EVELINE CATERINE SANDRI

EFEITO DA NUTRIÇÃO SOBRE O METABOLISMO DA GLÂNDULA MAMÁRIA DE VACAS E PORCAS LACTANTES

Tese apresentada ao Programa de Pós-graduação em Ciência Animal, da Universidade do Estado de Santa Catarina, como requisito parcial para obtenção do grau de Doutora em Ciência Animal

Orientador: Prof. Dr. Dimas Estrasulas de Oliveira

Co-orientador: Dr. Daniel E. Rico Navarrete

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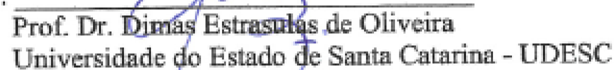

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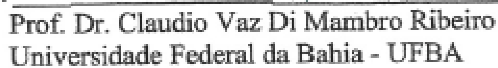

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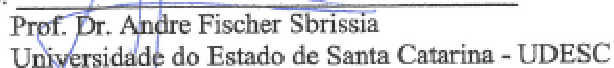

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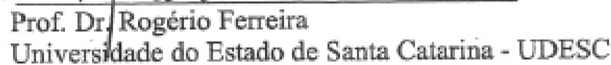

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Aos meus pais, Lauri e Justina, meus irmãos, Estefânia e Eduardo, e ao Rafael.

Dedico

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“Aquilo que marca a sua vida é o que você insiste em perseguir, em fazer melhor, em fazer dar certo.”

RESUMO

A gordura é responsável por muitas das características do leite e pode ser marcadamente afetada pela dieta. Em vacas leiteiras, mudanças na dieta que levam a distúrbios metabólicos, como acidose ruminal subaguda (ARS), causam depressão na gordura do leite (DGL) através da alteração na concentração de precursores ou inibidores da lipogênese mamária. Já em porcas, a redução no teor de gordura do leite tem o intuito de diminuir o custo energético da lactação e o catabolismo das reservas corporais, e a suplementação com o ácido linoleico conjugado (CLA) pode ser uma ferramenta nutricional, apesar de ainda se conhecer pouco sobre sua forma de ação nesses animais. O presente estudo visou avaliar as mudanças na síntese e composição da gordura do leite e as consequências metabólicas da ARS em vacas leiteiras e o efeito do CLA na composição do leite e expressão de genes lipogênicos na glândula mamária de porcas lactantes. No experimento 1, doze vacas foram distribuídas aleatoriamente, em um quadrado latino com 3 períodos de 21 dias, nos seguintes tratamentos: 1) Indução de ARS, 2) Recuperação, e 3) Controle. O teor de gordura do leite foi reduzido entre os dias 3 e 14 pela ARS ($P < 0,05$), enquanto que os teores de proteína e lactose foram aumentados nos dias 14 a 21 e 3 a 21, respectivamente ($P < 0,05$). A proporção de acetato:propionato e as concentrações de propionato e lactato foram maiores durante ARS em comparação com o Controle ($P < 0,05$). A concentração plasmática de insulina aumentou durante a ARS, enquanto que os ácidos graxos não-esterificados (AGNE) e β-hidroxibutirato (BHB) diminuíram ($P < 0,05$). A proporção de C18:1 *trans*-10:*trans*-11 no leite aumentou durante o período de indução de ARS ($P < 0,05$), mas o CLA *trans*-10, *cis*-12 não foi detectado. Os ácidos graxos ímpares foram aumentados e os ramificados foram reduzidos pela ARS ($P < 0,05$). No experimento 2, vinte porcas de uma linhagem comercial foram distribuídas aleatoriamente em um dos seguintes tratamentos, por 18 dias: 1) Controle (sem CLA), e 2) 1% de CLA misturado na ração. Comparado com o Controle, o tratamento com CLA diminuiu o teor de gordura do leite em 20% ($P = 0,004$) e reduziu o teor de proteína do leite em 11% ($P < 0,0001$). Apesar da redução no teor de gordura e proteína, o peso ao desmame dos leitões não foi diferente entre os tratamentos ($P = 0,60$). Na glândula mamária, o CLA reduziu a expressão de todos os genes avaliados (ACACAα, FASN, SCD1, LPL, AGPAT6 e DGAT1) exceto a proteína de ligação a ácido graxo 3 (FABP3). No tecido adiposo, o CLA não teve efeito na expressão de todos os genes avaliados. Como o tratamento com CLA reduziu o teor de proteína, consequentemente a expressão gênica da β-caseína e α-lactalbumina também foi reduzida. Estes resultados indicam que a ARS reduz o teor de gordura do leite e altera os metabólitos relacionados a DGL em vacas, porém esta não é associada somente à biohidrogenação ruminal e o CLA reduz o teor de gordura do leite das porcas sem afetar negativamente o desempenho da leitegada e seu efeito se dá sobre a expressão dos genes envolvidos em todas as vias lipogênicas.

Palavras-chave: Acidose. Expressão gênica. Lipogênese. Perfil de ácidos graxos.

ABSTRACT

Milk fat is responsible for many of the milk characteristics and can be markedly affected by diet. In dairy cows, dietary changes that lead to metabolic disorders, such as subacute ruminal acidosis (SARA), cause milk fat depression (MFD) by altering the concentration of precursors or inhibitors of mammary lipogenesis. In sows, the reduction in milk fat content is intended to decrease the energy cost of lactation and catabolism of body reserves, and supplementation with conjugated linoleic acid (CLA) can be used as a nutritional tool, although it is still known little about its action on these animals. The present study aims to evaluate changes in the synthesis and milk fat composition and the metabolic consequences of SARA in dairy cows and the effect of CLA on milk composition and lipogenic gene expression in the mammary gland of lactating sows. In experiment 1, twelve cows were randomly assigned in a Latin square design with 21-d periods, in the following treatments: 1) Induction of SARA, 2) Recovery, and 3) Control. The milk fat content was reduced on days 3 to 14 by SARA ($P < 0.05$), while protein and lactose contents were higher on days 14 to 21 and 3 to 21, respectively ($P < 0.05$). The proportion of acetate:propionate, and propionate and lactate concentrations were higher during SARA compared to Control ($P < 0.05$). Plasma insulin concentration increased during SARA, whereas non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) decreased ($P < 0.05$). The proportion of *trans*-10: *trans*-11 C18:1 in the milk increased during the induction period of SARA ($P < 0.05$), but the *trans*-10, *cis*-12 CLA was not detected. The odd fatty acids were increased, and the branched fatty acids were reduced by SARA ($P < 0.05$). In experiment 2, twenty sows from a commercial genotype were randomly assigned to one of the following treatments, for 18 days: 1) Control (without CLA), and 2) 1% of CLA mixed in the ration. Compared to Control, CLA treatment reduced milk fat content by 20% ($P = 0.004$) and reduced milk protein content by 11% ($P < 0.0001$). Despite the reduction in fat and protein content, weaning weight of piglets was not different between treatments ($P = 0.60$). In the mammary gland, CLA reduced the expression of all evaluated genes (ACACAα, FASN, SCD1, LPL, AGPAT6 and DGAT1) except the fatty acid binding protein 3 (FABP3). In adipose tissue, CLA had no effect on the expression of all evaluated genes. As the CLA treatment reduced the protein content, consequently the gene expression of β-casein and α-lactalbumin was also reduced These results indicate that SARA reduces milk fat content and alters MFD related metabolites in cows, but this is not only associated with ruminal biohydrogenation, and CLA reduces the fat content of milk in sows, without negatively affecting the performance of litter and its effect is on the expression of the genes involved in all lipogenic pathways.

Key-words: Acidosis. Fatty acid profile. Gene expression. Lipogenesis.

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LISTA DE ABREVIATURAS E SIGLAS

ACACAα	Acetil-CoA carboxilase alfa/ Acetyl-CoA carboxylase alpha
ACTB	Actin-beta
ADD-1	Adipocyte determination and differentiation factor-1
ADF	Acid detergent fiber
AGCL	Ácido graxo de cadeia longa
AGMI	Ácido graxo monoinsaturado
AGNE	Ácido graxo não-esterificado
AGPAT	Acil glicerol fosfato acil transferase/ Acyl glycerol phosphate acyltransferase
AGPI	Ácido graxo poliinsaturado
AGS	Ácido graxo saturado
aP2	Adipocyte fatty acid binding protein
ARS	Acidose ruminal subaguda
BHB	β-hidroxibutirato
BHBA	β-hydroxybutyrate
BW	Body weight
cDNA	Complementary DNA
CLA	Ácido linoleico conjugado/ Conjugated linoleic acid
CNF	Carboidratos não-fibrosos
CP	Crude protein
CSN1S1	Casein-α S1
CSN2	Casein-β
CSN3	Casein-κ
DGAT	Diacil glicerol acil transferase/ Diacylglycerol acyltransferase
DGL	Depressão da gordura do leite
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EDTA	Ethylenediamine tetraacetic acid
FA	Fatty acid
FABP	Proteína de ligação ao ácido graxo / Fatty acid binding protein

FASN	Ácido graxo sintase/ Fatty acid synthase
IDC	Intervalo desmame-cio
IGF	Insulin-like growth factor
LALBA	α-lactalbumin
LCFA	Long chain fatty acid
LFHO	Low-fiber, high-oil
LPL	Lipoproteína lipase/ Lipoprotein lipase
MFD	Milk fat depression
mTOR	Mammalian target of rapamycin
MUFA	Monounsaturated fatty acids
NDF	Neutral detergent fiber
NEFA	Non-esterified fatty acids
NRC	Nutrient requeriments council
OBCFA	Odd- and branched- chain fatty acids
OM	Organic matter
PBS	Phosphate-buffered saline
PPARγ	Receptores ativados por proliferadores de peroxissomo gama/ Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid
RT-qPCR	Quantitative real time - Polymerase chain reaction
RPS18	Ribosomal protein S18
SARA	Subacute ruminal acidosis
SCD1	Estearoil-CoA dessaturase 1/ Stearoyl-CoA desaturase 1
SFA	Saturated fatty acid
SREBP1	Proteína de ligação ao elemento regulatório esterol 1
TGFA	Triglyceride fatty acid
TMR	Total mixed ration
UDP	Uridine diphosphate
VFA	Volatile fatty acid
VLDL	Lipoproteína de muito baixa densidade/ Very low-density lipoprotein
WEI	Weaning-to-estrus interval

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1 INTRODUÇÃO

Maximizar a produção de leite tem sido o principal objetivo da indústria de lácteos por muitos anos. Recentemente, com a mudança no perfil da indústria e do consumidor, tem se dado maior ênfase a composição do leite com alta relação proteína:gordura (BEQUETTE et al., 2003).

Em ruminantes, principalmente vacas leiteiras, ao longo das décadas tem se visto um grande aumento na produtividade, resultado da seleção de animais superiores e melhorias nas práticas de manejo, incluindo a nutrição (OSORIO, LOHAKARE & BIONAZ, 2016).

A gordura do leite é o componente mais facilmente modificado pela dieta, exemplo clássico disso é a depressão da gordura do leite (DGL), e ao mesmo tempo é um dos principais desafios para os produtores, já que precisam se adaptar as demandas do mercado (MAXIN, RULQUIN & GLASSER, 2011). A fim de atender o potencial de produtividade das vacas, há a necessidade de se fornecer dietas de alta densidade energética, incluindo maiores quantidades de concentrados com consequente redução da quantidade de forragem, principalmente no início da lactação, que por serem de rápida degradação ruminal, levam ao acúmulo de ácidos orgânicos e queda do pH ruminal e consequente ocorrência da acidose ruminal subaguda (ARS). Além de problemas relacionados a saúde dos animais, a ARS afeta o teor de gordura do leite das vacas, e pela importância que esta tem para a indústria e o produtor, tem sido um dos principais assuntos de interesse por parte dos cientistas em estudar possíveis soluções para essa síndrome (KEUNEN et al., 2002).

Além de ser um dos componentes de maior importância para a indústria, a gordura representa a principal porção do custo energético da lactação para o animal (HARVATINE, BOISCLAIR & BAUMAN, 2009). Esse é um aspecto crítico por exemplo, em suínos, em que a produção e composição do leite das porcas é crucial para o crescimento e desempenho de seus leitões (FARMER, PALIN & HOVEY, 2010). As exigências nutricionais para a lactação em porcas são atendidas através da dieta ou mobilização das reservas corporais, o que consequentemente leva a redução da condição corporal (MULLAN & WILLIAMS, 1989). Porcas com menor peso corporal podem ter o ciclo reprodutivo subsequente prejudicado, com maior período de anestro pós-parto (intervalo desmame-cio, IDC), reduzindo assim a eficiência da produção. Para que esse período de anestro seja adequado, é necessário diminuir o catabolismo das reservas corporais, por meio da redução do custo energético da lactação, sem que o atendimento das exigências dos leitões seja prejudicado. Uma alternativa é a suplementação das porcas com o ácido linoleico conjugado

(CLA), que tem sido extensivamente estudado devido aos seus efeitos em humanos e animais (BELURY, 2002; PARIZA, 2004). O CLA *trans*-10, *cis*-12 foi identificado como o isômero responsável pela redução da síntese de gordura do leite em vacas (BAUMGARD et al., 2000) e da concentração de RNAm de várias enzimas lipogênicas na glândula mamária (BAUMGARD et al., 2002; PETERSON et al., 2003). O consumo de CLA por porcas durante a lactação tem mostrado que ele reduz as perdas de condição corporal bem como aumenta o peso ao desmame dos leitões (CORINO et al., 2009; CORDERO et al., 2011). Apesar dos estudos disponíveis com porcas em lactação comprovarem sua ação, os mecanismos específicos pelo qual o CLA reduz a gordura nesses animais não estão bem elucidados e podem ser melhor explorados.

Assim, independente da espécie animal e sua aptidão (leite, carne), aumentar a eficiência produtiva e qualidade dos produtos requer o conhecimento dos processos biológicos envolvidos no metabolismo dos nutrientes e desenvolvimento de soluções para problemas metabólicos que comumente afetam os animais. Nosso objetivo foi avaliar as mudanças nos parâmetros de lactação e a relação entre certos ácidos graxos do leite e as consequências metabólicas da ARS em vacas leiteiras e o efeito do CLA na composição do leite e expressão de genes lipogênicos na glândula mamária de porcas lactantes.

2 REVISÃO DE LITERATURA

2.1 COMPOSIÇÃO DO LEITE DOS ANIMAIS DOMÉSTICOS

O leite consiste de água, proteínas, lipídeos, carboidratos (principalmente lactose), minerais e vitaminas, e é produto da secreção das células epiteliais mamárias (REZAEI et al., 2016). As células do epitélio mamário possuem um alto nível de organização e uma marcante habilidade de converter os nutrientes circulantes nos componentes do leite (BAUMAN et al., 2006).

O leite é essencial para o crescimento, desenvolvimento e saúde dos neonatos. Nas espécies de interesse econômico (ex.: porcas e vacas), a produção de leite é um fator limitante para o máximo crescimento pré-desmame e sobrevivência da prole (AKERS, 2002; ARENDT & KUPERWASSER, 2015; KIM & WU, 2009).

A composição do leite varia entre espécies ou raças dentro de uma mesma espécie, entre estágios de lactação e diferentes intervalos de ordenha (JENNESS, 1986; JENNESS & SLOAN, 1970). A diferença entre espécies é o que mais afeta a quantidade dos principias componentes do leite. Animais com rápido crescimento, por exemplo, como coelhos e ratos, possuem altos teores de proteína no leite, enquanto que mamíferos marinhos, como golfinhos, focas, baleias e ursos polares, contém altos teores de gordura (PARK, HAENLEIN & WENDORFF, 2017). O componente mais constante no leite é a lactose, que varia de 3 a 7% entre as diferentes espécies (PARK, HAENLEIN & WENDORFF, 2017).

Independente da espécie e/ou raça, o efeito da dieta sobre a composição do leite ocorre principalmente sobre a gordura. Em ruminantes domésticos (ex.: vacas, ovelhas e cabras), o teor de lipídeos do leite varia de 4 a 9% (DEVLE et al., 2012); já não-ruminantes podem apresentar desde quantidades muito baixas de gordura, como asininos e equinos, que possuem de 0,14 a 1,3% de gordura, respectivamente (PARK, HAENLEIN & WENDORFF, 2017; DEVLE et al., 2012; GANTNER et al., 2015), até elevados teores, como os suínos, que apresentam em torno de 7,6% de gordura (HURLEY, 2015).

2.2 GORDURA DO LEITE

A gordura é o principal componente energético do leite e é responsável por muitas das propriedades físicas, características industriais e qualidade organoléptica do leite e seus derivados. É também o componente mais variável e fatores ambientais e fisiológicos que a afetam têm sido extensivamente caracterizados. A nutrição é o fator predominante que altera o teor e a produção de gordura e representa uma ferramenta prática para modificar a composição de ácidos graxos. O maior exemplo dos efeitos nutricionais sobre a gordura no leite é a síndrome da baixa gordura no leite, referida como depressão da gordura do leite (DGL), que ocorre em animais ruminantes (BAUMAN et al., 2006).

Os ruminantes lactantes têm sido utilizados como modelo animal para estudos sobre a síntese de gordura no leite. Trabalhos sobre captação de nutrientes pela glândula mamária, vias de biossíntese e a relação entre dieta e composição da gordura têm sido primeiramente realizados nestes animais e os resultados extrapolados e comparados com outros mamíferos (BAUMAN & GRIINARI, 2003; NEVILLE & PICCIANO, 1997).

