	6.32	19.69	12.84						*
5-oxoETE	**Treat mean**	9.30	9.52	13.80							
9-HODE	0	80.61	102.27	61.99	81.63	19.80	0.03	0.01	0.43		
9-HODE	5	105.17	105.52	72.26b	94.32						
9-HODE	10	153.98	115.34	97.49b	122.27						
9-HODE	**Treat mean**	113.26	107.71	77.25							*
9-oxoODE	0	47.72	53.27	36.78	45.93	8.68	0.03	0.01	0.09		
9-oxoODE	5	63.18	46.06	45.68	51.64						
9-oxoODE	10	102.22	52.01	58.73	70.99						
9-oxoODE	**Treat mean**	71.04	50.45	47.07						*	
11-HETE	0	10.63	17.21	8.53	12.13	4.56	0.12	0.68	0.02		
11-HETE	5	6.94	9.32	13.57	9.95						
11-HETE	10	7.69	6.71	20.58	11.66						
11-HETE	**Treat mean**	8.42	11.08	14.23							*
13-HODE	0	70.61	92.97	59.23	74.27	12.91	0.03	0.01	0.49		
13-HODE	5	95.99	91.41	65.95	84.45						
13-HODE	10	129.55	104.9	83.85	106.10					*	
13-HODE	**Treat mean**	98.72	96.43	69.68							*
13-oxoODE	0	7.25	9.57	5.72	7.51	1.27	0.07	0.12	0.28		
13-oxoODE	5	9.43	8.28	7.20	8.30						

13-oxoODE	10	12.76	9.02	7.74	9.84					
13-oxoODE	**Treat mean**	9.81	8.96	6.89						
15-HETE	0	13.49	20.85	11.28	15.20	5.94	0.01	0.20	0.003	
15-HETE	5	10.86	13.54	24.39	16.26					
15-HETE	10	13.18	12.19	38.83	21.40					
15-HETE	**Treat mean**	12.51	15.52	24.83						*
15-oxoETE	0	0.87	1.86	0.82	1.18	0.48	0.01	0.15	0.002	
15-oxoETE	5	0.87	1.05	2.02	1.31					
15-oxoETE	10	0.89	0.99	3.63	1.84					*
15-oxoETE	**Treat mean**	0.88	1.30	2.16						
17-HDoHE	0	4.37	11.42	1.50	5.76	2.16	0.01	0.41	0.001	
17-HDoHE	5	1.89	4.03	14.19	6.70					*
17-HDoHE	10	3.97	4.12	18.23	8.77					*
17-HDoHE	**Treat mean**	3.41	6.52	11.31						
COX[9]										
TXB2	0	0.48	0.74	0.23	0.48	0.37	0.24	0.48	0.39	
TXB2	5	0.48	0.35	0.27	0.37					
TXB2	10	2.80	0.34	0.21	1.12					
TXB2	**Treat mean**	1.25	0.48	0.23						
CYP450[10]										
8,9-DHET	0	1.11	1.26	1.26	1.21	0.45	0.13	0.003	0.29	
8,9-DHET	5	1.49	1.50	1.79	1.60					
8,9-DHET	10	1.97	1.79	3.38	2.38					
8,9-DHET	**Treat mean**	1.52	1.52	2.14						
9,10-DiHOME	0	11.05	12.63	12.57	12.08	4.52	0.13	0.003	0.29	
9,10-DiHOME	5	14.94	15.00	17.92	15.96					
9,10-DiHOME	10	19.68	17.88	33.82	23.79					
9,10-DiHOME	**Treat mean**	15.22	15.17	21.44						
9,10-EpOME	0	4.66	5.55	3.79	4.67	0.92	0.06	0.06	0.18	
9,10-EpOME	5	5.41	4.80	3.97	4.73					

9,10-EpOME	10	9.16	5.30	4.81	6.43						
9,10-EpOME	**Treat mean**	6.41	5.22	4.19							
11,12-DHET	0	4.31	5.21	3.74	4.42	1.12	0.99	0.08	0.42		
11,12-DHET	5	5.56	6.42	5.67	5.88						
11,12-DHET	10	6.42	5.02	7.34	6.26						
11,12-DHET	**Treat mean**	5.43	5.55	5.58							
12,13-DiHOME	0	1.56	2.06	1.68	1.77	0.34	0.45	0.01	0.71		
12,13-DiHOME	5	2.17	1.31	1.16	1.55						
12,13-DiHOME	10	3.56	2.68	2.79	3.01						
12,13-DiHOME	**Treat mean**	2.43	2.02	1.88							
12,13-EpOME	0	4.42	5.73	3.59	4.58	1.14	0.21	0.005	0.92		
12,13-EpOME	5	5.67	5.98	3.73	5.13						
12,13-EpOME	10	8.42	7.73	6.84	7.67						
12,13-EpOME	**Treat mean**	6.17	6.48	4.72							
14,15-DHET	0	1.73	2.11	1.68	1.84	0.43	0.84	0.12	0.95		
14,15-DHET	5	2.68	2.75	2.35	2.59						
14,15-DHET	10	2.26	2.27	2.42	2.32						
14,15-DHET	**Treat mean**	2.22	2.38	2.15							
17,18-DiHETE	0	521.35	526.01	343.06	463.48	101.52	<0.0001	0.002	<0.0001		
17,18-DiHETE	5	535.89	493.90	1471.22	833.67						
17,18-DiHETE	10	454.37	281.64	2537.50	1091.17					*	
17,18-DiHETE	**Treat mean**	503.87	433.85	1450.59							
19,20-DiHDPA	0	5.64	6.27	0.46	4.13	2.68	<0.0001	0.002	<0.0001		
19,20-DiHDPA	5	5.32	6.14	22.89	11.45						*
19,20-DiHDPA	10	4.90	4.44	40.59	16.64						*
19,20-DiHDPA	**Treat mean**	5.29	5.62	21.31							
19,20-EpDPE	0	0.80	1.01	0.36	0.73	0.56	0.001	0.05	0.003		
19,20-EpDPE	5	0.72	0.53	2.88	1.38						*
19,20-EpDPE	10	0.70	0.67	4.24	1.87						*
19,20-EpDPE	**Treat mean**	0.74	0.74	2.49							