Embora muito se sabe sobre a bioquímica da síntese de gordura no leite, os sistemas regulatórios e de sinalização celular da lipogênese ainda não são bem compreendidos. (BAUMAN et al., 2008).

2.2.1 Síntese e composição da gordura do leite

A gordura do leite é composta de aproximadamente 98% de triglicerídeos, constituídos principalmente de ácidos graxos e, em menor proporção, de fosfolipídios, colesterol, glicerol e ácidos graxos livres (BERNARD, LEROUX & CHILLIARD, 2008). Os ácidos graxos são sintetizados a partir de duas vias: da síntese *de novo* na glândula mamária ou a partir dos ácidos graxos circulantes, provenientes da dieta ou das reservas corporais.

A diferença entre espécies na composição da gordura do leite reflete a importância da fonte de ácidos graxos usada para a síntese. Em ruminantes, os ácidos graxos sintetizados *de novo* são provenientes do acetato e butirato produzidos pela fermentação ruminal dos carboidratos, resultando em ácidos graxos de cadeia curta e média (C4:0 a C16:0), que representam 40 a 50% dos ácidos graxos secretados no leite desses animais (BERNARD, LEROUX & CHILLIARD, 2008). Os demais ácidos graxos, de cadeia longa (C18:0) e parte do C16:0, são derivados dos lipídeos dietéticos circulantes, na forma de quilomícron ou lipoproteína de muito baixa densidade (VLDL), ou dos ácidos graxos não-esterificados

(AGNE) mobilizados do tecido adiposo. Neste último caso, a mobilização dos AGNEs ocorre com maior intensidade em animais em balanço energético negativo (especialmente no início da lactação; BAUMAN & GRIINARI, 2003).

Em não-ruminantes, a glicose é utilizada para a síntese *de novo* dos ácidos graxos (BAUMAN & GRIINARI, 2003), contudo, boa parte dos lipídeos do leite são provenientes da dieta ou das reservas corporais (BEE, 2000b). Como exemplo disso, Hartmann & Holmes (1989) reportaram que a maioria dos ácidos graxos do leite de porcas refletem aqueles presentes nos triglicerídeos circulantes, que por sua vez são influenciados pelo tipo de gordura ingerida pela porca. Dessa forma, o leite de porcas possui pequenas quantidades de ácidos graxos de cadeia curta e média (CSAPO et al., 1996; LAURIDSEN & DANIELSEN, 2004).

Prévios estudos demonstraram que as vias bioquímicas da glândula mamária relacionadas a biossíntese e secreção de lipídeos são controladas por um complexo de genes e fatores de transcrição (ANDRES & DJONOV, 2010; RUDOLPH, NEVILLE & ANDERSON, 2007) e como a composição da gordura e ácidos graxos do leite é diferente entre as espécies (ex.: ruminantes e não-ruminantes), sugere-se que pode haver diferenças também na regulação desses genes (SHI et al., 2015).

Entre os principais genes que codificam enzimas lipogênicas e que foram primeiramente estudados estão a acetil-CoA carboxilase alfa (ACACAα) e ácido graxo sintase (FASN), envolvidas na via da síntese *de novo*; lipoproteína lipase (LPL) e proteína de ligação ao ácido graxo (FABP), que fazem a captação e transporte intracelular (respectivamente) dos ácidos graxos circulantes. Além disso, a estearoil-CoA dessaturase 1 (SCD1) faz a dessaturação dos ácidos graxos saturados, resultando na síntese de ácidos graxos monoinsaturados *cis*-9, e a acil glicerol fosfato acil transferase (AGPAT) e diacil glicerol acil transferase (DGAT), que formam os triglicerídeos (BERNARD, LEROUX & CHILLIARD, 2008).

Ainda de acordo com Bernard, Leroux & Chilliard (2008), a mudança na expressão gênica devida a ação dos nutrientes da dieta envolve o controle de eventos que ocorrem em nível transcricional, pós-transcricional, traducional e pós-traducional. Estudos sobre a regulação da expressão dos genes lipogênicos mamários são disponíveis principalmente em ruminantes, principalmente com dietas que causam a DGL. Ao contrário, em suínos, pouco se sabe sobre os efeitos dos ácidos graxos dietéticos sobre a regulação dos genes (DURAN-MONTGÉ et al., 2009), e os estudos existentes são realizados no fígado (THEIL & LAURIDSEN, 2007), músculo e tecido adiposo de animais em crescimento ou adipócitos cultivados *in vitro* (HSU et al., 2004; LIU et al., 2005).

2.2.2 Depressão da gordura no leite (DGL)

A DGL representa um dos principais desafios que envolvem a interrelação entre os processos digestivos e o metabolismo dos tecidos. Ela ocorre naturalmente quando os animais são alimentados com dietas contendo carboidratos altamente fermentáveis e/ou suplementados com óleos vegetais e/ou de peixe (BAUMAN & GRIINARI, 2003).

Descrita há muito tempo, a DGL pode reduzir até 50% a produção de gordura no leite e esse decréscimo envolve os ácidos graxos de todos os tamanhos de cadeia. Muitas teorias têm sido propostas para explicar as causas da DGL, mas muitas delas se tornaram inadequadas ao longo do tempo (BAUMAN & GRIINARI, 2003; GRIINARI & BAUMAN, 2006). Entre elas, há as teorias que consideram a depressão como consequência da diminuição dos precursores lipídicos para a glândula mamária e aquelas que atribuem a DGL a direta inibição das etapas da síntese de gordura (BAUMAN & GRIINARI, 2001). Duas dessas teorias serão melhor discutidas nesta revisão.

2.2.2.1 Teoria glicogênica-insulínica

A teoria glicogênica-insulínica é a principal teoria que envolve a falta de precursores lipogênicos como causa da DGL e sugere que a insulina tem papel importante no processo de depressão da síntese de gordura (DAVIS & BROWN, 1970). Ela é baseada na competição de nutrientes entre a glândula mamária e outros tecidos lipogênicos (ex.: tecido adiposo) e a diferença de resposta entre eles (BAUMAN & GRIINARI, 2003).

McClymont & Vallance (1962) sugeriram que a alta concentração ruminal de propionato e o aumento das taxas de gliconeogênese hepática podem resultar no aumento da insulina circulante e, consequentemente, reduzir a mobilização de ácidos graxos de cadeia longa das reservas corporais, os quais são precursores para a lipogênese mamária (BAUMAN & GRIINARI, 2003).

Contudo, estudos com infusão abomasal e/ou venosa de propionato, glicose ou insulina, mostraram resultados controversos entre si com relação a redução do teor de gordura no leite (CORL et al., 2006; MCGUIRE et al., 1995). Acredita-se que essas diferenças com relação a DGL se devem ao balanço energético dos animais. Isso é suportado por Corl et al. (2006), que observaram que a redução na gordura do leite poderia ser atribuída a mobilização das reservas corporais devido as vacas avaliadas estarem no início da lactação.

2.2.2.2 Teoria da biohidrogenação

A teoria da biohidrogenação é atualmente a mais aceita e sugere que a síntese de gordura no leite é inibida por ácidos graxos *trans*, os quais são resultado de alterações na biohidrogenação ruminal dos ácidos graxos poliinsaturados (AGPI) da dieta.

Griinari et al. (1998) propuseram que a suplementação dos animais com AGPI modifica os processos microbianos do rúmen e isso envolve alterações na via de biohidrogenação, que resulta em um aumento na formação de C18:1 *trans*-10 e intermediários relacionados. Esses mesmos autores observaram que em vacas recebendo dietas com baixa fibra e altos teores de ácidos graxos insaturados, apresentaram maior concentração de ácidos graxos *trans* no leite com a concomitante redução no teor de gordura no leite, suportando a hipótese de que os ácidos graxos *trans* estão envolvidos na DGL.

Em uma condição normal de biohidrogenação do ácido linoleico (C18:2 *cis*-9, *cis*-12), a primeira etapa é a isomerização, que forma o ácido linoleico conjugado (CLA) *cis*-9, *trans*-11. Ela é seguida por duas reduções que formam o C18:1 *trans*-11 e então o ácido esteárico (C18:0). Consistente com isso, o CLA *cis*-9, *trans*-11 e o C18:1 *trans*-11 são os principais ácidos graxos encontrados tipicamente na gordura do leite. Contudo, em condições já citadas, a biohidrogenação pode ser alterada e neste caso a etapa inicial de isomerização formaria o CLA *trans*-10, *cis*-12 (GRIINARI & BAUMAN, 1999).

Ácido linoleico conjugado (CLA) é um termo genérico usado para descrever os isômeros posicionais e geométricos, com duas duplas ligações conjugadas, do ácido linoleico (C18:2). Os dois isômeros mais estudados pelos seus efeitos biológicos são o *cis*-9, *trans*-11, o isômero predominante em produtos de ruminantes, e o *trans*-10, *cis*-12.

O CLA *trans*-10, *cis*-12 foi o isômero identificado como efetivo agente inibidor da síntese de gordura na glândula mamária e no tecido adiposo em várias espécies (BAUMGARD et al., 2000; OSTROWSKA et al., 2003). Com relação a animais lactantes, vacas apresentaram uma redução de 25, 40 e até mais de 50% no teor de gordura do leite quando receberam o isômero CLA *trans*-10, *cis*-12 (BAUMGARD et al., 2000; PETERSON et al., 2003; PIPEROVA et al., 2000). A DGL ocasionada pelo CLA *trans*-10, *cis*-12 foi associada com o efeito negativo do isômero sobre a atividade enzimática ou abundância de mRNA dos genes codificadores das enzimas envolvidas nos processos de síntese *de novo* (ACACAα e FASN), dessaturação (SCD1), captação e transporte de ácidos graxos (LPL e FABP) e síntese de triglicerídeos (AGPAT e DGAT; AHNADI et al., 2002; PETERSON et al., 2003; PIPEROVA et al., 2000).

A expressão desses genes é regulada por fatores de transcrição já caracterizados, como a proteína de ligação ao elemento regulatório esterol 1 (SREBP1) e os receptores ativados por proliferadores de peroxissomo gama (PPARγ). A SREBP1 é altamente expressa na glândula mamária, onde está fortemente correlacionado com a expressão da FASN e LPL (HARVATINE & BAUMAN, 2006). Estudos reportaram que houve redução na sua expressão durante a cultivo de células epiteliais mamárias com o CLA *trans*-10, *cis*-12 ou em vacas recebendo CLA ou dietas que induziam a DGL (PETERSON, MATITASHVILI & BAUMAN, 2004; HARVATINE & BAUMAN, 2006), bem como dos genes envolvidos na síntese de lipídeos que são regulados por ela. Ainda, tem se especulado o papel do PPARγ sobre a DGL, e o que tem se observado é que ele parece ter sua expressão reduzida pelo CLA nas células epiteliais mamárias, assim como seus possíveis genes alvos (KADEGOWDA et al., 2009).

2.3 ACIDOSE RUMINAL SUBAGUDA (ARS) E DGL EM VACAS

2.3.1 Caracterização e diagnóstico

A acidose ruminal subaguda (ARS) é uma das principais desordens digestivas em criações intensivas de vacas leiteiras (GUO et al., 2013). A ARS ocorre quando o pH ruminal é menor que 5,6 durante longos períodos (mais de 3 horas/dia), causado pela alimentação com altas quantidades de carboidratos não-fibrosos (CNF) que alteram o perfil de fermentação ruminal, com o acúmulo de ácidos orgânicos e redução na capacidade de tamponamento (NRC, 2001). Os principais problemas relacionados a acidose são a redução na degradação ruminal do alimento e consequente redução no consumo, mudança na produção e composição do leite, ocasionando a DGL, e em casos mais sérios pode levar a problemas como laminite, abcessos de fígado e inflamações (PLAIZIER et al., 2008).

A medida do pH do fluido ruminal é a forma mais confiável e acurada para o diagnóstico da ARS. Por essa razão, várias técnicas são disponíveis para medir o pH ruminal, seja em condições experimentais ou nas fazendas comerciais (PENNER, BEAUCHEMIN & MUTSVANGWA, 2006), tais como ruminocentese, sondagem oro-ruminal e sondas para medir o pH diretamente no rúmen. Contudo, a ruminocentese e as sondas oro-ruminais possuem alguns problemas, como flutuações diárias na fermentação ruminal e problemas em se obter amostras representativas do fluido ruminal para a medida do pH (GIANESELLA et al., 2010). Além disso, a ruminocentese tem sido associada a abscessos e peritonite e a determinação do

pH por sonda oro-ruminal é menos precisa, pois as amostras são susceptíveis à contaminação por saliva (DUFFIELD et al., 2004).

A melhor forma de avaliar o pH do rúmen é inserir uma sonda diretamente no rúmen e registrar o pH em tempo real (DADO & ALLEN, 1993). Porém, quando uma sonda é liberada via boca do animal, é mais provável que ela permaneça no retículo e comparado ao pH ruminal, o pH reticular é relativamente maior e mais estável devido a diluição pela saliva (DUFFIELD et al., 2004). Curiosamente, a possibilidade da ARS ser diagnosticada pela medida contínua do pH reticular em comparação ao pH ruminal foi avaliada por Kimura et al. (2012), que observaram uma correlação positiva entre o pH reticular e ruminal, o que permitiria utilizar, nesse caso, as medidas de pH reticular para detectar a ARS em vacas.

Além das mudanças no pH ruminal, a ARS tem efeitos metabólicos que consequentemente alteram a composição do leite e a saúde animal. A mudança nesses parâmetros metabólicos podem ser interessantes métodos não-invasivos de se diagnosticar a acidose em vacas em lactação.

2.3.2 Efeitos da ARS sobre a composição do leite e metabolismo

Há um crescente interesse no perfil de ácidos graxos do leite como uma potencial ferramenta de diagnóstico da atividade ruminal (COLMAN et al., 2010). Sabe-se que o baixo pH do rúmen causado pela alimentação com dietas de baixa fibra ou alto concentrado, o qual pode levar a ARS, resulta na incompleta biohidrogenação dos ácidos graxos e aumento nos ácidos *trans*-octadecadienóicos, especialmente o CLA *trans*-10, *cis*-12, que causa a DGL e altera o perfil de ácidos graxos do leite (GRIINARI et al., 1998; ENJALBERT et al., 2008).

Recentemente, Mitchell et al. (2016) suplementaram vacas em lactação com uma dieta com baixa fibra para investigar o efeito da acidose sobre o perfil de ácidos graxos do leite. Os autores encontraram que o tratamento com baixa fibra aumentou a concentração de ácidos graxos de cadeia ímpar e reduziu a concentração de ácidos graxos ramificados no leite. A produção de propionato é fortemente aumentada nessa situação (BAUMAN & GRIINARI, 2001) e é reconhecido que ele é um precursor dos ácidos graxos de cadeia ímpar no leite (MASSART-LEËN et al., 1983) e a redução nos ácidos graxos de cadeia ramificada é provavelmente devido ao declínio na relação forragem:concentrado e consequente mudança no ambiente e nos microrganismos ruminais. Maiores concentrações de ácidos graxos ramificados são tipicamente um resultado de dietas com alta forragem, devido a maior população de

bactérias celulolíticas que produzem acetato e este é precursor dos ácidos graxos ramificados (PATEL, WREDLE & BERTILSSON, 2013).

Vários trabalhos mostram que a ARS reduz as concentrações do C18:0, C18:1 *trans*-11 e do CLA *cis*-9, *trans*-11 e aumenta os teores de C18:2n-6, C18:3n-3, C18:1 *trans*-10 e CLA *trans*-10, *cis*-12 (ENJALBERT et al., 2008; MITCHELL et al., 2016). Esses resultados sugerem que a ARS é resultado de modificações em diferentes etapas da biohidrogenação, onde o baixo pH ruminal é conhecido por modificar principalmente a etapa de isomerização (TROEGELER-MEYNADIER, BRET-BENNIS & ENJALBERT, 2006).

Embora a teoria da DGL mais aceita é a que envolve o papel específico do CLA *trans*-10, *cis*-12 (como mencionado anteriormente), Enjalbert et al. (2008) observaram que uma dieta com a inclusão de 34% de trigo comparada com 20% de trigo fornecida para vacas em lactação, significantemente aumentou a proporção do CLA *trans*-10, *cis*-12 na gordura do leite mas ambas as dietas (com 20 e 34% de trigo) diminuíram o teor de gordura no leite. Além disso, o retorno a dieta com 0% de trigo resultou na completa recuperação do teor de gordura no leite, mas, numericamente, os animais apresentaram uma maior proporção do CLA *trans*-10, *cis*-12 que durante o período sem adição de trigo. Isso sugere que o CLA *trans*-10, *cis*-12 pode não ser responsável por todas as modificações do teor de gordura do leite de vacas com ARS, como apontado por Loor et al. (2005).

Como a relação entre ARS e a DGL é controversa e complexa, os metabólitos do plasma (ex.: AGNE e insulina) são frequentemente usados para monitorar a saúde e o status metabólico de vacas leiteiras, e Ametaj et al. (2009) indicaram que vacas alimentadas com dietas com alta concentração de carboidratos rapidamente fermentáveis poderiam ter consideráveis alterações nos padrões de metabólitos plasmáticos.

Os níveis de AGNE no plasma têm sido usados como indicador do status energético de vacas em lactação (STAPLES, THATCHER & CLARK, 1990) e a liberação no plasma é baseada na sua mobilização do tecido adiposo. Guo et al. (2013) alimentaram vacas com dietas com diferentes proporções de trigo finamente moído para induzir a acidose e observaram que a alta proporção de trigo estimulou a produção de propionato e isso aumentou os níveis de insulina. Por sua vez, a insulina estimula a lipogênese e inibe a lipólise no tecido adiposo, diminuindo assim o fluxo de AGNE para a glândula mamária (BAUMAN & GRIINARI, 2003).