20-HETE	0	1.56	2.67	1.63	1.95	0.97	0.41	0.24	0.15
20-HETE	5	3.08	1.95	3.32	2.78				
20-HETE	10	4.38	1.42	3.37	3.06				
20-HETE	**Treat mean**	3.01	2.01	2.77					
Non-enzymatic pathway									
8-iso-PGA2	0	0.27	0.25	0.33	0.28	0.10	0.34	0.06	0.35
8-iso-PGA2	5	0.19	0.21	0.15	0.19				
8-iso-PGA2	10	0.25	0.29	0.55	0.36				
8-iso-PGA2	**Treat mean**	0.24	0.25	0.34					
8,12-iso-iPF2alpha-VI	0	0.34	0.48	0.33	0.38	0.09	0.74	0.06	0.37
8,12-iso-iPF2alpha-VI	5	0.12	0.23	0.34	0.23				
8,12-iso-iPF2alpha-VI	10	0.28	0.20	0.19	0.22				
8,12-iso-iPF2alpha-VI	**Treat mean**	0.25	0.30	0.29					
5-iPF2alpha-VI	0	0.15	0.20	0.14	0.16	0.06	0.28	0.32	0.18
5-iPF2alpha-VI	5	0.15	0.14	0.21	0.17				
5-iPF2alpha-VI	10	0.24	0.12	0.31	0.22				
5-iPF2alpha-VI	**Treat mean**	0.18	0.15	0.22					
Ratio[11]									
5 HETE:5 oxoETE	0	24.63	20.18	21.48	22.10	1.49	0.64	0.49	0.45
5 HETE:5 oxoETE	5	21.50	21.27	18.42	20.40				
5 HETE:5 oxoETE	10	18.79	22.14	19.62	20.19				
5 HETE:5 oxoETE	**Treat mean**	21.64	21.20	19.84					
9 HODE:9 oxoODE	0	1.70	2.10	1.79	1.86	0.29	0.23	0.48	0.72
9 HODE:9 oxoODE	5	1.67	2.34	1.85	1.95				
9 HODE:9 oxoODE	10	1.56	2.48	2.71	2.25				
9 HODE:9 oxoODE	**Treat mean**	1.64	2.31	2.12					
13 HODE:13 oxoODE	0	10.12	10.54	11.07	10.58	0.66	0.42	0.42	0.66
13 HODE:13 oxoODE	5	10.19	11.91	9.97	10.69				
13 HODE:13 oxoODE	10	10.47	11.84	12.19	11.50				
13 HODE:13 oxoODE	**Treat mean**	10.26	11.43	10.26					

15 HETE:15 oxoETE	0	18.43	17.71	17.66	17.94	1.47	0.36	0.04	0.75
15 HETE:15 oxoETE	5	14.07	12.80	12.46	13.11				
15 HETE:15 oxoETE	10	17.36	13.26	10.39	13.67				
15 HETE:15 oxoETE	**Treat mean**	13.51	14.59	16.62					