A redução de AGNE pode contribuir principalmente para a redução na síntese de ácidos graxos de cadeia longa (AGCL), os quais são derivados das reservas corporais (CORL et al., 2006). Contudo, a mobilização das reservas corporais para a síntese de gordura no leite é maior no início da lactação. Nesse período, quando o consumo voluntário é inadequado, as vacas estão

em um substancial balanço energético negativo, e os AGNEs circulantes são elevados. Nessa situação, as reservas corporais mobilizadas representam a principal fonte de ácidos graxos para a gordura do leite e a insulina marcadamente reduz as taxas de lipólise, como indicado pelas mudanças nos níveis de AGNE plasmáticos, e resulta em uma redução em torno de 35% na produção de gordura no leite. Em contraste, vacas no meio da lactação estão em balanço energético positivo e os AGNEs circulantes baixos. A lipólise é também inibida pelo aumento na insulina como indicado pela redução nos AGNE, mas neste caso, ácidos graxos derivados das reservas corporais representam uma menor fonte de ácidos graxos para o leite, de modo que a redução de gordura pode ser em torno de 6% ou menos (BAUMAN & GRIINARI, 2003).

Por isso, as alterações nas concentrações de metabólitos relacionados ao metabolismo de lipídeos e carboidratos (ex.: AGNE e insulina) no plasma são alternativas para o diagnóstico de ARS em vacas sob mesmo status fisiológico e ambiente (GUO et al., 2013).

2.4 ÁCIDO LINOLEICO CONJUGADO (CLA) E DGL EM PORCAS

Como mencionado anteriormente, o consumo do CLA *trans*-10, *cis*-12 durante a lactação modifica a composição dos ácidos graxos do leite (CHIN et al., 1994; BEE, 2000a), e sua infusão no abomaso, na forma protegida ou não da biohidrogenação ruminal, tem mostrado deprimir o teor de gordura no leite de ruminantes (CHOUINARD et al., 1999). Assim como em ruminantes, em porcas os isômeros dietéticos são excretados no leite e dessa forma são disponíveis para os leitões (BEE, 2000a), alterando também a composição corporal de ácidos graxos e o metabolismo lipídico de animais em crescimento.

Em suínos, o período de lactação representa a fase em que grande parte da energia consumida é direcionada para a produção de leite e desempenho dos leitões, em detrimento à reprodução (BUTLER, 2005; MELLAGI, 2011). Dessa forma, as perdas corporais são inevitáveis e a energia oriunda das reservas corporais são frequentemente usadas para atender o total das exigências energéticas da lactação (NOBLET et al., 1998). Assim, é importante minimizar as perdas de reservas corporais durante a lactação bem como manter o máximo crescimento dos leitões e o desempenho reprodutivo subsequente (LEE et al., 2014).

O fornecimento do CLA pode ser uma alternativa, conforme mostrado no estudo de Harrel et al. (2000), que forneceram uma dieta com 1% de CLA para porcas em lactação com o intuito de reduzir o conteúdo de gordura do leite e constataram que as reservas corporais não foram alteradas pelo CLA. Esses autores sugeriram que pode haver uma diminuição no custo

energético de lactação pela redução da energia total do leite (menor teor de gordura), sem afetar o desempenho da porca e da leitegada.

Em não-ruminantes, mecanismos pelo qual o CLA altera a lipogênese mamária não estão bem definidos (HAYASHI et al, 2007). O efeito do CLA sobre a composição e produção de gordura no leite de vacas já está melhor estabelecido (BERNAL-SANTOS et al., 2003; DE VETH et al., 2004), sendo aceito que o CLA marcadamente afeta o metabolismo lipídico e reduz a concentração de gordura (CHOUINARD et al., 1999). O limitado número de experimentos com porcas lactantes indica uma resposta similar (HARRELL et al., 2000; POULOS, AZAIN & HAUSMAN, 2004). Contudo, em porcas, a redução no teor de gordura do leite é menor que em vacas, podendo chegar a 36% (HARRELL et al., 2000). Esses mesmos autores explicaram que essa diferença pode ser devida as formas de resposta das espécies ou pela fonte de ácidos graxos utilizados para a síntese de gordura.

Em suínos em crescimento, estudos que avaliam o efeito do CLA *trans*-10, *cis*-12 sobre o desempenho e a composição corporal têm sido mais explorados pelo interesse em se melhorar a qualidade dos produtos (ex.: redução de gordura e fonte de CLA para alimentação humana). A seleção genética na suinocultura tem resultado em carcaças mais magras, porém, mais estudos são necessários para melhorar a qualidade da carne, que está associada com os níveis de gordura intramuscular. A adição de CLA nas dietas é uma forma potencial de aumentar a gordura intramuscular e concomitantemente reduzir a gordura subcutânea (MOREL et al., 2008). Contudo, os efeitos do CLA sobre suínos em crescimento são controversos (CORINO et al., 2006; DUGAN, AALHUS & KRAMER, 2004) e estão relacionados ao peso dos animais e/ou duração da suplementação do CLA.

Andretta et al. (2009) realizaram uma meta-análise com o intuito de determinar a relação entre o fornecimento de CLA na dieta e o desempenho e características da carcaça e da carne em suínos. A base de dados utilizada contemplou 15 artigos publicados entre 1999 e 2006 e o que se observou foi que não houve alteração no consumo de ração, no ganho de peso e na eficiência alimentar dos suínos, porém o CLA aumentou o conteúdo de carne magra na carcaça e seu consumo variou a espessura média de toucinho. Ou seja, o CLA alterou a composição corporal, sem influenciar o desempenho dos suínos.

Além de redirecionar a síntese de gordura nos tecidos, o CLA modifica a composição dos ácidos graxos que a constituem. Bee (2000a) conduziu um trabalho para avaliar os efeitos do CLA sobre o desempenho e composição da gordura subcutânea de leitões desmamados de porcas que tinham sido suplementadas ou não com CLA durante a gestação e lactação e que passaram a receber também CLA após o desmame, durante 35 dias. O CLA aumentou os níveis

de C12:0, C18:0 e os ácidos graxos saturados (AGS) totais, enquanto que o C16:1n-7, C20:1n-9, ácido linoleico e os ácidos graxos monoinsaturados (AGMI) totais foram reduzidos no tecido adiposo.

A partir desse mesmo trabalho, Bee (2000b) avaliou a composição da gordura do tecido adiposo e do leite das porcas suplementadas com CLA e os resultados foram similares: ele aumentou os níveis de AGS, principalmente o ácido esteárico (C18:0) e reduziu os AGMI no tecido e no leite, o que foi observado posteriormente em outro trabalho (CORDERO et al., 2011). Por se tratar de uma mistura de isômeros, Bee (2000a) observou que a incorporação e eficiência de captação nos tecidos foi maior para o isômero *cis*-9, *trans*-11, e isso deve ser considerado sobre os efeitos observados no perfil de ácidos graxos. Apesar de não ter sido avaliada a expressão gênica ou atividade enzimática, esses resultados evidenciam também a ação do CLA sobre a expressão gênica da SCD1, responsável pela dessaturação dos ácidos graxos e dos genes da síntese *de novo* (BEE, 2000b; CORINO et al., 2003).

Sabe-se que o CLA altera o metabolismo lipídico atuando direta ou indiretamente na expressão de genes que codificam as enzimas lipogênicas responsáveis pelo aumento e/ou diminuição da síntese, deposição e mobilização de ácidos graxos e genes relacionados ao metabolismo da glicose (JOSÉ, 2008). Contudo, trabalhos que avaliem a ação do CLA sobre a expressão dos genes envolvidos na lipogênese no tecido adiposo e glândula mamária em animais não-ruminantes ainda são escassos. Animais em crescimento suplementados e células adiposas cultivadas com o CLA têm mostrado o seu efeito negativo sobre a expressão das enzimas ACACAα e LPL (CORINO et al., 2003; CORL et al., 2008; ZHOU et al., 2007), inferindo que a ação do CLA nesse tecido se dá sobre ambas as vias de síntese: *de novo* e captação dos ácidos graxos circulantes.

Essa autora não tem conhecimento de trabalhos com expressão de genes lipogênicos na glândula mamária de porcas lactantes. Em ratas e ovelhas lactantes se observou que os ácidos graxos sintetizados *de novo* tiveram uma redução mais pronunciada com a concomitante redução na expressão dos genes da ACACAα, FASN e SCD1 (HAYASHI et al., 2007; HUSSEIN et al., 2013). Pelas particularidades das espécies, futuros trabalhos auxiliarão na explicação da ação do CLA em nível molecular em porcas lactantes.

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4 ARTIGO

INDUCTION AND RECOVERY FROM SUBACUTE RUMINAL ACIDOSIS (SARA): EFFECTS ON MILK FAT SYNTHESIS AND METABOLIC PROFILE OF DAIRY COWS

ABSTRACT

Subacute ruminal acidosis (SARA) is one of the main metabolic diseases observed in modern dairy cows. Importantly, SARA can sometimes be accompanied by milk fat depression, which might depend on diet polyunsaturated fatty acid (PUFA) content. The present study evaluated the timing of metabolic changes and milk fat synthesis during induction and recovery from SARA in high concentrate diets. Twelve ruminally cannulated cows were randomly assigned to treatment in a Latin square design with 21-d periods. Treatments were 1) SARA Induction, 2) Recovery, and 3) Control. SARA was induced by feeding a diet containing 29.36% starch, 24.03% neutral detergent fiber (NDF), and 2.8% fatty acids (FA), whereas the Recovery and Control diets contained 19.95% starch, 31.03% NDF, and 2.57% FA. Experimental sampling took place on days 0, 3, 7, 10, 14, 17, and 21 of each period. Data were analyzed as repeated measures using the MIXED procedure of SAS. Dry matter intake (DMI) and milk yield were increased by SARA on days 14 to 21 and 10 to 21, respectively ($P < 0.05$). Milk fat content was reduced on days 3 to 14 by SARA induction ($P < 0.05$), while greater protein and lactose content were observed on days 14 to 21 and 3 to 21, respectively ($P < 0.05$). The acetate-to-propionate ratio, and the concentrations of propionate and lactate were greater during SARA as compared with the Control ($P < 0.05$). Plasma insulin concentrations increased during SARA, whereas plasma non-esterified fatty acids (NEFA) and milk β-hydroxybutyrate (BHBA) decreased ($P < 0.05$). The ratio of *trans*-10 to *trans*-11 C18:1 increased during the SARA induction period ($P < 0.05$), but concentration of *trans*-10 C18:1 remained below 0.5% of FA, and *trans*-10, *cis*-12 conjugated linoleic acid (CLA) was not detected. Odd-chain fatty acids were increased, and the branched-chain fatty acids were reduced by SARA induction ($P < 0.05$). SARA reduced milk fat synthesis transiently, however, such reduction was not associated with ruminal biohydrogenation, but rather with a reduced supply of preformed FA.

Keywords: Acidosis. Fatty acid profile. Insulin. Milk fat depression.

4.1 INTRODUCTION

High production animals require high-grain diets that are rich in starch to increase energy intake and meet the energy requirements of lactation (ABDELA, 2016), however, these conditions increase the risk of subacute ruminal acidosis (SARA).

The SARA phenotype is characterized by low rumen pH and is one of the most important metabolic disorders in dairy cows (COLMAN et al., 2015). During SARA, rumen pH is depressed for several hours per day due to accumulation of organic acids and insufficient rumen buffering (PLAIZIER et al., 2008). There are no typical clinical signs in SARA affected cows (KRAUSE & OETZEL, 2005; MUTSVANGWA et al., 2002; TAJIK & NAZIFI, 2011). However, SARA consequences are diverse and complex, which include feed intake depression, reduced ruminal digestion, milk fat depression, gastrointestinal damage, liver abscesses and lameness (KRAUSE & OETZEL, 2006; PLAIZIER et al., 2008; RADOSTITS et al., 2007).

The low pH of the rumen caused by diets with low fiber or high concentrate, which can lead to SARA, results in the incomplete biohydrogenation of fatty acids and increased *trans*-octadecadienoic acids, especially *trans*-10, *cis*-12 conjugated linoleic acid (CLA), which causes milk fat depression (MFD) and changes the milk fatty acid (FA) profile (GRIINARI et al., 1998; ENJALBERT et al., 2008). At the same time, the high ruminal concentration of propionate may result in increased circulating insulin, which consequently reduces the mobilization of long chain fatty acids (LCFA) from body reserves, which are precursors for mammary lipogenesis (BAUMAN & GRIINARI, 2003).

Maxin et al. (2011a) infused acetate and propionate in the rumen and the isomer *trans*-10, *cis*-12 CLA in the duodenum of lactating cows, separated and together, to evaluate their effect on milk fat yield and composition, and the interaction between them. The authors observed that when propionate and *trans*-10, *cis*-12-CLA were infused together, their effects on milk fat secretion cumulated, leading to a greater reduction (22%) in milk fat content than when infused separately (9 and 15%, respectively). These results show that these 2 inhibiting nutrients acted simultaneously on mammary lipogenesis. The milk FA profile was also altered additively, exhibiting a decrease in even short- and medium-chain FA, and an increase in odd-chain FA.

Easily accessible and inexpensive markers of SARA are needed for the diagnosis of ruminal health problems (DANSCHER et al., 2015). Various analyses of blood, urine, feces, and milk have been considered and evaluated for this purpose (PLAIZIER et al., 2008; LI et

al., 2012; ENEMARK, JORGENSEN & KRISTENSEN, 2004), but the results of these studies are conflicting.

Odd- and branched- chain fatty acids (OBCFA) in milk fat are largely derived from rumen bacteria. OBCFA in milk already show potential as biomarkers of rumen function. This indicates milk OBCFA might give most scope for rumen VFA predictions in research units in which a limited number of fistulated lactating animals could be used to assess the experimental effect or under practical conditions to monitor dietary or management changes over time. Furthermore, OBCFA in milk fat are candidates for the early detection of ruminal acidosis and duodenal flow of microbial protein (FIEVEZ et al., 2012). Recently, Jing et al. (2018) differentiated cows susceptible or not to SARA according to the milk FA pattern and distinguished differences not only in *trans* fatty acid concentrations, but also in *iso* C14:0, *iso* C16:0 and C15:0 FA.

The objective of this study was to evaluate temporal changes in the synthesis and milk fat composition and metabolites related to SARA induction and recovery in high concentrate diets.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design and treatments

Twelve ruminally cannulated Holstein cows subdivided into 2 groups and randomly assigned according to their stage of lactation (A: 89,8 ± 42 days in milk [DIM], 40,8 ± 5,8 kg milk/d and B: 170,3 ± 25,4 DIM, 30,2 ± 5,9 kg milk/d) from the permanent herd of the Centre de recherche en sciences animals de Deschambault (CRSAD, Deschambault, QC) were used in this study. The experiment was conducted from December 2016 to March 2017. Animals were housed individually in tie stalls with continuous access to water. Two diets were utilized: 1) a diet containing a high proportion of concentrates to induce the SARA; 2) a conventional ration with lower levels of concentrates (Control and Recovery diets). The experimental period was three 21-day periods, preceded by an adjustment period of 21 days. Depending on the period, cows received alternate treatments followed by a recovery period (Table 1).

Table 1 - Treatment assignment of a repeated design to study the induction and recovery of SARA

Assignment	Pre-experiment	Period 1	Period 2	Period 3
1	Control	Control	Acidosis	Recovery
2	Acidosis	Recovery	Control	Acidosis
3	Control	Acidosis	Recovery	Control

Source: author production, 2018

4.2.2 Management, feeding and milk sampling and analysis

Cows were fed once daily (10h00) at 100% of expected intake with total mixed ration (TMR). The quantities offered were adjusted daily according to the previous day's intake, to provide 10% of refusals. The ingredients level was weekly adjusted according the corn and alfalfa silage dry matter (DM). The diets were composed of corn and alfafa silage, concentrate mixture containing soybean meal, gluten and ground corn and a commercial vitamin/mineral mix (Table 2). The dry matter intake (DMI) was determined according the offered DM and the refusal DM on days 0, 3, 7, 10, 14, 17 and 21 of each period.

Table 2 - Ingredient and chemical composition of experimental diets

Item	Control	Acidosis
Ingredients, % of DM		
Corn silage	44.74	34.55
Alfalfa silage	24.51	22.22
Ground corn	4.67	21.73
Grass hay	10.49	5.1
Gluten	7.86	6.41
Soybean meal	5.08	7.31
Limestone	0.61	0.62
Mineral and vitamins mix 18-5[1]	2.04	2.06
Chemical composition, % of DM		
DM[2]	46.4	48.9
OM[3]	92.71	93.4
CP[4]	17.18	16.05
NDF[5]	31.03	24.03
ADF[6]	20.51	15.81
Total FA[7]	2.57	2.80
Starch	19.95	29.36
Fatty acids, g/Kg		
C16:0	4.30	4.40
cis-9 C18:1	4.30	5.50
cis-9, *cis*-12 C18:2	11.00	13.00
cis-9, *cis*-12, *cis*-15 C18:3	4.50	3.30

Source: author production, 2018
[1] Contained (DM basis): 18,0% Ca, 5,0% P, 9,5% Na, 6,0% Mg, 45 mg/kg I, 3620 mg/kg Fe, 600 mg/kg Cu,2000 mg/kg Mn, 3000 mg/kg Zn, 20 mg/kg Co, 480 mg/kg F, 25 mg/kg Se, 300000 IU/kg vitamin A – Retinol acetate, 100000 IU/kg vitamin D - D3 cholecalciferol, 1500 IU/kg vitamin E - DL-alpha tocopherol acetate (La coop fédérée). [2] DM: Dry matter. [3] OM: Organic matter. [4] CP: crude protein. [5] NDF: Neutral detergente fiber. [6] ADF: Acid detergente fiber. [7] FA: Fatty acids.