Treatment: HS/n3: Heat stress + fish oil (HS/n3; 159 g/d of fish oil; 8.3% 20:5 n-3, 19% 22:6 n-3; THI = 84), HS/n6: Heat stress + corn oil (HS/n6; 159 g/d cor
THI = 84), TNPF/n6: thermoneutrality in pair feeding + corn oil (159 g/d corn oil; 55% 18:2 n-6; THI =64). [1]Treatment x Day: mean correlations between treatm
and day (time). [2]Treatment mean: TNPF/n6, HS/n6 and HS/n3. [3]Day: collection days 0, 5 and 10. [4]Mean between days: 0, 5 and 10. [5]Standard Error of the M
[6]*P* value: Significant with P < 0.05, for tendency Treatment and Day (P < 0.10) and interaction (P < 0.15). [7]Contrasts: Treatments were compared as follows: H
vs. HS/n6, and HS/n6 vs. TNPF/n6. [8]Lipoxygenase pathway. [9]Cyclooxygenase pathway. [10]Cytochrome 450 pathway. [11]Ratio: Relationship of two metabolites
pathway, precursor and derivative. The symbol * means the difference between pre-planned contrasts (*P* < 0.05). Abbreviations: EPA: Eicosapentaenoic acid, D
Docosahexaenoic acid, DPA: Docosapentaenoic acid, ALA: Linolenic acid, LA: Linoleic acid, AA: Arachidonic acid, 5-HETE: 5-Hydroxyeicosatetraenoic aci
oxoETE: 5-Oxoeicosatetraenoic acid, 9-HODE: 9-Hydroxyoctadecadienoic acid, 9-oxoODE: 9-Oxooctadecadienoic acid, 11-HETE: 11-5-Hydroxyeicosatetrae
acid, 13-HODE: 13-Hydroxyoctadecadienoic acid, 13-oxoODE: 13-Oxooctadecadienoic acid, 15-HETE: 15-5-Hydroxyeicosatetraenoic acid, 15-oxoETE
Oxoeicosatetraenoic acid, 17-HDoHE: 17-Hydroxydocosahexaenoic acid, TXB2: Thromboxane B2, 8,9-DHET: 8,9-Dihydroxyeicosatrienoic acid, 9,10-EpOME:
Epoxyoctadecenoic acid, 11,12-DHET: 11,12-Dihydroxyeicosatrienoic acid, 12,13-DiHOME: 12,13-Dihydroxyoctadecenoic acid, 12,13-EpOME: 1
Epoxyoctadecenoic acid, 14,15-DHET: 14,15-Dihydroxyeicosatrienoic acid, 17, 18-DiHETE: 17,18-Dihydroxyeicosatetraenoic acid, 19,20-DiHPA: 1
Dihydroxydocosapentaenoic acid, 19,20-EpDPE: 19,20-Epoxydocosapentaenoic acid, 20-HETE: 20-5-Hydroxyeicosatetraenoic acid, 8-iso-PGA2: 8
Prostaglandin A2, 8,12-iso-iPF2alpha-VI: 8,12-iso-Isoprostane-F2α-VI, 5-iPF2alpha-VI: 5-iso Prostaglandin F2α-VI. Unit of measurement: nmol/mL.

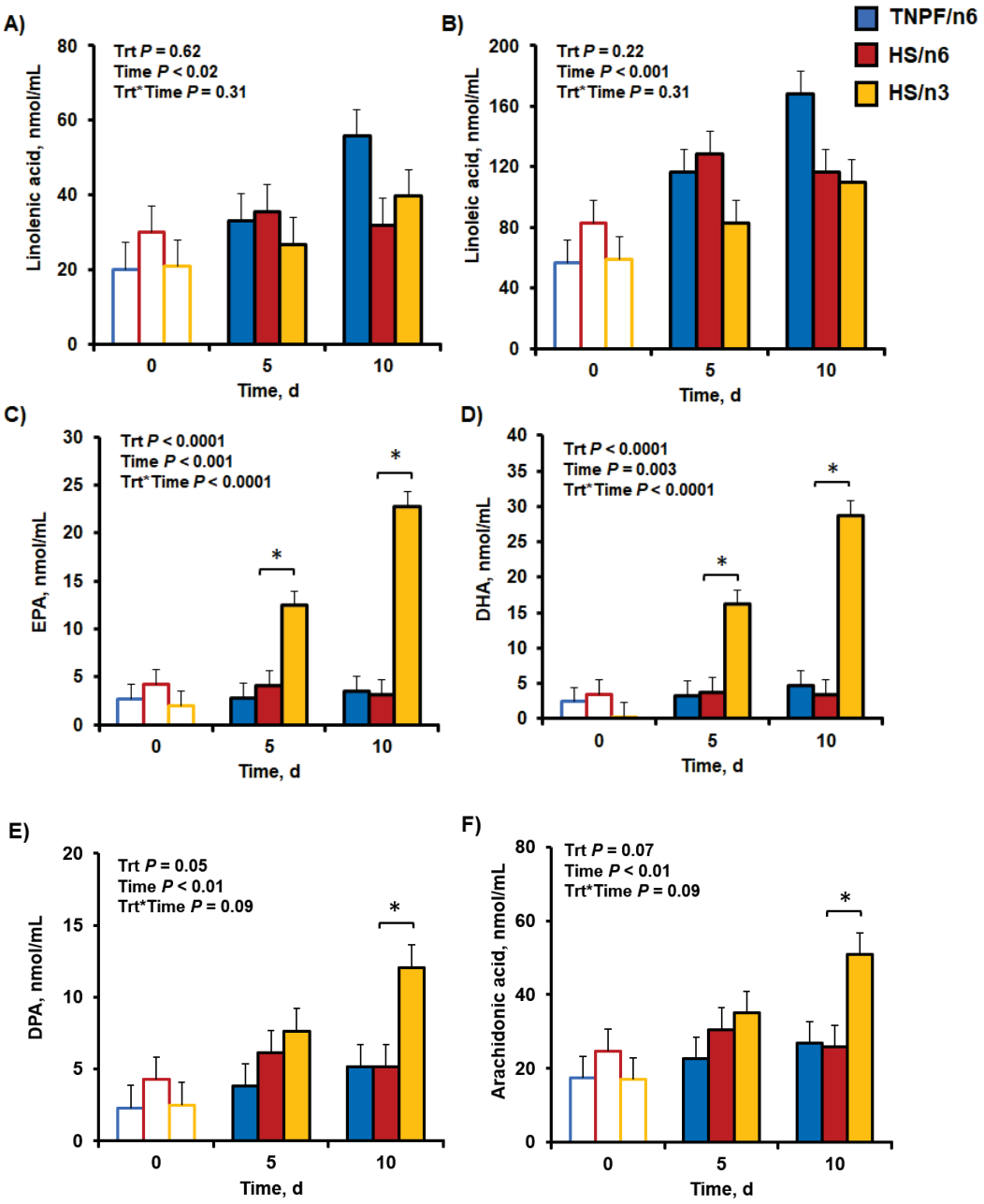

Figure 1: Effects of experimental treatments on plasma concentrations of omega-3 and omega-6 fatty acids. HS/n3: Heat stress + fish oil (HS/n3; 159 g /d of fish oil; 8.3% 20:5 n-3, 19% 22:6 n-3; THI = 84), HS/n6: Heat stress + corn oil (HS/n6; 159 g/d corn oil; THI = 84), TNPF/n6: thermoneutrality in pair feeding + corn oil (159 g/d corn oil; 55% 18:2 n-6; THI =64). A) Linolenic acid, B) Linoleic acid, C) EPA (Eicosapentaenoic acid), D) DHA (Docosahexaenoic acid), E) DPA (Docosapentaenoic acid), F) Arachidonic acid. Treatments were compared as follows: HS/n3 vs. HS/n6, and HS/n6 vs. TNPF/n6. P value: Significant with $P < 0.05$ and interaction with time ($P < 0.15$). The symbol * represents significant results for the pre-planned contrasts. Error bars represent SEM.

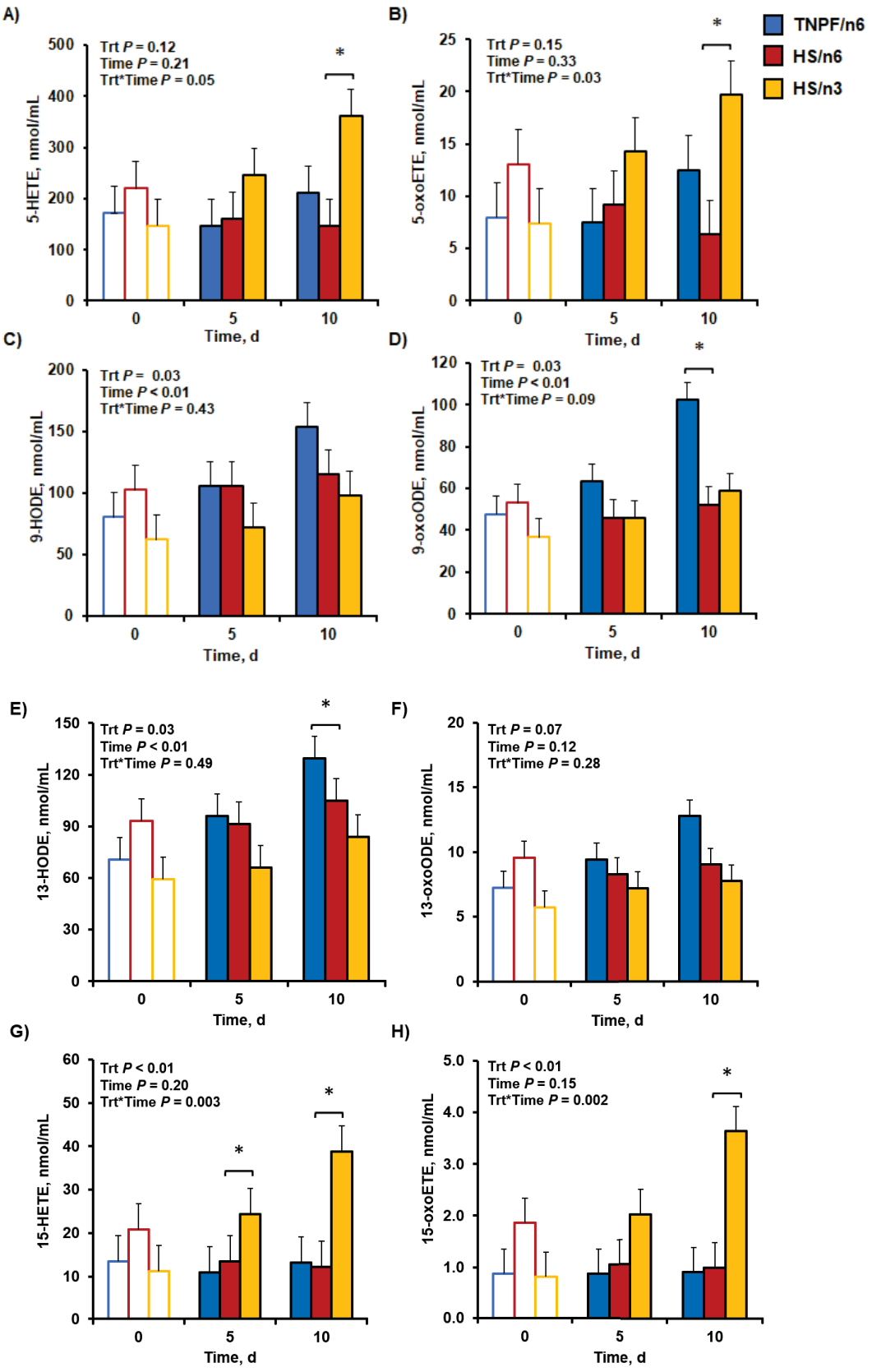
A)
Trt P = 0.12
Time P = 0.21
Trt*Time P = 0.05
5-HETE, nmol/mL
Time, d
B)
Trt P = 0.15
Time P = 0.33
Trt*Time P = 0.03
5-oxoETE, nmol/mL
Time, d
TNPF/n6
HS/n6
HS/n3
C)
Trt P = 0.03
Time P < 0.01
Trt*Time P = 0.43
9-HODE, nmol/mL
Time, d
D)
Trt P = 0.03
Time P < 0.01
Trt*Time P = 0.09
9-oxoODE, nmol/mL
Time, d
E)
Trt P = 0.03
Time P < 0.01
Trt*Time P = 0.49
13-HODE, nmol/mL
Time, d
F)
Trt P = 0.07
Time P = 0.12
Trt*Time P = 0.28
13-oxoODE, nmol/mL
Time, d
G)
Trt P < 0.01
Time P = 0.20
Trt*Time P = 0.003
15-HETE, nmol/mL
Time, d
H)
Trt P < 0.01
Time P = 0.15
Trt*Time P = 0.002
15-oxoETE, nmol/mL
Time, d