Each TMR was sampled once per week and stored at -20°C, dried in a forced-air oven at 55°C for 96 h, and ground to a size of 1-mm using a Wiley Mill (A.H. Thomas, Philadelphia, PA). Samples of TMR were composited by period and analyzed for DM, Ash, and CP according to AOAC International (2000), for NDF and ADF according to Van Soest, Robertson & Lewis (1991), for starch according to Hall et al. (2001) and fatty acid concentration and profile by gas chromatography after direct methylation (SUKHIJA & PALMQUIST, 1988). Methyl esters

were quantified in a gas chromatograph (Agilent 7820A, Agilent Technologies, Santa Clara, CA) equipped with a HP-INNOWax Column (30 m length × 0.32 mm internal diameter × 0.25 μm film thickness; Agilent Technologies Canada Inc., Mississauga, ON) and a flame ionization detector with hydrogen was the carrier gas. Initial oven temperature was 100°C, which increased by 5°C/min to 220°C and held for 38 min. Inlet and detector temperatures were 240°C and 250 °C, respectively. The split ratio was 1:50.

Cows were milked twice daily at 7h30 and 16h30 and milk yield determined by an integrated milk meter (Flomaster Pro, DeLaval, Tumba, Sweden) on days 0, 3, 7, 10, 14, 17 and 21, in each period. Milk was sampled on days 0, 3, 7, 14 and 21 of the both milking. One subsample was stored at 4ºC with preservative Bronopol (2-bromo-2-nitropropane-1,3-dio) until analyzed for fat, protein, lactose, and β-hydroxybutyrate (BHBA) (Valacta, Sainte-Anne-de-Bellevue, QC, Canada). The analysis of the milk components was performed by infrared absorption spectroscopy with a Foss MilkoScan FT 6000 instrument (Foss, Hillerød, Denmark). The same sample was used for somatic cell counting with Fossomatic FC (Foss). Another subsample was stored at -20°C without preservative until analyzed for FA profile.

4.2.3 Ruminal and reticular pH

Ruminal and reticular pH were monitored using indwelling probes (eCow Devon Ltd, Devon, UK) placed in the reticulum and ventral sac of the rumen through the rumen cannula. Boluses were placed inside the cows before feeding (at approximately 09h30) and pH was recorded every 5 minutes during 24 h on days 0, 3, 7, 14 and 21 of each period. The calibration of the boluses was verified daily (pH 4.0 and 7.0 at 39 °C) before inserting them in the cows and at the end of the measurement period. Data were discarded when outside of ± 0.1 pH units from either calibration point at the time of removal from the cow.

4.2.4 Milk fatty acid profile analysis

Milk fat was extracted with hexane:isopropanol according to Hara & Radin (1978) and FA methyl esters obtained by base-catalyzed transmethylation according to Chouinard et al. (1999). Fatty acid methyl esters were determined according to Boivin et al. (2013) using a gas chromatograph (Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a CP-Sil-88 capillary column (100 m length × 0.25 mm internal diameter × 0.20 mm film

thickness, Agilent Technologies Canada Inc., Mississauga, ON, Canada) and a flame ionization detector.

4.2.5 Rumen volatile fatty acids (VFA) and lactate analysis

On day 21 of each period, ruminal liquid was sampled from each cow at 0, 2, 4 and 6 hours after the morning feeding. Rumen fluid was sampled through the fistula using a metallic tube equipped with a 1-mm screen. The liquid was thus aspirated with a 60 mL syringe and immediately transferred into 20 mL glass vials containing 200 µL of H_2SO_4 (50%) and stored at -20°C for subsequent analysis of the VFA concentrations and lactate. The samples were centrifuged at 10,000 RPM for 15 minutes. The supernatant was transferred to microtubes and the VFA profile and the lactate content were determined by liquid phase chromatography.

4.2.6 Blood parameters – insulin and NEFA

The blood samples of all cows were collected on days 0, 3, 7, 14 and 21 of each period, in a 10 mL Vacutainer tube containing an anticoagulant ethylenediamine tetraacetic acid (EDTA). The blood samples were put on ice and immediately centrifuged at 1,800 × g for 15 min at 4ºC. The supernatant plasma samples were stored in plastic tubes at -20 °C until analysis of insulin and NEFA.

The concentration of plasma components was determined by commercial kits. Plasma concentration of insulin was measured using the Mercodia Bovine Insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and NEFA by the Wako NEFA-HR (2) reagent (Wako Chemicals GmbH, Neuss, Germany).

4.2.7 Statistical analysis

Data were analyzed using the MIXED procedure of SAS with repeated measures (version 9.3, SAS Institute, Cary, NC). The model included sequence and period as random effects, and the fixed effects of treatment, time, and their interaction. Time was the repeated variable, cow by treatment was the subject, the Kenward-Rogers method was used for adjustment of denominator degrees of freedom. Preplanned contrasts tested Control vs. Induction and Control vs. Recovery at each time point. Significance and tendencies of main effects and preplanned contrasts were declared at $P < 0.05$ and $P < 0.10$, respectively, and interactions at $P < 0.10$ and $P < 0.15$, respectively. Data were log transformed if distribution of

residuals was non-constant and back transformed. Least-square means and standard error of the means are reported.

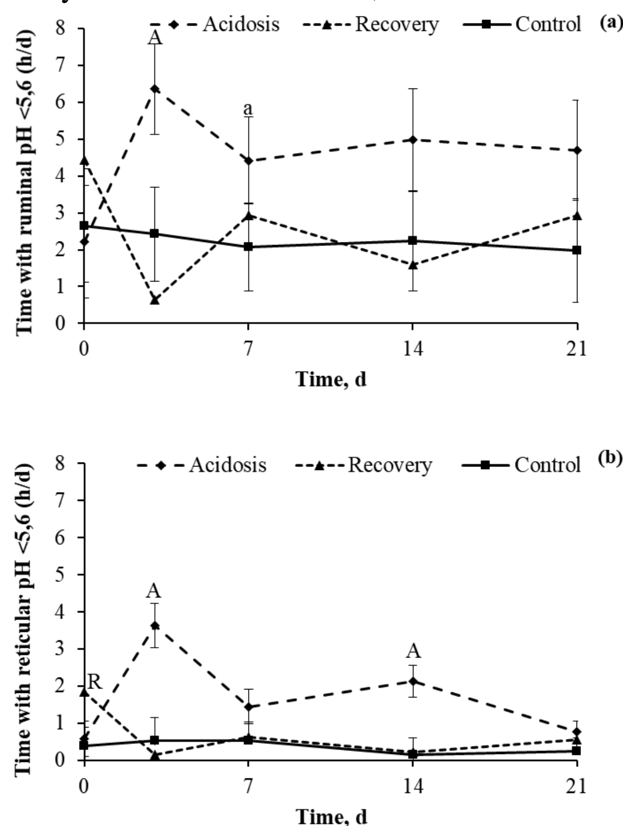
4.3 RESULTS

4.3.1 Ruminal and reticular pH

For this study, the threshold used for the diagnosis of SARA was pH below 5.6 for more than 180 minutes. The duration of ruminal pH below 5.6 of the cows fed the acidogenic ration was higher compared to the Control group on day 3 ($P < 0.05$) and tended to be higher on day 7 ($P < 0.10$), with an average duration of 270 minutes/day, indicating that the animals were in acidosis (Figure 1a).

The same data measured in the reticulum, the pH duration below 5.6 was higher in the animals that received the acidogenic ration on days 3 and 14 ($P < 0.05$), however, the average time in the evaluated days was of 102 minutes/day (Figure 1b).

Figure 1 - Number of hours below the pH 5.6 threshold measured at the ventral sac of the rumen (a) and reticulum (b) according to the diets applied (A: Acidosis vs. Control = $P < 0.05$; R: Recovery vs. Control = $P < 0.05$; a: Acidosis vs. Control = $P < 0.10$)



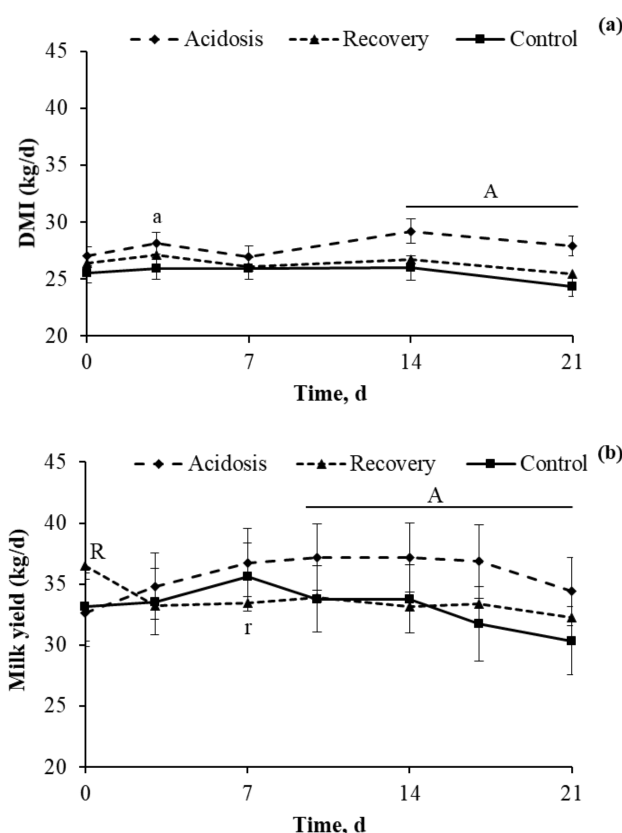
Source: author production, 2018

4.3.2 Dry matter intake (DMI) and milk production

The DMI tended to be higher on day 3 ($P < 0.10$) and was higher on days 14 to 21 ($P < 0.05$; Figure 2a) in cows fed acidogenic ration, comparing with the Control.

Milk production was also higher in cows receiving the acidogenic ration on days 10 to 21 ($P < 0.05$; Figure 2b).

Figure 2 - Evolution of DMI and milk yield according to the diets applied (A: Acidosis vs. Control = $P < 0.05$; a: Acidosis vs. Control = $P < 0.10$; R: Recovery vs. Control = $P < 0.50$)



Source: author production, 2018

4.3.3 Milk composition

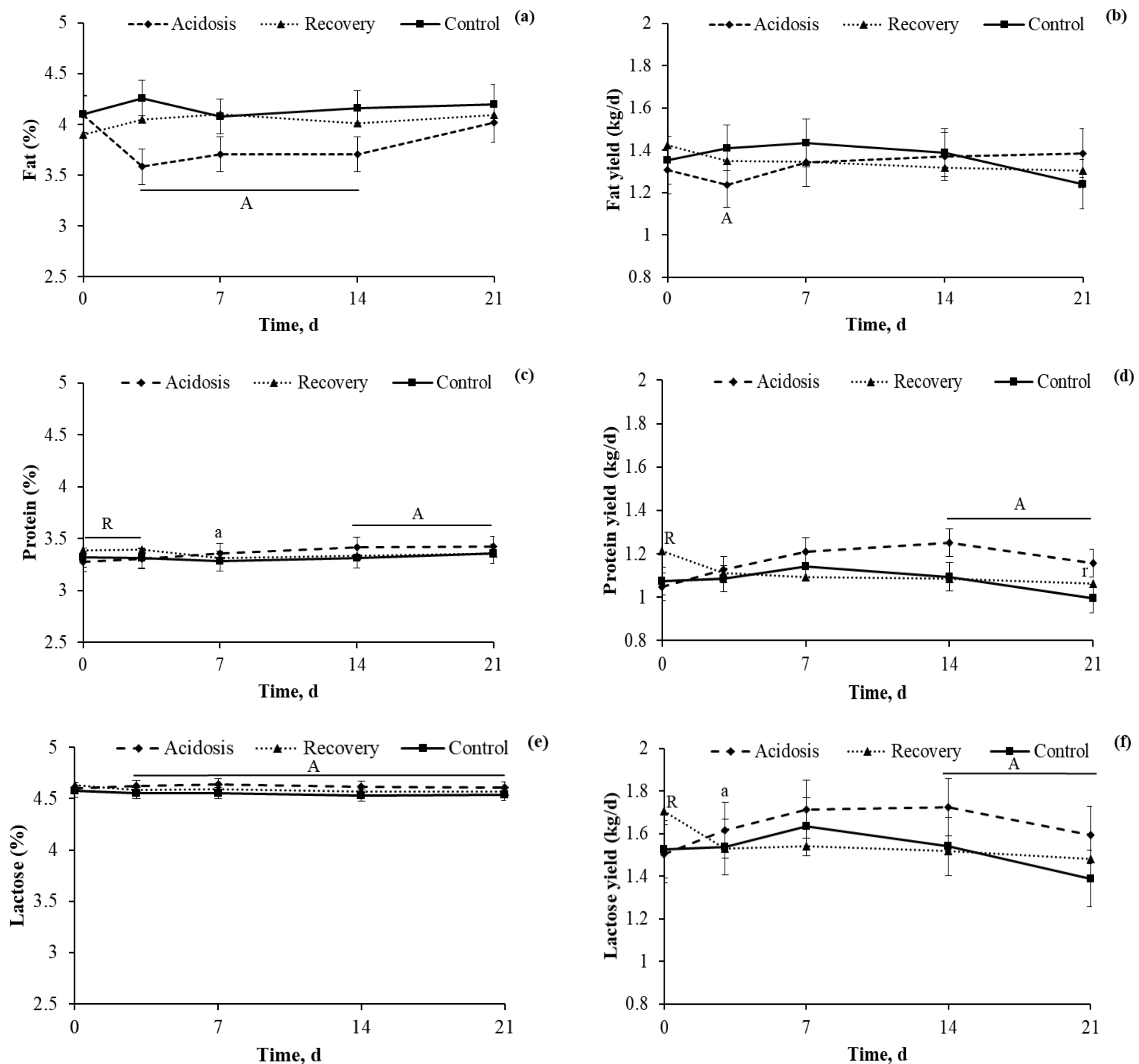
Cows receiving the acidogenic diet produced a lower fat content in the milk than the cows that received the Control diet on days 3, 7 and 14 of the experimental period ($P < 0.05$, Figure 3a). Milk fat yield was lower in cows fed the acidogenic diet only on day 3 ($P < 0.05$; Figure 3b).

In contrast to fat, there was an increase in protein and lactose contents at days 14 and 21 ($P < 0.05$) and 3 to 21 ($P < 0.05$), respectively, in the cows receiving the acidogenic diet (Figure

3c and e). As for the daily yield of these two components, it also increased on days 14 and 21 ($P < 0.05$) compared to the Control (Figure 3d and f).

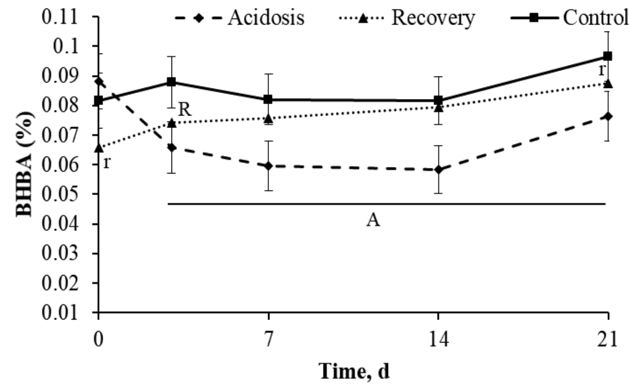
The BHBA level in the milk of the cows that received the acidogenic diet were decreased on days 3 to 21 ($P < 0.05$; Figure 4).

Figure 3 - Evolution of the milk fat (a), protein (c) and lactose (e) contents and the daily yield of fat (b), protein (d) and lactose (f) according to the diets applied (A: Acidosis vs. Control = $P < 0.05$; a: Acidosis vs. Control = $P < 0.10$; R: Recovery vs. Control = $P < 0.05$)



Source: author production, 2018

Figure 4 - Evolution of the milk BHBA content according to the diets applied (A: Acidosis vs. Control = $P < 0.05$; R: Recovery vs. Control = $P < 0.05$; r: Recovery vs. Control = $P < 0.10$)

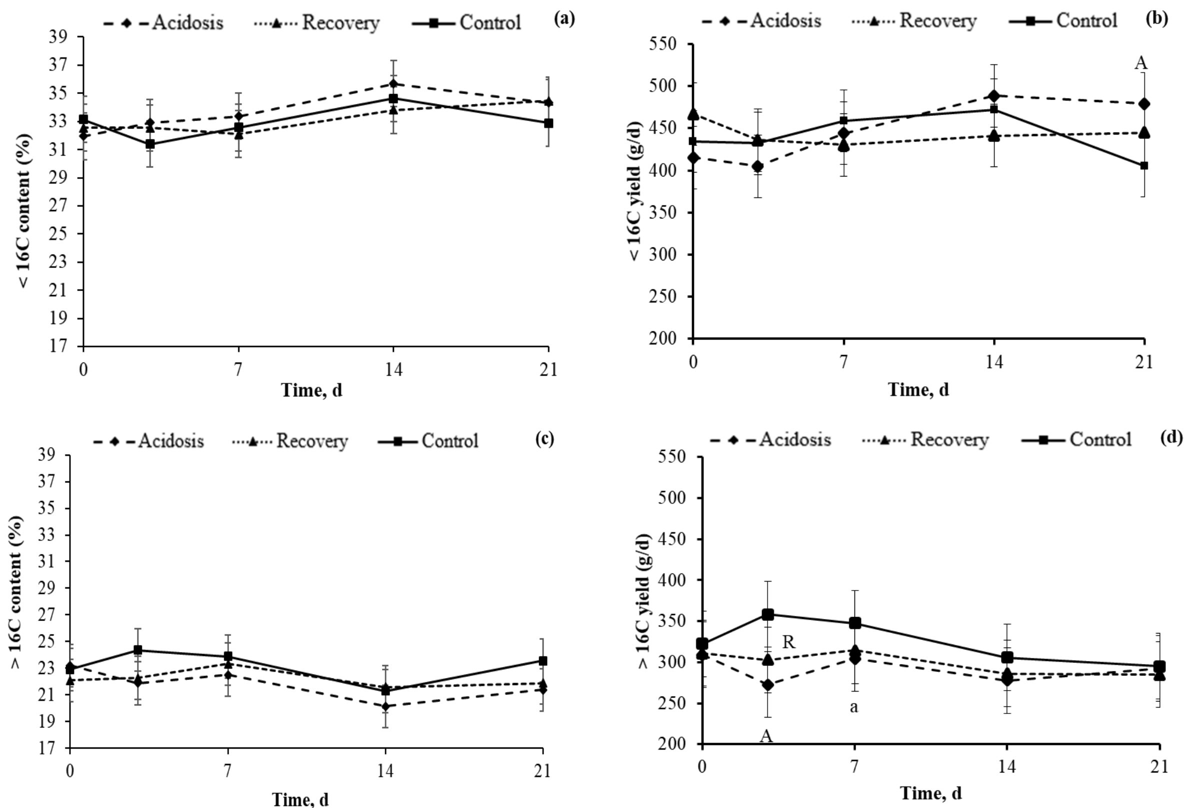


Source: author production, 2018

4.3.4 Milk fatty acid profile

In our study, fatty acids profile was affected by acidosis. The content of fatty acids with less than 16 carbons, synthesized entirely in the mammary gland, and the fatty acids with more than 16 carbons, that come from the bloodstream and represent the preformed fatty acids derived from adipose tissue and food, was not affected by the treatments (Figure 5a and c). The fatty acids yield with less than 16 carbons was increased on day 21 and the fatty acids yield with more than 16 carbons was reduced on day 3 ($P < 0.05$; Figure 5b) and trended to reduce on day 7 ($P < 0.10$; Figure 5d).

Figure 5 - Evolution of milk fatty acids according to the diets applied: <16 carbons content (a) and yield (b), >16 carbons content (c) and yield (d) (A: Acidosis vs. Control = $P < 0.05$; a: Acidosis vs. Control = $P < 0.10$; R: Recovery vs. Control = $P < 0.05$)

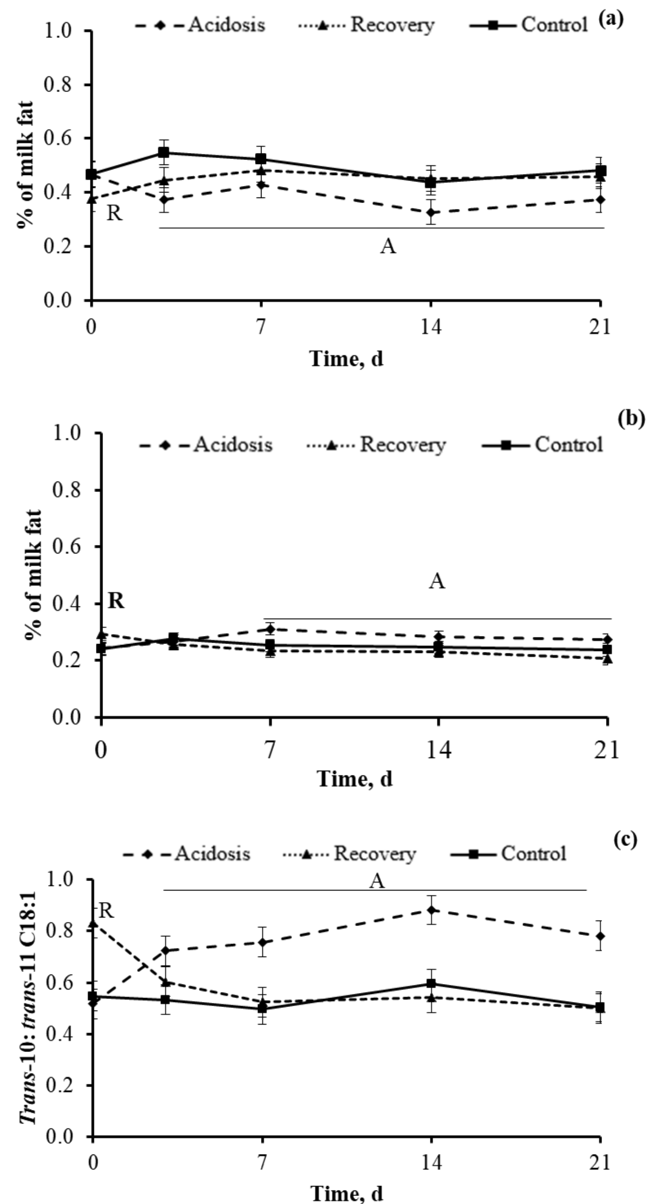


Source: author production, 2018

The *trans*-11 C18:1 fatty acid (normal pathway of biohydrogenation) was decreased with this metabolic disorder, on days 3 to 21 ($P < 0.001$; Figure 6a), while the *trans*-10 C18:1 fatty acid increased on days 7 to 21 ($P < 0.01$; Figure 6b). The *trans*-10, *cis*-12 CLA was not detected.

Compared to Control cows, there was an increase in the ratio of *trans*-10: *trans*-11 C18:1 fatty acids in the milk of cows that consumed an acidogenic ration during days 3 to 21 of the period ($P < 0.001$, Figure 6c). When transferring to a Recovery diet, this ratio decreased gradually and became similar to that of the Control by day 3.

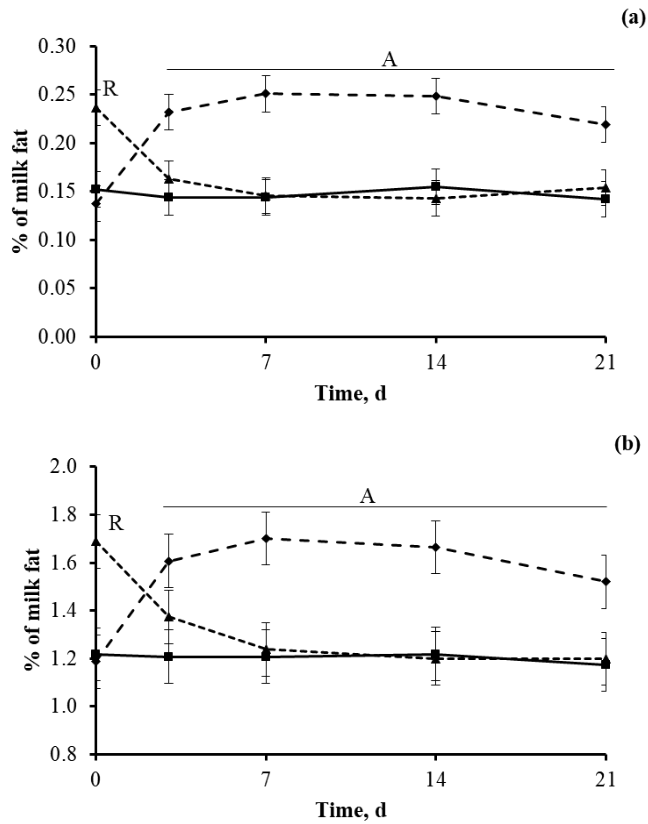
Figure 6 – *Trans*-11, C18:1 (a), *trans*-10, C18:1 (b) and ratio *trans*-10: *trans*-11 C18:1 (c) according to the diets applied. (A: Acidosis vs. Control = $P < 0.05$; R: Recovery vs. Control = $P < 0.05$)



Source: author production, 2018

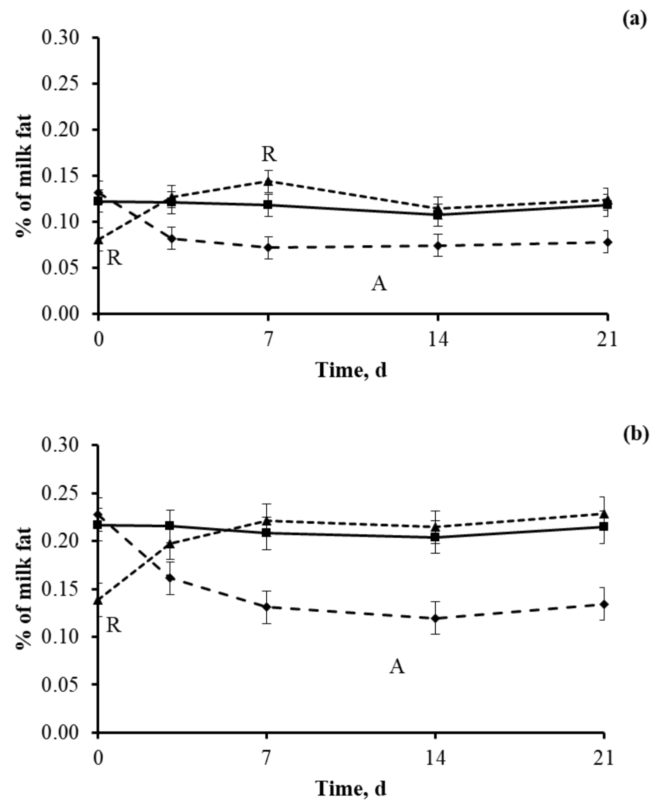
SARA increased the contents of the odd fatty acids C13:0 ($P < 0.001$) and C15:0 ($P < 0.001$), compared to Control treatment (Figure 7a and b), on days 3 to 21. The branched fatty acids *iso* C14:0 ($P < 0.001$) and *iso* C16:0 ($P < 0.001$) were reduced in the acidosis, on days 3 to 21 (Figure 8a and b), and the branched fatty acids *anteiso* C15:0 ($P < 0.001$) and *anteiso* C17:0 (P = 0.02) were reduced on days 3, 7 and 21 and 14, respectively (Figure 9a and b).

Figure 7 – Odd C13:0 (a) and C15:0 (b) fatty acids in the milk, according to the diets applied. (A: Acidosis vs. Control = $P < 0.05$; R: Recovery vs. Control = $P < 0.05$)



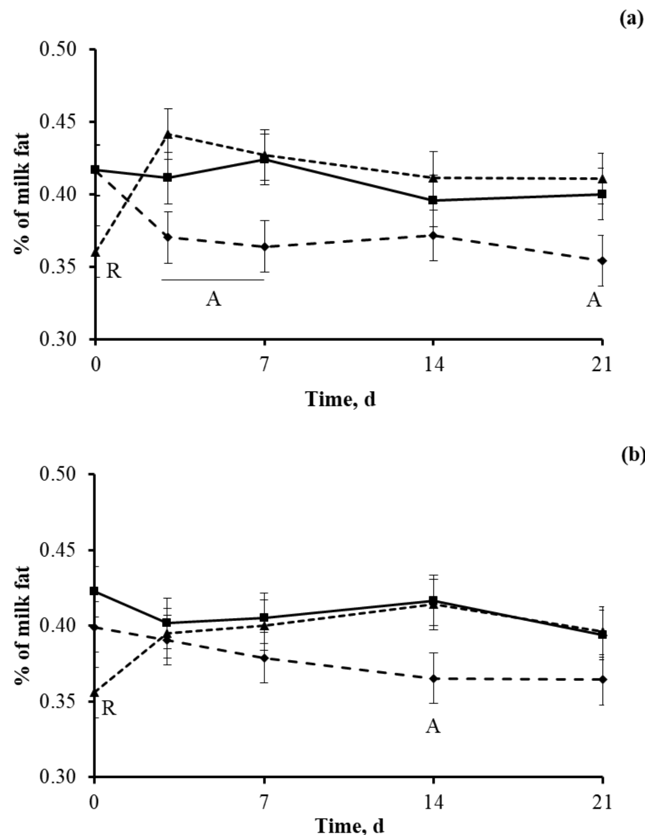
Source: author production, 2018

Figure 8 – Branched *iso* C14:0 (a) and *iso* C16:0 (b) fatty acids in the milk, according to the diets applied. (A: Acidosis vs. Control = $P < 0.05$; R: Recovery vs. Control = $P < 0.05$)



Source: author production, 2018

Figure 9 – Branched *anteiso* C15:0 (a) and *anteiso* C17:0 (b) fatty acids in the milk, according to the diets applied. (A: Acidosis vs. Control = $P < 0.05$; R: Recovery vs. Control = $P < 0.05$)



Source: author production, 2018

4.3.5 Rumen volatile fatty acids (VFAs) and lactate

The lactate content of the ruminal fluid of cows receiving an acidogenic ration is 14 times higher than that of the Control cows ($P = 0.02$, Table 5), whereas no difference is observed between the Control and Recovery groups.

Total VFA and propionate concentrations were also approximately 6% ($P < 0.01$) and 21% ($P < 0.001$) higher, respectively, in cows receiving an acidogenic ration compared to the Control, while the acetate content was reduced by 7% ($P < 0.001$). As a result, the acetate: propionate ratio of cows in acidosis is 24% lower than Control ($P < 0.001$). As expected, no difference was observed between Control and Recovery groups since the rations offered were the same. Finally, the butyrate content of the ruminal fluid (13% of total VFA on average) was not influenced by the treatments (Table 3).

Table 3 – Lactate and VFA contents of the ruminal liquid according to the treatments

Variable	Acidosis	Recovery	Control	SEM	***P* – value**	
					A vs. C	**R vs. T**
Lactate, ng/µL	0.044[a]	0.011[b]	0.003[b]	0.017	0.02	0.81
Total VFA, ng/µL	9.52[a]	9.24[b]	8.92[b]	0.33	<0.01	0.23
VFA, %						
Acetate	53.4[b]	57.0[a]	57.7[a]	0.54	<0.001	0.37
Propionate	27.2[a]	22.4[b]	22.4[b]	0.54	<0.001	0.73
Butyrate	12.5	13.6	12.9	0.31	0.23	0.15
Acetate:propionate ratio	2.02[b]	2.64[a]	2.66[a]	0.07	<0.001	0.81

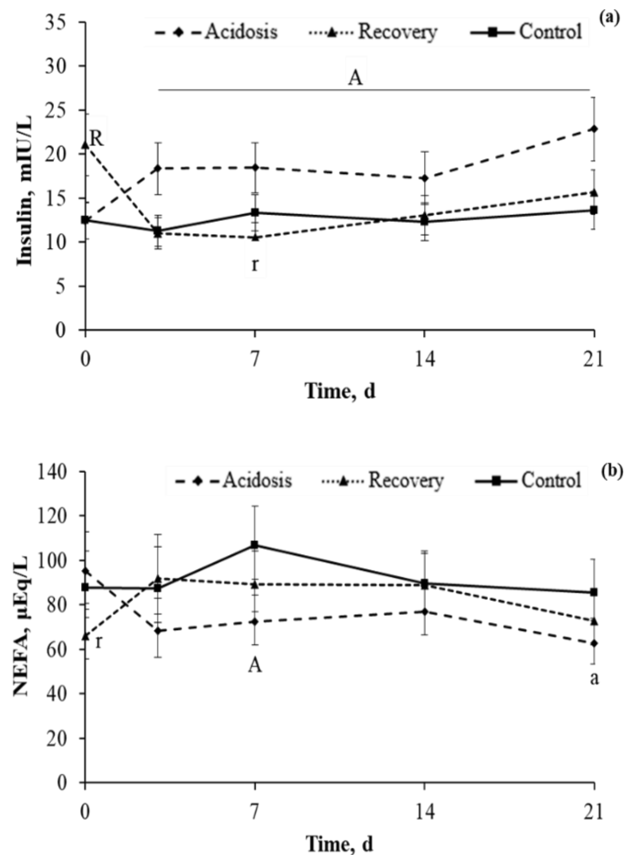
Source: author production, 2018

4.3.6 Blood parameters – insulin and NEFA

In the present study, cows in acidosis exhibited higher plasma insulin concentrations than the Control group on days 3 to 21 ($P < 0.05$, Figure 10a). As a result, insulin content in Recovery cows were higher on day 0 than those in Control but decreased rapidly thereafter. Indeed, a downward trend in insulin plasma concentrations of recovering cows is observed at day 7 whereas for the rest of the period, there was no significant difference compared to the Control.

As expected, the plasma concentration of non-esterified fatty acids (NEFA) was lower in cows that consumed the acidogenic diet than Control, particularly on days 7 ($P < 0.05$) and 21 ($P < 0.10$, Figure 10b).