Figure 2: Lipoxygenase pathway oxylipids detected by LC-MS/MS analysis.
HS/n3: Heat stress + fish oil (HS/n3; 159 g /d of fish oil; 8.3% 20:5 n-3, 19% 22:6 n-3; THI = 84), HS/n6: Heat stress + corn oil (HS/n6; 159 g/d corn oil; THI = 84), TNPF/n6: thermoneutrality in pair feeding + corn oil (159 g/d corn oil; 55% 18:2 n-6; THI =64). A) 5-HETE: 5-Hydroxyeicosatetraenoic acid, B) 5 oxo-ETE: 5-Oxoeicosatetraenoic acid, C) 9-HODE: 9-Hydroxyoctadecadienoic acid, D) 9-oxoODE: 9-Oxooctadecadienoic acid, E) 13-HODE: 13-Hydroxyoctadecadienoic acid, F) 13-oxoODE: 13-Oxooctadecadienoic acid, G) 15-HETE: 15-5-Hydroxyeicosatetraenoic acid and H) 15-oxoETE: 15-Oxoeicosatetraenoic acid. Treatments were compared as follows: HS/n3 vs. HS/n6, and HS/n6 vs. TNPF/n6. P value: Significant with $P < 0.05$ and interaction with time ($P < 0.15$). The symbol * represents significant results for the pre-planned contrasts. Error bars represent SEM.