Figure 10 - Evolution of plasma insulin (a) and NEFA (b) content according to the diets applied (A: Acidosis vs. Control = $P < 0.05$; a: Acidosis vs Control = $P < 0.10$; R: Recovery vs Control = $P < 0.05$; r: Recovery vs Control = $P < 0.10$)



Source: author production, 2018

4.4 DISCUSSION

Our model of induction and recovery served to test the effects of SARA by exemplifying a scenario where the variation in the concentrate content of the diets, common in high production systems, may cause acidosis and consequently MFD and other metabolic alterations. Importantly, the experimental diets varied in fermentability (starch and NDF content), but were formulated to provide low quantities of fat. This allows to study the role of concentrates and SARA on milk fat synthesis while minimizing the known influence of high PUFA. The experimental design was successful in induction of SARA and recovery within the experimental periods.

In relation to the time of change in ruminal pH and milk composition, at 3 days of acidosis induction there was already a difference in pH and reduction in milk fat content, while the recovery time course was similar. Rico & Harvatine (2013), following a similar experimental design with low-fiber, high-oil (LFHO), observed reductions in milk fat

concentration around day 9 and recovery around day 15. Induction of MFD through dietary modification has been shown to require 11 to 19 days (SHINGFIELD et al., 2006; HE et al., 2012) because changes in the rumen environment and a shift in the microbial population must occur to result in synthesis of sufficient quantities of milk-fat-depressing intermediates to affect mammary lipid synthesis. Satter & Bringe (1969) abruptly changed the diet and simultaneously the ruminal content between two cows and they observed that 70% of the maximal reduction in milk fat in cows fed low forage was achieved by day 3 and complete MFD within 5 to 6 days. This demonstrates that ruminal adaptation is a limiting factor, that is, the faster reductions were possible by the change in the microbial population.

Unlike this study, a decrease in DMI is usually seen and this has been used as a clinical SARA diagnosis (KLEEN et al., 2003; OETZEL, 2003). Reasons for the feed intake depression can include reduced fiber digestibility and increases in VFAs, and in the osmolarity in the rumen (ALLEN, 2000). The difference between these studies and ours are unclear, but may be explained by variations in the SARA induction protocol, the severity of SARA, or the individual differences of the cows (GUO et al., 2013). Furthermore, the higher passage rate of the concentrate and lower physical limit of the acidogenic diet may have allowed animals to feed more (ALLEN, 2000), regardless of ruminal pH reduction (Figure 1a) due to the increase in the proportion of lactate and VFA (especially propionate). Consequently, milk yield was increased by the higher energy intake of the high concentrate diet.

Similar to the present study, experimentally induced SARA, either by adding pellets to the diet or by replacing alfalfa with alfalfa pellets, reduced milk fat percentage, but increased milk protein percentage (FAIRFIELD et al., 2007; KHAFIPOUR, KRAUSE & PLAIZIER, 2007). Milk protein synthesis is sensitive to energy level in the diet due to the increase in insulin and energy available for the process of assembling amino acids into proteins.

Bionaz, Hurley & Loor (2012) suggest that insulin actives the mammalian target of rapamycin (mTOR), promoting the regulation of translation and consequently allows the mammary gland to fine-tune regulate the milk protein synthesis based on energy availability. In addition to the possible direct effect of insulin demonstrated that alterations in some components of the insulin-like growth factor (IGF) system promote the temporal increases in milk protein yield (MCGUIRE et al., 1995; GRIINARI et al., 1997). Another explanation is that a diet containing an appropriate amount of rapidly fermentable starch may improve the efficiency of nitrogen utilization by ruminal microorganisms (HERRERA-SALDANA & HUBER, 1989) and this may have contributed to the higher milk protein percentage in the present study. This occurs because the availability of energy and nitrogen increases the

production of microbial protein, the main source of protein for ruminants (VAGNONI & BRODERICK, 1997). In addition, reduction of milk fat by *trans*-10, *cis*-12 CLA may increase milk production and/or milk protein during early lactation in certain situations, as reported by Medeiros et al. (2010) and Lock et al. (2006). However, how *trans*-10, *cis*-12 CLA was not detected, we do not have reason to believe this mechanism played a role in our study.

Milk fat yield was rapidly decreased and then progressively increased during induction, which is related to the milk production increase caused by the acidogenic diet. However, for the farmer the interesting thing is the fat content, since currently part of his remuneration is based on it.

As previously described in several studies, and that has also been observed in our work, one of the main effects of reducing ruminal pH is the decrease in milk fat content (NOCEK, 1997; KLEEN et al., 2003; OETZEL, 2003; STONE, 2004) and consequent change in FA profile. Fatty acids with more 16 carbons were more affected by SARA, which may be inferred that the synthesis pathway of preformed FA was altered by it, since the intermediaries were also reduced (e. g. NEFA).

During biohydrogenation-induced MFD, yield of both *de novo* synthesized, and preformed FA are decreased in milk, but a greater reduction occurs in *de novo* synthesized FA. The main precursor for *de novo* synthesis of FA in the bovine mammary gland is the VFA acetate (URRUTIA & HARVATINE, 2017).

According to the most accepted theory for MFD, changes in the milk FA profile are due to alteration on the ruminal biohydrogenation of the polyunsaturated fatty acids (PUFA), with the production of intermediate FA, such as the *trans*-10, *cis*-12 CLA, characterized as the main anti-lipogenic agent in the mammary gland (PETERSON, MATITASHVILI, & BAUMAN, 2003).

The increase in the *trans*-10: *trans*-11 C18:1 ratio demonstrates that ruminal lipid biohydrogenation in acidosis cows was modified and this is associated with the decrease of milk fat (BAUMAN & GRIINARI, 2003). However, the concentration of *trans*-10 C18:1 in the milk of cows fed an acidogenic ration represented approximately 0.2% of total fatty acids, which is very low compared to what is observed during BH-induced MFD. According to several authors, the decline in milk fat is observed when the concentrations of *trans*-10 C18:1 is > 2% of total fatty acids (RICO & HARVATINE, 2013, RICO et al., 2015). In addition, the *trans*-10, *cis*-12 CLA in milk was not detected, indicating that the SARA intensity was probably not high enough to increase the production of this fatty acid, and the low quantity of C18:2n-6

available (1.3 and 1.1% of DM, Acidosis vs. Control, Table 2) prevented it from attaining detectable levels.

This leads us to deduce that the MFD observed in the acidosis treatment involves other factors, such as change in the proportion of VFA and consequently of the concentrations of insulin and NEFA. This same issue had been raised by Roy et al. (2006) that proposes the quantities of *trans*-10, *cis*-12 CLA isomer produced *in vivo* in the rumen are insufficient to fully explain the milk fat reductions observed during MFD, and because of this, other nutrients or mechanisms are also involved in MFD (SHINGFIELD et al., 2010). Maxin, Rulquin & Glasser (2011b) compiled a database from studies involving digestive infusions of different nutrients in dairy cows and observed that *trans*-10, *cis*-12 CLA, propionate and glucose are all capable of decreasing milk fat synthesis.

During MFD, there is also a change in the milk FA profile caused by changes in the microbial profile and concentration of precursors. This can be verified by the evaluation of the profile of odd and branched fatty acids in the milk of the experimental animals. Ruminant lipids contain non-branched fatty acids with an odd carbon number and branched-chain fatty acids with an odd or even carbon number, collectively termed odd- and branched-chain fatty acids (OBCFA; FRENCH, BERTICS & ARMENTANO, 2012).

The increase and reduction of odd and branched fatty acids, respectively, is accompanied by the increase and reduction in proportions of propionate and acetate. An increase in starch content, forage digestibility as well as a decrease in forage:concentrate ratio or NDF content, promote the growth of amylolytic bacteria (e.g. *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis* and *Succinomonas amylolytica*) and limits the growth of cellulolytic bacteria (e.g. *Ruminococcus albus*, *Butyrivibrio fibrisolvens* and *Ruminococcus flavefaciens*). This is expected to increase propionate while decreasing acetate proportions (BEAUCHEMIN et al., 2008). Vlaeminck et al. (2006) reported that diets rich in starch reduced the *iso* C14:0, *iso* C15:0 and *iso* C16:0 fatty acids in milk fat and this occurs by changing the precursor (e. g. acetate) and microbial profile of the rumen (ENJALBERT et al., 2008; VLAEMINCK et al.; 2006). In turn, the ruminal infusion of propionate increases the content of odd fatty acids C15:0 and C17:0.

Variation in the OBCFA profile leaving the rumen is expected to reflect changes in the relative abundance of specific bacterial populations in the rumen rather than an altered bacterial fatty acid synthesis (FIEVEZ et al., 2012), so it is possible that changes in these groups of milk FA could be used to identify shifts in microbial populations that are associated with SARA. Moreover, in the experiment described by Colman et al. (2010), dairy cows were subjected to

an acidosis induction trial with five induction weeks. Acidosis, based on rumen pH measurements, was observed in weeks 5 and 6, but some milk OBCFA showed changes before rumen acidosis was observed. Indeed, C15:0 and C17:0 in milk fat increased already in week 4, while *iso* C14:0 and *trans*-10 C18:1 changed in weeks 5 and 6, respectively. In this experiment, *trans*-10 C18:1 showed more potential as indicator of acute acidosis whereas C15:0 and C17:0 could be indicators of SARA. For this, we suggested the predictive potential of these milk OBCFA based on feeding trials with non-fistulated animals and prior to clinical symptoms.

Interestingly, *de novo* synthesized fatty acids were negatively related to branched C:17-fatty acids and showed a positive correlation with linear odd-chain fatty acids (VLAEMICK et al., 2006). The negative correlation between branched C:17- and *de novo* synthesized fatty acids might indicate a direct inhibitory effect of these fatty acids on fatty acid synthesis in the mammary gland, as reported in breast cancer cells (WONGTANGTINTHARN et al., 2004). However, it is more likely that increased amounts of branched C17-fatty acids are associated with rumen conditions favoring the formation and accumulation of specific hydrogenation intermediates (BAUMAN & GRIINARI, 2003).

Our results corroborate with Enjalbert et al. (2008) and Mitchell et al. (2016) who supplemented cows with increasing starch quantities (26.0, 39.2 and 43.6% of starch and 36.1, 32.4 and 28.5% of NDF) and low-fiber diets (38% chopped grass hay and 57% grain mix, containing 32.3% NDF and 32.1% starch), respectively, to induce acidosis and to evaluate the effect on milk FA profile and observed that the pH reduction increased the concentration of the odd fatty acids and reduced the branched fatty acids.

It is known that lactate, which is produced during the degradation of starch in the rumen, increases in cows with acidosis and contributes to the fall of ruminal pH (PLAIZIER et al., 2008). Furthermore, a ration containing high quantity of starch, such as that used to induce ruminal acidosis in this study, is one way to increase the proportion of ruminal propionate, which is the major source of carbon for glucose in ruminants (SEAL & REYNOLDS, 1993). Increased propionate uptake when cows are fed with a high starch diet promotes increased plasma glucose as well as insulin secretion (ANNISON, BICKERSTAFFE & LINZELL, 1974).

Insulin, in turn, is an anabolic hormone that reduces the lipolysis (mobilization) of adipose tissue. By preventing the release of NEFA from adipose tissue, insulin reduces the amount of these preformed fatty acids (those that are not synthesized in the mammary gland) that reach the mammary gland.

Based on this assumption, Corl et al. (2006) infused insulin and glucose in cows immediately postpartum to test the effect of insulin on MFD and found that fat content in milk

decreased by 27% and concomitantly the concentration of NEFA and the proportion of LCFA were decreased. This indicates that the decrease in milk fat synthesis was a consequence of reduced availability of plasma fatty acids derived from the mobilization of body fat reserves. On the other hand, a just 5% decrease in milk fat production was observed in advanced lactating cows whose plasma insulin levels were artificially increased (hyperinsulinemia) and whose blood glucose levels were maintained stable (GRIINARI et al., 1997, MACKLE et al., 1999). Because of these differences, the effect of insulin may partly explain MFD, since the variability in the proportion of milk fatty acids derived from body fat reserves is a consequence of differences in the net energy balance of animals (e.g. beginning and established lactation). This can help explain our results, but the alterations of concentrations of selected metabolites related to carbohydrate and lipid metabolism (e. g. NEFA and insulin) in the plasma are alternative candidates for diagnosis of SARA in cows of the same physiological state and environment (GUO et al., 2013).

In our study, we observed that MFD in SARA was transient, whereas insulin remained high until d 21. This seemingly contradictory observation may be explained by the progressive increase in DMI. Despite higher insulin and lower NEFA during SARA, the total flux of nutrients (dietary FA, acetate and butyrate) is expected to increase when intake increases during SARA, which can be related to the increased yield of *de novo* synthesized FA by the end of the period. In addition, we observed that yields of milk, lactose, protein, fat yields and specifically *de novo* FA yields increase progressively during SARA.

4.5 CONCLUSION

SARA reduced the milk fat synthesis transiently and modified milk fatty acid profile, as well as altered other metabolites, which may be potential indicators of SARA.

The reduction in milk fat synthesis was not strongly associated with ruminal biohydrogenation intermediates such as *trans*-10, *cis*-12 CLA and *trans*-10 C18:1, which suggests that other mechanisms such as reduced preformed FA availability were at play.

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5 ARTIGO

TRANS-10, *CIS*-12 CONJUGATED LINOLEIC ACID (CLA) REDUCES MILK FAT CONTENT AND GENE EXPRESSION IN THE MAMMARY GLAND OF SOWS WITHOUT ALTERING LITTER PERFORMANCE

ABSTRACT

As shown in dairy cows, ewes and goats, *trans*-10, *cis*-12 conjugated linoleic acid (CLA) decreases milk fat synthesis in lactating sows and it can be an option to minimize the energy costs of lactation, but without compromising the piglet performance. Milk fat depression caused by *trans*-10, *cis*-12 CLA involves, at least in part, the down-regulation of genes involved in the lipogenesis and the objective of this study was evaluated the effect of CLA on sow milk composition and the lipogenic gene expression. Twenty multiparous sows from a commercial genotype in their 1st to 5th parities and weighing (BW) 200 ± 10 kg were randomly assigned to one of the two treatments (n = 10/treatment) for 18 days: 1) Control (no CLA added) and; 2) 1% of CLA (29.9% of *trans*-10, *cis*-12 and 29.8% of *cis*-9, *trans*-11) mixed in the ration. Sows were kept in a controlled environment and the CLA treatment was administered from d 7 through d 25 of lactation. Milk samples were collected from all sows from d 7 and d 25 to evaluate the milk composition and mammary and adipose tissue biopsies were taken on day 25 of experimental period for the subsequent analysis of gene expression. Data were analyzed as a complete randomized design using the Mixed Procedure of SAS. Compared to Control, CLA treatment decreased milk fat content by 20% ($P = 0.004$). In addition, CLA reduced milk protein content by 11% ($P = 0.0001$). Despite the reduction in fat and protein content, the weight of piglets at weaning was not different between treatments ($P = 0.60$). Dietary CLA increased the saturated fatty acids (SFA) proportions ($P <.0001$) and decreased the monounsaturated fatty acids (MUFA) proportions ($P <.0001$). In the mammary gland, CLA reduced the gene expression in 37 ($P = 0.003$), 64 ($P = 0.002$), 52 ($P = 0.003$), 26 ($P = 0.03$), 15 ($P = 0.02$) and 27% ($P = 0.02$) of ACACAα, FASN, SCD1, LPL, AGPAT6 and DGAT1, respectively, whereas the expression of FABP3 tended to be altered by CLA treatment ($P = 0.09$), and in adipose tissue CLA treatment had no effect on the expression of all genes evaluated. In addition, the β-casein and α-lactalbumin genes were reduced by CLA 68 ($P = 0.0004$) and 62% ($P = 0.005$), respectively, compared to Control. These results indicate that CLA reduces the milk fat content without negatively affecting litter performance and its effect is on the gene expression involved in all lipogenic pathways.

Keywords: Fatty acids profile. Lipogenesis. Milk composition.

5.1 INTRODUCTION

Conjugated linoleic acid (CLA) is a generic term used to describe positional and geometric isomers of octadecadienoic fatty acids with a conjugated double bond (BAUMAN et al., 2008). Naturally, CLA originate mainly from bacterial isomerization and biohydrogenation of polyunsaturated fatty acids (PUFA) in the rumen and from the desaturation of vaccenic acid (*trans*-11 C18:1) in the adipose tissue and mammary gland (GRIINARI & BAUMAN, 1999). Although there are many isomers, two of them (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) have received most attention due to their known biological effects (HAYASHI et al., 2007).

Studies have revealed that *trans*-10, *cis*-12 CLA isomer is responsible for milk fat depression (MFD) in lactating cows (BAUMGARD et al., 2000), ewes (OLIVEIRA et al., 2012; SANDRI et al., 2017), goats (BALDIN et al., 2013; FERNANDES et al., 2014), mice (LOOR, LIN & HERBEIN, 2003) and pigs (BONTEMPO et al., 2004; POULOS, AZAIN & HAUSMAN, 2004; LEE et al., 2014).

In lactating sows, milk is the major source of nutrients for suckling piglets, and their maximal growth performance and survival largely depend on enough milk production by sows (WU et al., 2006). It has been documented that sows mobilize sufficient energy from their body tissue stores for milk production (KING & WILLIAMS, 1984; KING & DUNKIN, 1986; NOBLET & ETIENNE, 1989). The milk production from sows has been shown to reduce body weight (BW) during lactation (QUESNEL, ETIENNE & PÈRE, 2007) and several studies have reported that a BW loss during lactation reduced reproductive performance in the subsequent parity (CLOWES et al., 2003; THAKER & BILKEI, 2005). Thus, it is important to minimize BW loss in sows during lactation as well as maintain both maximal growth of piglets and subsequent reproductive performance (LEE et al., 2014).

Feeding rumen-protected *trans*-10, *cis*-12 CLA supplements presents an opportunity to manipulate milk fat synthesis, since these supplements may improve energy balance (EB) in lactating animals by reducing their energy requirements for milk synthesis (GRIINARI & BAUMAN, 2006). Spared energy can be partitioned toward the synthesis of other milk components (MEDEIROS et al., 2010) or alternatively, depending on the lactation period, it could also be used to replenish body fat reserves (HARVATINE, PERFIELD & BAUMAN, 2009), which may improve reproductive performance.

In ruminants, research at cellular levels demonstrated a coordinated downregulation in transcript abundance of genes involved in milk fat synthesis in the mammary gland of lactating cows (BAUMGARD et al., 2002), mammary line cell (KADEGOWDA et al., 2009), ewes (TICIANI et al., 2016) and goats (SHI et al., 2017), caused by supplementation with the *trans*-10, *cis*-12 CLA.

However, molecular mechanisms regarding the inhibitory effect of *trans*-10, *cis*-12 CLA on mammary lipid synthesis in sows remains unclear. Therefore, we focused to evaluate not only the effect of CLA on milk composition but also the lipogenic gene expression in mammary gland and adipose tissue of lactating sows. We hypothesized that the CLA would act differently on the gene expression of the different lipogenic pathways, affecting mainly the genes involved in the metabolism of circulating fatty acids.

5.2 MATERIALS AND METHODS

5.2.1 Animals, design and treatments

All procedures were approved by the Santa Catarina State University Ethical Committee, protocol no. 3162250216 and were performed at a commercial farm in Concórdia City, SC (27° 14' 03" S and 52° 01' 40" W). Twenty multiparous sows from a commercial genotype (Aurora Genetic) in their 1st to 5th parities and weighing (BW) 200 ± 10 kg were randomly assigned to one of the following treatments: 1) Control, without CLA added in the diet; 2) 1% CLA (containing 4.1% palmitic acid, 3.6% stearic acid, 27.4% oleic acid, 1.2% linoleic acid, 29.8% *cis*-9, *trans*-11 CLA, 29.9% *trans*-10, *cis*-12 CLA and 3.0% others FA) mixed in the diet. The amount of CLA fed was based on the paper of Lee et al. (2014).

5.2.2 Management, feeding, experimental period, sampling and analysis

The sows were moved into farrowing rooms after 108 days of gestation and were housed individually in pens (2.2 m × 1.6 m) with slatted floors and temperature and relative humidity controlled. Experimental diets were formulated to meet the recommended amounts of nutrients as required by the animals and contained ground corn (68.8%), soybean meal (23.2%), a commercial vitamin/mineral mix (5%), and soybean oil (3%). The soybean oil was replaced by CLA in the treatment (1% of the total amount). Sows were fed twice a day and received 7.4 ± 0.1 kg/day (as fed) of ration. The water access was *ad libitum*.

The experimental period was 18 days and the CLA feeding started at 7 days of lactation and was maintained up to 25 days of lactation.

The sizes of litters were adjusted (twelve piglets per sow) by cross-fostering piglets within 24 hours after birth. The litters weights were recorded at day 0 (7th day) and day 25th and they were weaned at 28 days. In the first 5 days old, the litters were subjected to normal management procedures, including cutting of teeth and tails, ear notching, iron shots and the males were castrated. At two weeks old, the litters began to receive a ration for adaptation to solid diet. The litters from both treatments received the same ration at two weeks of age and the intake of all them was approximately 0.80 kg (as fed) during the period.

On day 7 and 25 of lactation, milk samples were collected. Approximately 50 mL of milk was obtained after intravenous injection of 0.5 mL of oxytocin (Ocitovet©, Ceva Santé Animal, Paulínia, SP, Brazil). The samples were stored at 4°C with a preservative (Bronopol tablet; D & F Control Systems Inc., San Ramon, CA, USA). Milk fat, protein, lactose, and total solids were determined by infrared analysis (method 972.160, AOAC, 2000).

Weaning-to-estrus interval (WEI) was determined by monitoring estrus from 3 to 7 days after weaning.

5.2.3 Milk fatty acid profile analysis

On day 25 of lactation, milk samples were collected to determine the milk fatty acids profile. The fat cake was obtained by milk centrifugation at 3,000 RPM for 15 min at 4°C. Approximately 50 mg was then methylated according to O'Fallon et al. (2007). The resulting fatty acid methyl ester were determined using a gas chromatograph (model Focus GC; Thermo Scientific, Milan, Italy), equipped with flame ionization detector and fused silica capillary column SP-2560 (100 m x 25 mm x 0.2 µm of film thickness; Supelco, Bellefonte, Pennsylvania). Hydrogen was used as a carrier gas (1 ml/min) and nitrogen as an auxiliary gas. Detector and injector temperatures were set at 250°C, with split ratio 15:1. Oven temperature was set for 70°C for 4 min, increased by 13°C/min to 175°C, held for 27 min, increased by 4°C/min to 215°C and held for 31 min (KRAMER et al., 1997). The FAME were identified by comparing three FAME references (Supelco FAME mix # C4-C24, *trans*-9, *cis*-11 CLA # 16413, and *trans*-10, *cis*-12 CLA # 04397; Sigma Aldrich). The *cis*/*trans*-18:1 isomer were identified according to their order of elution reported under the same chromatographic conditions (KRAMER et al., 1997).

5.2.4 Mammary and adipose tissue biopsies

Mammary biopsies were taken on day 25 of experimental period. A tranquilizer was administered (2 mL/sow, intramuscular, and 6 mL/sow, intravenous of Destress injectable, Des-Far Laboratories LDTA, São Paulo, SP, Brazil) to immobilize the animals and then lidocaine hydrochloride subdermal (2 mL/sow) was administered above the incision site. A coaxial needle with a trocar was introduced in the first or second thoracic mammary glands. The biopsy was collected using a Bard Max-Core Disposable Core Biopsy Instrument (Bard Biopsy Systems, Covington, GA, USA). Briefly, a 16-gauge biopsy needle was partially inserted through the coaxial needle and two tissue samples (~35 mg tissue/biopsy) were collected, inspected to verify tissue homogeneity, rinsed with saline solution, placed in cryotubes containing 1 mL of Dulbecco's phosphate-buffered saline (PBS) (Gibco Laboratories, Grand Island, NY, USA) and immediately stored in liquid nitrogen until RNA extraction. The biopsy procedure resulted in minimal bleeding and no intra-mammary infections were observed.

The adipose tissue biopsy was taken from the tail head region immediately cranial and lateral to the last lumbar vertebra (dorsal subcutaneous depot). Prior to the biopsy, lidocaine hydrochloride subdermal was administered in a circular pattern surrounding the incision site (2 mL/sow). A small incision was made in the skin and adipose tissue was dissected. Two samples of adipose tissue (~100 mg) from the same site were obtained, rinsed with sterile saline solution, placed in cryotubes with PBS and snap frozen in liquid nitrogen until RNA extraction. The incision was closed with number 1 Nylon using a blanket stitch. After biopsies of adipose and mammary tissues an antinflammatory was administered (flunixin meglumine; 1.1 mg/kg of BW).

5.2.5 RNA extraction, synthesis of complementary DNA (cDNA) and quantitative real time PCR (RT-qPCR)

Total RNA extraction, synthesis of complementary DNA (cDNA) and quantitative real time polymerase chain reaction (RT-qPCR) were carried out at Santa Catarina State University biochemistry laboratory, according to Sandri et al. (2017). Total RNA was extracted from both mammary and adipose tissues samples using the RNeasy Lipid Tissue Mini Kit (Qiagen Sciences, Germantown, MD, USA) with column DNase treatment (RNase-free DNase set, Qiagen Sciences, Germantown, MD, USA). The RNA concentration was measured using a spectrophotometer (NanoDrop ND-2000; NanoDrop Technologies, Wilmington, DE, USA)

and the quality was evaluated by the $A_{260/280}$ ratio, which was ~2.03 (± 0.01). Total RNA was transcribed to complementary DNA (cDNA) using the GoScript™ Reverse Transcription Mix (Promega Corporation, Madison, WI, USA) with random primers. PCR amplification was performed in triplicates in a 48 well reaction plate (MicroAmp™, Applied Biosystems, Waltham, MA, USA) with 15 µL volume reaction, in a StepOne Real-Time machine (Applied BioSystems, Foster City, CA, USA). The data were analyzed with StepOne software version 2.1 (Applied Biosystems, Foster City, CA, USA). Dissociation curves were generated at the end each run to verify the presence of a single product.

5.2.6 Primer design

Gene sequences for primer designs were obtained from the gene bank of the National Center for Biotechnology Information (NCBI, USA). All primers were synthesized at Invitrogen™ (Carlsbad, CA, USA) and were tested for their efficiency before use. Gene expression of the following genes was measured: acetyl-CoA carboxylase-α (ACACAα), fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), lipoprotein lipase (LPL), fatty acid binding protein 3 (FABP3), acyl glycerol phosphate acyltransferase 6 (AGPAT6), diacylglycerol acyltransferase 1 (DGAT1), casein-α S1 (CSN1S1), casein-β (CSN2), casein-κ (CSN3) and α-lactalbumin (LALBA). The primer sequences of measured genes are listed in Table 4.

Table 4 - Swine primers used in the real-time PCR analysis

Symbols	Forward (F) and reverse (R) primer[1]	R^2	Efficiency
RPS18 [2]	F: CTGGCCAACGGTCTGGATAA	99.9	90.0
	R: GGACACGCAGTCCCCAGAAG		
ACTB[3]	F: TCGCCGACAGGATGCAGAA	99.5	108.0
	R: CCGATCCACACGGAGTACTTG		
ACACAα	F: CCTGCCCTAGCTTTCCAGTTAGAG	99.9	93.6
	R: TGGCTGCCCCAAGGTACA		
FASN	F: GAACCTGGAGGAGTTCTGGGC	99.9	90.0
	R: ATCGTGTTCGCCTGCTTGGA		
SCD1	F: GTGACCCTGGGCAAGTCATTTA	99.7	99.9
	R: ACGCCTCAAAACTGCCCTTT		
LPL	F: AGATGTGGACCAGCTCGTGAA	99.9	93.6
	R: GCACCGGTAGGCCTTACTAGGA		
FABP3	F: CAAGCTGGGAGTGGAGTTTGAT	99.0	99.0
	R: CCACTTCTGCACGTGGACAA		
AGPAT6	F: CTCCCCACGTCTGGTTCGAA	99.0	95.0
	R: AGGATGGGCAGCTTGCTTTT		
DGAT1	F: GCCTGCAGGATTCTTTGTTCAG	99.0	88.0
	R: AGCCGTGCATTGCTCAAGAC		
CSN1S1	F: GCCATGAGCAAAGGGGATCT	99.0	110.1
	R: AGGCTCTCCCTGTTGGGTAT		
CSN2	F: GCCATGAAGCTCCTCATCCT	99.0	98.0
	R: AGGCTTTCCACAGTCTCACC		
CSN3	F: TTTGGGTGCAGAGGAGCAAA	99.0	99.0
	R: AGCTACAACTGGCCTTGCAT		
LALBA	F: ATGTGAACACCCGCTGTCTT	99.0	91.0
	R: GCACCAGTCACGCATCTCTA		

Source: author production, 2018

[1] Primers are reported as 5' to 3' sequence.

[2] Ribosomal protein S18.

[3] Actin-beta.

5.2.7 Statistical analysis

Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC, 2009). Milk yield and concentration and yield of milk components were analyzed by the MIXED procedure with day 'zero' as a covariate (removed if not significant), treatment as a fixed effect and the animal as random effect. The data for all genes of interest were normalized by the geometric mean of the housekeeping genes ribosomal protein S18 (RPS18) and actin-beta (ACTB; VANDESOMPELE et al., 2002) and the model for gene expression included the fixed effect of treatment and the animal as random.

Data points with Studentized residuals outside of ±2.5 were considered outliers and excluded from analysis. When necessary data were log2 transformed and the original data is reported. LSMEANS were used to compare treatments and significance was declared at $P < 0.05$ and a trend at $P < 0.10$.

5.3 RESULTS

5.3.1 Milk composition, litter performance and weaning-to-estrus interval (WEI)

Milk composition, piglet weaning weight and WEI are presented in Table 5. Milk fat concentration was decreased 20% ($P = 0.004$) by CLA treatment, compared to Control. Also, milk protein concentration was 11% lower for CLA compared to Control ($P < 0.0001$) and casein content decreased 17% in the CLA treatment ($P < 0.0001$). Total solids content was 9.6% ($P = 0.0004$) lower for CLA and there was a trend to increase the lactose content in CLA treatment ($P = 0.10$). There was no treatment effect on WEI and piglet weight.

Table 5 - Effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on milk composition, piglet weaning weight and weaning-to-estrus interval (WEI) of lactating sows

Variable	Treatments		SEM[1]	*P*-value[2]
	Control	CLA		
Fat (%)	6.24[a]	4.99[b]	0.084	0.004
Protein (%)	4.98[a]	4.42[b]	0.020	<0.0001
Lactose (%)	5.68	5.81	0.018	0.10
Casein (%)	4.11[a]	3.40[b]	0.017	<0.0001
Total solids (%)	18.07[a]	16.34[b]	0.087	0.0004
Piglet weaning weight (kg)	7.80	7.90	0.043	0.60
WEI (days)	4.1	4.4	0.109	0.50

Source: author production, 2018
[1] Standard error mean
[2] Overall effect of treatment

5.3.2 Milk fatty acid profile analysis

Milk fatty acid composition are shown in Table 6. Dietary CLA increased the C14:0 (*P* = 0.003), C18:0 (*P* <.0001), C18:1 *trans* (*P* = 0.0004), C20:0 (*P* <.0001), C20:4n-6 (*P* = 0.03), *cis*-9, *trans*-11 CLA (*P* = 0.0004), and saturated fatty acids (SFA) proportions (*P* <.0001) and reduced the C14:1 (*P* = 0.0002), C16:1 (*P* = 0.0001), C18:1n-9 (*P* = 0.03), C18:2n-6 (*P* = 0.03), C18:3n-3 (*P* = 0.03), and monounsaturated fatty acids (MUFA) proportions (*P* <.0001). No alteration in total polyunsaturated fatty acids (PUFA) concentration was observed with the CLA treatment.

As expected, *trans*-10, *cis*-12 isomer was not detectable in the Control treatment, however, in the CLA treatment the mean concentration was 0.58%.

Table 6 - Effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on fatty acid composition (% total fatty acids)

	Treatments			
	Control	CLA	SEM[1]	*P*-value[2]
C8:0	0.08	0.09	0.073	0.59
C10:0	0.47	0.52	0.070	0.66
C12:0	0.60	0.63	0.017	0.66
C14:0	4.27[b]	5.21[a]	0.058	0.003
C14:1	0.28[a]	0.15[b]	0.006	0.0002
C16:0	31.78	33.84	0.314	0.16
C16:1	8.69[a]	4.90[b]	0.169	0.0001
C18:0	4.02[b]	6.62[a]	0.062	<.0001
C18:1 *trans*	0.09[b]	0.61[a]	0.026	0.0004
C18:1 n-9	22.70[a]	20.30[b]	0.013	0.03
C18:2 n-6	20.33[a]	17.44[b]	0.282	0.03
C18:3 n-3	1.73[a]	1.39[b]	0.032	0.03
C20:0	0.11[b]	0.14[a]	0.001	<.0001
C20:1n-9	0.17	0.17	0.046	0.89
C20:2n-6	0.21	0.24	0.044	0.30
C20:4n-6	0.29[b]	0.31[a]	0.008	0.03
cis-9, *trans*-11 CLA	0.20[b]	0.90[a]	0.036	0.0004
trans-10, *cis* 12 CLA	0.00[3]	0.58	0.027	-
∑ SFA[4]	41.39[b]	48.00[a]	0.266	<.0001
∑ MUFA[4]	32.12[a]	26.49[b]	0.201	<.0001
∑ PUFA[4]	22.93	20.83	0.314	0.15

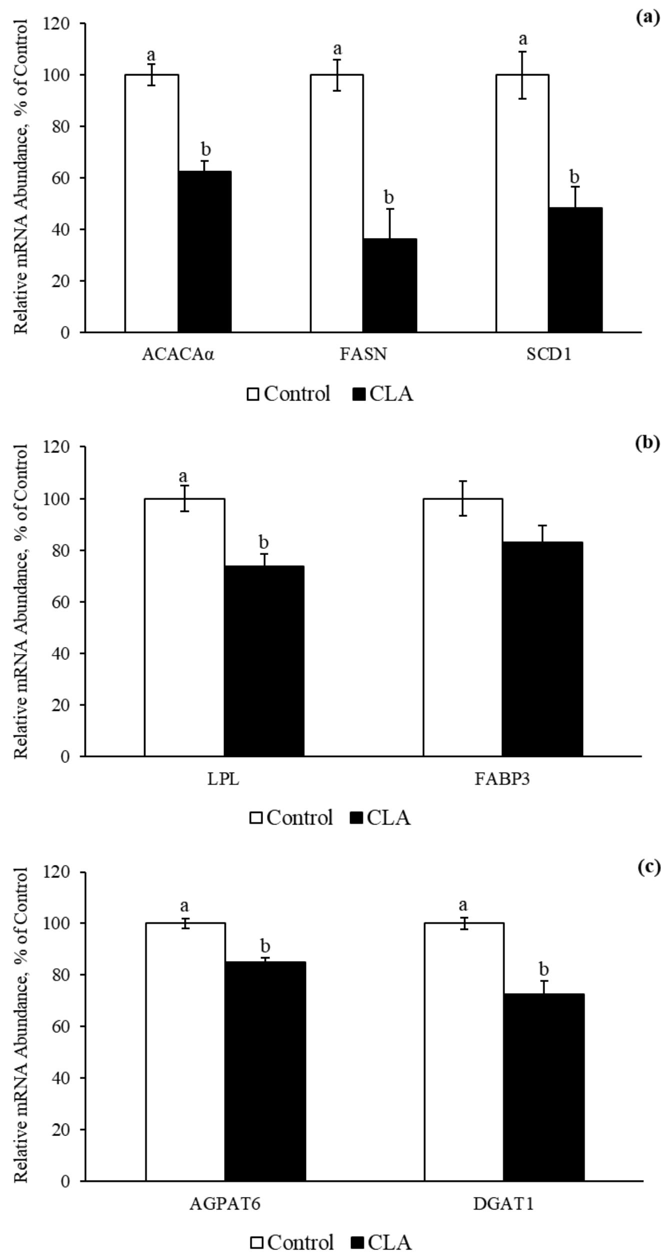
Source: author production, 2018
[1] Standard error mean
[2] Overall effect of treatment
[3] *trans*-10, *cis*-12 CLA was not detected in Control treatment
[4] ∑ SFA, ∑ MUFA, ∑ PUFA: sum of saturated, monounsaturated and polyunsaturated fatty acids, respectively

5.3.3 Expression of lipogenic genes in mammary gland and adipose tissue

In the mammary gland, the CLA reduced the gene expression in 37 ($P = 0.003$), 64 ($P = 0.002$) and 52% ($P = 0.003$) of ACACAα, FASN and SCD1, respectively (Figure 11a). In relation to the genes for the uptake and transport of fatty acids, LPL expression was reduced by CLA by 26% ($P = 0.03$, Figure 11b) whereas the expression of FABP3 tended to reduce by the

treatment (*P* = 0.09, Figure 1b). Finally, the triglyceride synthesis genes, AGPAT6 and DGAT1, were reduced in 15 (*P* = 0.02) and 27% (*P* = 0.02), respectively (Figure 11c).