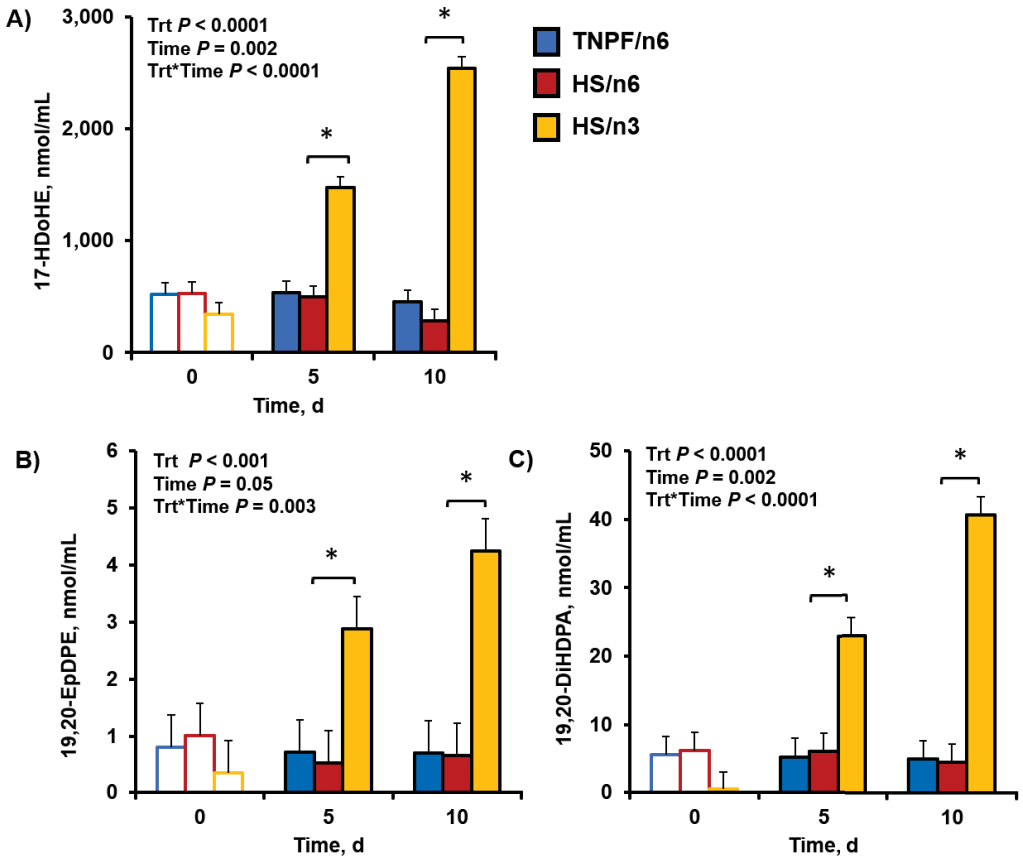

Figure 3: Oxylipids derived from EPA and DHA pathway the Lipoxygenase (LOX) and Cytochrome 450 (CYP450) pathway detected by LC-MS/MS analysis.
HS/n3: Heat stress + fish oil (HS/n3; 159 g /d of fish oil; 8.3% 20:5 n-3, 19% 22:6 n-3; THI = 84), HS/n6: Heat stress + corn oil (HS/n6; 159 g/d corn oil; THI = 84), TNPF/n6: thermoneutrality in pair feeding + corn oil (159 g/d corn oil; 55% 18:2 n-6; THI =64). A) 17-HDoHE: 17-Hydroxydocosahexaenoic acid (LOX), B) 19,20-EpDPE: 19,20-Epoxydocosapentaenoic acid (CYP450), and C) 19, 20-DiHDPA: 19,20-Dihydroxydocosapentaenoic acid (CYP450). Treatments were compared as follows: HS/n3 vs. HS/n6, and HS/n6 vs. TNPF/n6. P value: Significant with $P < 0.05$ and interaction with time ($P < 0.15$). The symbol * represents significant results for the pre-planned contrasts. Error bars represent SEM.

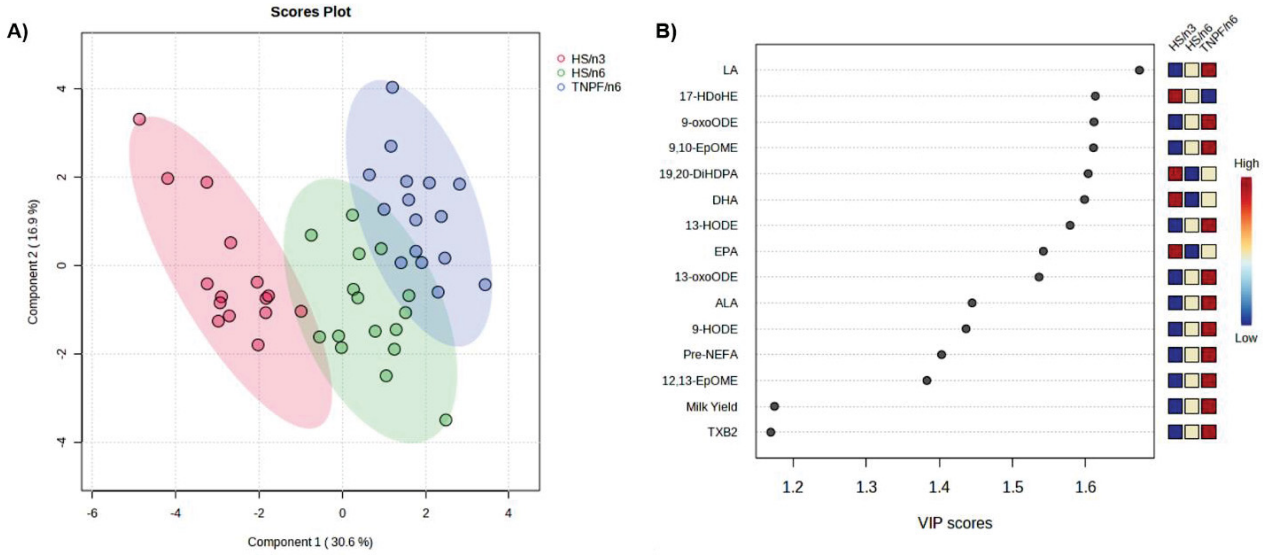

Figure 4: Non-directed PLS-DA metabolomics analysis.
HS/n3: Heat stress + fish oil (HS/n3; 159 g /d of fish oil; 8.3% 20:5 n-3, 19% 22:6 n-3; THI = 84), HS/n6: Heat stress + corn oil (HS/n6; 159 g/d corn oil; THI = 84), TNPF/n6: thermoneutrality in pair feeding + corn oil (159 g/d corn oil; 55% 18:2 n-6; THI =64). A) The PLS-DA analysis showed a relevant separation between the groups. B) Relative concentrations of metabolites in each study group are represented by the colored columns on the right. Red indicates a high contribution, while blue indicates a low contribution to the variation observed in the model (A). The Projection Variable Importance Value (VIP) is calculated as the weighted sum of the squares of the PLS loadings, taking into account the amount of variation Y explained in each dimension, being more expressive when VIP is greater than 1.

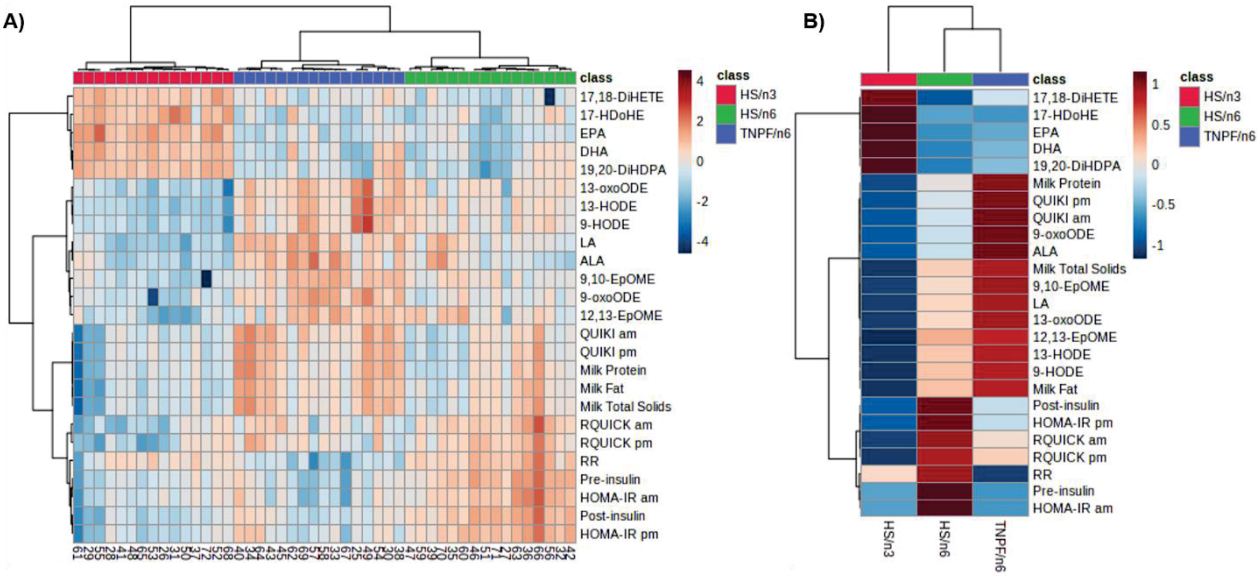

Figure 5: Non-directed metabolomic analysis of heatmap clustering.
HS/n3: Heat stress + fish oil (HS/n3; 159 g/d of fish oil; 8.3% 20:5 n-3, 19% 22:6 n-3; THI = 84), HS/n6: Heat stress + corn oil (HS/n6; 159 g/d corn oil; THI = 84), TNPF/n6: thermoneutrality in pair feeding + corn oil (159 g/d corn oil; 55% 18:2 n-6; THI =64). A) The heatmap cluster analysis results are displayed with a total of 72 samples and the clusters are demarcated by different colors. Each colored cell on the map reflects a concentration value, with samples arranged in columns and the variable of interest in rows. The visualization displays the top 25 components identified through the t-test. B) Similar to the previous representation (Figure A), the heat map displays the average of the samples from each group (n=24) using different colors. The 25 most significant features selected after testing the average of each group were highlighted. The milk components are arranged in kg/day.

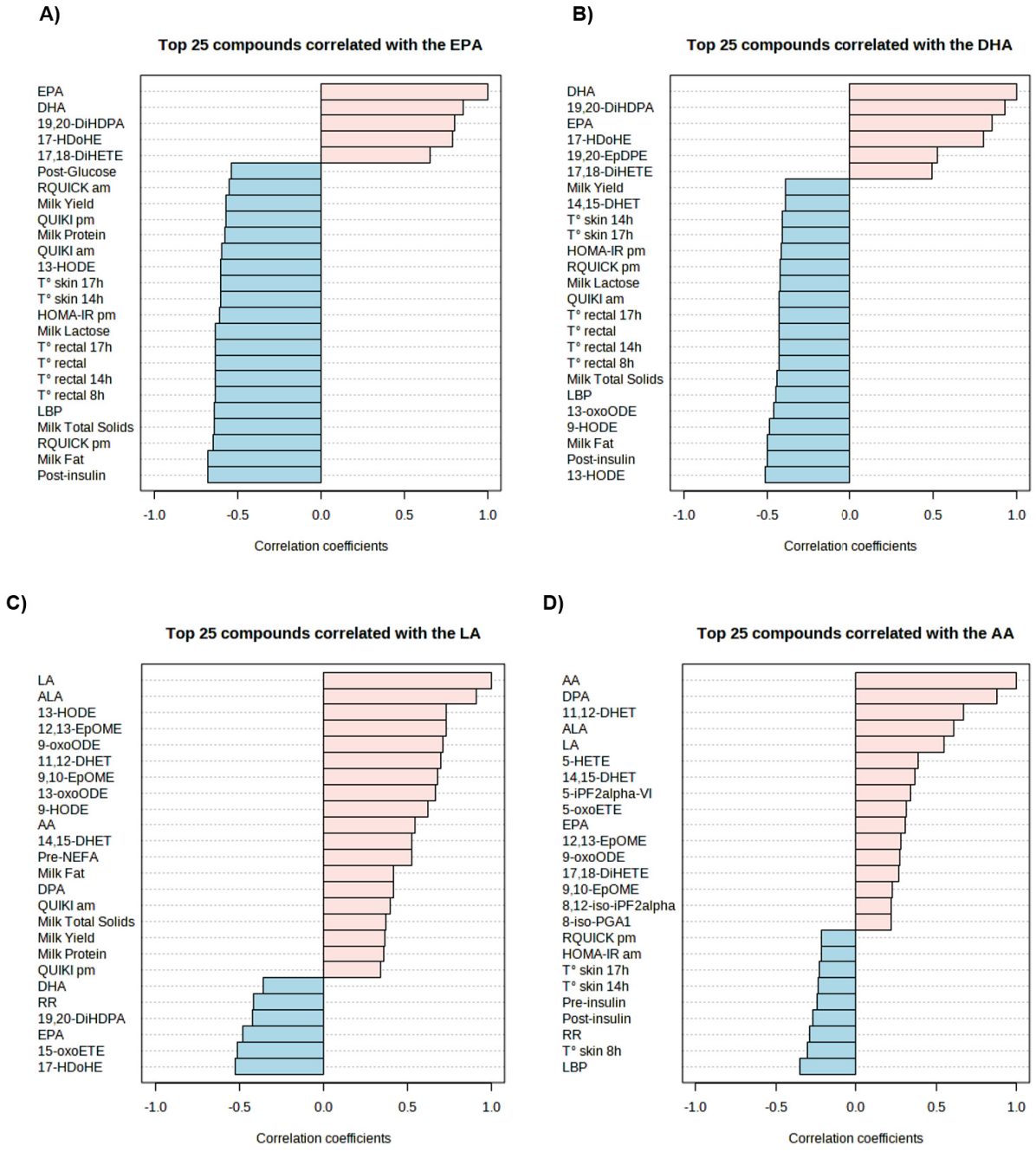

Figure 6: Non-directed metabolomics analysis of correlations.
Positive correlations are shown on the right in shades of pink, while negative correlations are shown on the left in shades of blue. The correlations shown in this illustration were calculated using the Pearson correlation coefficient on the Metaboanalyst platform. A) The 25 components that correlated positively or negatively with EPA (eicosapentaenoic acid), B) DHA (docosahexaenoic acid), C) LA (linoleic acid), and D) AA (arachidonic acid). The milk components are arranged in kg/day.

8 CONSIDERAÇÃO FINAL

Esta tese apresentou um estudo abrangente sobre os efeitos da suplementação lipídica em vacas leiteiras durante períodos de desafio fisiológico, com foco nos ácidos graxos de cadeia média (AGCM) e nos ácidos graxos ômega-3 e ômega-6. Os principais achados indicam que a suplementação com AGCM, embora não tenha influenciado significativamente a produção de leite e os metabólitos sanguíneos, afetou a expressão gênica e o perfil de ácidos graxos do leite. Além disso, a suplementação com ômega-3 mostrou um potencial significativo para modular mediadores lipídicos durante o estresse calórico, promovendo uma resposta anti-inflamatória.

Esses resultados contribuem para o conhecimento sobre a nutrição de vacas leiteiras, sugerindo que a suplementação lipídica pode ser uma estratégia eficaz para melhorar a saúde e a produtividade, especialmente sob condições de estresse. As implicações práticas deste estudo incluem o desenvolvimento de dietas mais eficientes para vacas leiteiras, potencialmente melhorando a qualidade do leite e a saúde animal.

No entanto, o estudo apresenta algumas limitações. A dose de AGCM utilizada pode ter sido insuficiente para provocar efeitos mais significativos, mas uma dose mais alta poderia causar efeitos negativos, como a redução do consumo e da digestibilidade da FDN. Futuros estudos devem explorar diferentes dosagens e combinações de ácidos graxos, além de investigar os mecanismos moleculares subjacentes às respostas observadas.

Em conclusão, esta tese fornece novas perspectivas sobre a suplementação lipídica em vacas leiteiras, com implicações para a prática agrícola e para a ciência da nutrição animal. O desenvolvimento contínuo neste campo poderá levar a práticas de manejo mais sustentáveis e produtivas, beneficiando produtores e animais.

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