Figure 11 – ACACAα, FASN, SCD1 (a), LPL, FABP3 (b), AGPAT6 and DGAT1 (c) gene expression in the mammary gland of sows supplemented with *trans*-10, *cis*-12 conjugated linoleic acid (CLA), compared to Control [1, 2]

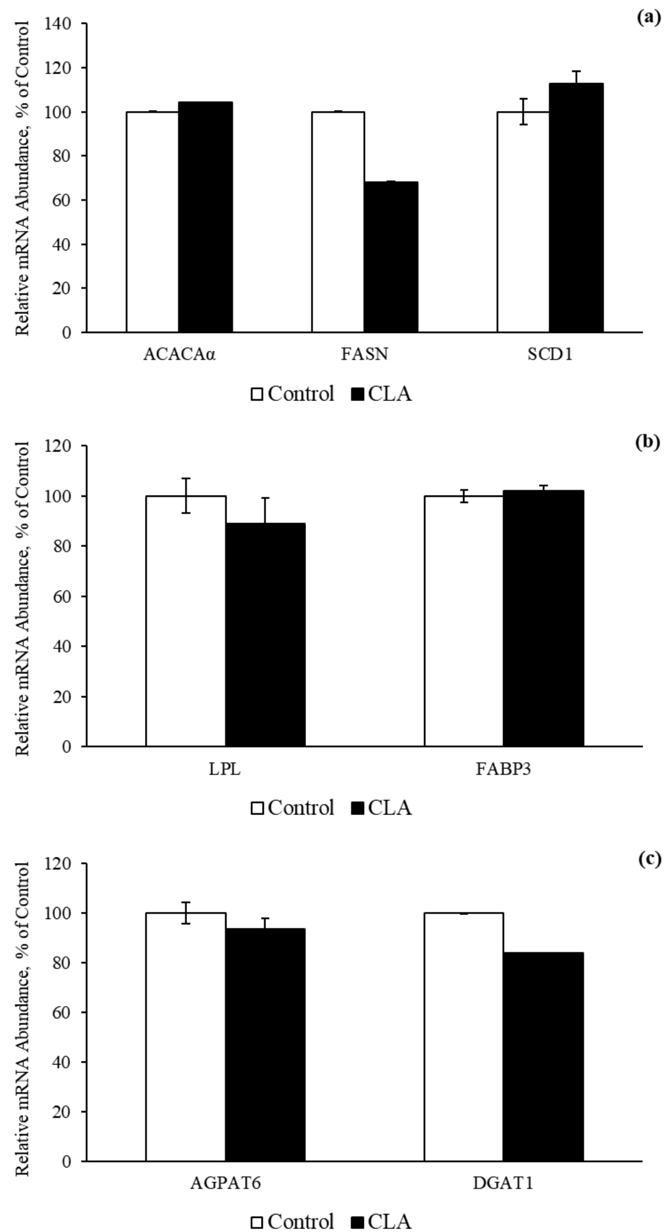


Source: author production, 2018
[1] Values are means ± SEM
[2] Different letters denote significant differences (*P* < 0.05)

In adipose tissue, CLA treatment had no effect on the gene expression of all lipogenic genes evaluated (ACACAα, *P* = 0.92; FASN, *P* = 0.67 and SCD1, *P* = 0.80, Figure 12a; LPL,

$P = 0.73$ and FABP3, $P = 0.88$, Figure 12b; and AGPAT6, $P = 0.69$ and DGAT1, $P = 0.40$, Figure 12c).

Figure 12 - ACACAα, FASN, SCD1 (a), LPL, FABP3 (b), AGPAT6 and DGAT1 (c) gene expression in the adipose tissue of sows supplemented with *trans*-10, *cis*-12 conjugated linoleic acid (CLA), compared to Control [1, 2]

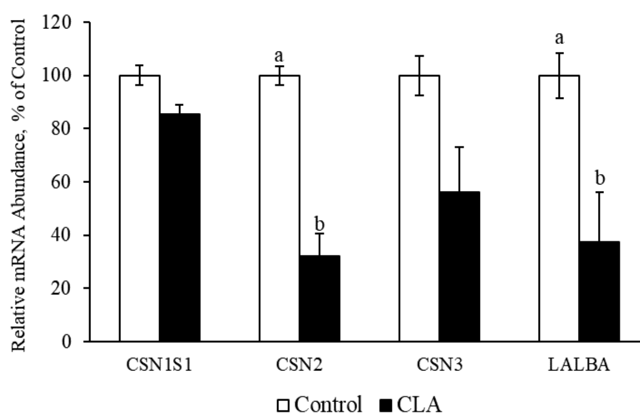


Source: author production, 2018
[1] Values are means ± SEM
[2] Different letters denote significant differences ($P < 0.05$)

5.3.4 Expression of genes codifying protein milk in mammary gland

Since milk protein was modified by treatment (Table 5), the expression of individual milk protein genes was determined (Figure 13). Among the caseins, the CSN2 gene expression was reduced by the CLA treatment in 68% (*P* = 0.0004), compared to Control. In addition, serum protein LALBA was reduced by CLA in 62% (*P* = 0.005).

Figure 13 – CSN1S1, CSN2, CSN3, and LALBA gene expression in the mammary gland of sows supplemented with *trans*-10, *cis*-12 conjugated linoleic acid (CLA), compared to Control [1, 2]



Source: author production, 2018
[1] Values are means ± SEM
[2] Different letters denote significant differences (*P* < 0.05)

5.4 DISCUSSION

As in previous studies, supplementation of lactating sows with CLA promoted the reduction of fat content in milk. In our study, this reduction was approximately 20%, in accordance with found in other studies (~ 14 to 36%; CORDERO et al., 2011; HARREL et al., 2000). These different responses may be due to the level and time of supplementation in the diet, dominant isomer, genotype and physiological state (LEE et al., 2014). A negative effect of CLA on milk protein content was observed. As milk protein concentration is positively associated with dietary energy content (BOCQUIER & CAJA, 2001), since CLA reduces the energy level, this may have reflected in the milk protein reduction. In addition, the *trans*-10, *cis*-12, but not *cis*-9, *trans*-11 isomer, stimulates the mammalian target of rapamycin (mTOR) expression in the mammary gland of the sows before initiating lactation, and it has effect on protein synthesis in this species. As a mixture of isomers was used, a possible explanation for protein reduction in the CLA-treated animals may be due to the isomer-specific action of *cis*-9, *trans*-11 on the mTOR expression (CHUNG et al., 2005; MANJARÍN et al., 2012).

Unlike other studies, the weight of the piglets was not different between the treatments. Corino et al. (2009) and Cordero et al. (2011) found an increase in weaning weight of pigs from sows receiving CLA, which may be a reflection of the increase in milk yield of the animals that was not measured in our study. Moreover, the ration intake of the piglets was the same between the treatments and there was a trend in increasing the lactose content (Table 5) and as it is the drive for milk synthesis, we suggest that milk yield was increased by CLA treatment. Regarding the WEI of the sows, this was not affected by the treatments, corroborating with the recent work of Lee et al. (2014).

Supplementation with CLA had a marked effect on the milk FA profile, with an increase of total SFA, a decrease of MUFA and no effect on total polyunsaturated fatty acids (PUFA), in agreement with previous researches (CORDERO et al., 2011, BEE, 2000).

The distinct shift towards higher deposition of SFA and lower deposition of MUFA indicated a potential down-regulation of SCD1 activity by dietary CLA (SMITH et al., 2002), as can be evidenced by the reduction of its gene expression (Figure 1a) and the effect observed on C16:1, product of C16:0 desaturation process through the SCD1, and the reduction of LPL expression (Figure 1b), which hydrolyzes and captures the fatty acids from the diet, may indicate a lower presence of these fatty acids and, consequently, a greater mobilization of the reserve fatty acids, composed of SFA.

Another important aspect is that the FA synthesized *de novo* in the mammary gland did not change or increase their concentration with the CLA (e. g., C8:0, C10:0 and C12:0 were not modified and C14:0 was increased by CLA), while the expression of the ACACAα and FASN genes, responsible for this synthesis pathway, were reduced by CLA treatment. This discrepancy between the results can be explained by the presence of the enzyme thioesterase II in the mammary tissue of the non-ruminant. Thioesterase II appears to function identically to thioesterase I to terminate the chain to form the MUFA (SMITH, 1994). However, thioesterase II is not associated to FASN and its specificity differs, producing C8:0 to C14:0 fatty acids, that is, fatty acid synthesis in the mammary gland is terminated at the level of myristic acid (C14:0) rather than palmitic acid (C16:0), as in other tissues (CHEN et al., 1995). Moreover, Duttaroy et al. (2003) investigated the effects of fatty acids on thioesterase activity in placental choriocarcinoma (BeWo) cells and its activity was increased by CLA, what could help explain the higher concentration of C14 in CLA treatment.

Regarding the CLA isomers, the presence of *trans*-10, *cis*-12 isomer was not detectable in the Control treatment and because of this it was not able to be analyzed statically, but was present in the CLA treatment, what caused the MFD. The *cis*-9, *trans*-11 isomer increased more

than 4-fold its concentration with CLA treatment. Given that the dietary CLA supplement contained the two isomers in similar proportion, studies show that the *trans*-10, *cis*-12 isomer is linked to a less efficient mechanism of incorporation into tissues than the *cis*-9, *trans*-11 isomer (FIEGO et al., 2005; OSTROWSKA et al., 2003).

To our knowledge, this was the first study that evaluated the gene expression in mammary gland and adipose tissue of lactating sows, therefore, the comparisons are made with ruminants or growing pigs. As in our work, studies with cows, ewes and goats, which have already been established the antilipogenic action of the *trans*-10, *cis*-12 CLA isomer, showed that its supplementation caused a reduction in the expression of the genes involved in all synthesis pathways: ACACAα and FASN, SCD1, LPL, AGPAT and DGAT (BAUMGARD et al., 2002; HUSSEIN et al., 2013; SHI et al., 2017).

ACACAα carboxylates acetyl-CoA to form malonyl-CoA, followed by successive addition of two carbon atoms to the growing chain of the fatty acids by FASN (PALMQUIST et al., 2006). The SCD1 enzyme is responsible for the oxidation reaction converting SFA to MUFA, particularly *cis*-9 C18:1, or MUFA to PUFA (C18:1 *trans*-11 to *cis*-9, *trans*-11 CLA) by the addition of a *cis* double bond (KELSEY et al., 2003; BERNARD et al., 2005). In the biochemical pathway for the use of preformed fatty acids, LPL hydrolyzes the circulating triglycerides in the form of chylomicron and very low-density lipoproteins (VLDL), and FABP3 does the intracellular transport of fatty acids (HUSSEIN et al., 2013). Milk fatty acids are mainly secreted as triacylglycerols, synthesized in the endoplasmic reticulum of mammary epithelial cells (EMERY, 1973). In this process, the genes of AGPAT and DGAT are involved.

Our results revealed a more pronounced reduction in the expression of *de novo* synthesis genes (e. g. more than 60% reduction in FASN), whereas the genes for triglyceride synthesis and uptake and transport of preformed fatty acids were less affected, contrary to our hypothesis that genes related to preformed acids would be more affected by the supplementation with CLA.

Spincer, Rook & Towers (1969) used the arteriovenous difference technique to identify milk precursors in lactating sows between 5th and 6th week of lactation. Nutrients that had a high percentage of extraction by mammary gland included glucose (31%), essential amino acids (22 to 38%) and the triglyceride fatty acids (TGFA) oleate (23%) and palmitate (19%). Percentage extraction of NEFA (-6%) and BHBA (11%) suggest a minor role for these metabolites in milk synthesis. The authors propose that the incorporation of preformed fatty acids appears to be less important quantitatively, suggesting that *de novo* synthesis of milk fat is greater in pigs, and from glucose as opposed to acetate and BHBA for ruminants (SPINCER, ROOK & TOWERS, 1969). Therefore, it is worth emphasizing that the synthesis from the

reserve fatty acids is more intense at the beginning of the lactation and how we evaluate the gene expression at the end of the lactation, consequently, in this period, *de novo* synthesis playing an important role in mammary lipogenesis and this can explain the greater response of the ACACAα and FASN genes to CLA. Furthermore, this may have implicated in the lower magnitude of the reduction of LPL expression and on the tendency to reduce FABP3, compared to *de novo* synthesis genes.

The expression of the FABP3 gene was the only one unaffected by CLA in the mammary gland and this corroborates with Peterson, Matitashvili & Bauman (2003) and Hussein et al. (2013), who observed the same result in lactating cows and ewes receiving diets that induced MFD (high concentrate/low forage) and supplemented with CLA, respectively.

Among the FABP isoforms, Bionaz & Loor (2008) confirmed the predominance of FABP3 in cows' mammary gland, which was also found in mouse (RUDOLPH et al., 2007) and other species (HAUNERLAND &, SPENER, 2004). According to Bionaz & Loor (2008), the expression of FABP3, FABP4, and FABP5, the most abundant among FABP isoforms, were upregulated by the onset of lactation and had large increases (1 to 78-fold) relative to prepartum contents. Among them, FABP3 has the main role in bovine mammary lipid synthesis. We suggest that, just as the FABP3 is highly expressed in lactating cows, in sows it could also be at high quantities, to the point that the amount and time of CLA supplementation were not sufficient to cause difference in its expression or it may be due to the fact that the animals were in a positive energy balance and the fatty acid mobilization from the reserves was not as important.

In the adipose tissue, the increased lipid synthesis during MFD may be an indirect response due to the reduction in energy required for milk fat synthesis (SANDRI et al., 2017). Harvatine, Perfield & Bauman (2009) observed that cows abomasally infused with *trans*-10, *cis*-12 CLA had increased gene expression of enzymes involved in lipid synthesis (FASN, SCD1 and FABP1) and lipid synthesis regulatory transcription factors (peroxisome proliferator-activated receptor γ - PPARγ) in adipose tissue whereas fat synthesis decreased in the mammary gland. However, it is worth noting that in addition to reducing milk fat, there was a reduction in voluntary intake, resulting in an excess of available energy that was directed to adipose tissue. In our study, the voluntary intake was not measured, but was not observed ration refusals, so there may not have been an excess of energy capable of stimulating a greater fat synthesis in the adipose tissue observed by the non-modification in the lipogenic genes expression in this tissue.

More specifically in pigs, some aspects must be taken into account: 1) in these animals, adipose tissue is the main site of lipogenesis (O'HEA & LEVEILLE, 1969) and Duran-Montgé et al. (2009) in their experiment observed that SFA were equivalent (or more potent) inhibitors of lipogenesis in the adipose tissue in pigs than unsaturated FA, since the animals fed the diet with highest contents in SFA tended to decrease the mRNA abundance of ACACAα, FASN and SCD1 relative to other diets. Therefore, the CLA in the adipose tissue of the sows in our experiment would not have an effect as potent as in other species; 2) Zhou et al. (2007) observed that *trans*-10, *cis*-12 CLA but not *cis*-9, *trans*-11 CLA reduced the mRNA expression of adipocyte determination and differentiation factor-1 (ADD-1), PPARγ, adipocyte fatty acid binding protein (aP2) and LPL genes in subcutaneous adipose tissue cultures with a 6 d treatment, that is, when a mixture of isomers is used (as in our case), different effects should be considered.

As a reduction in protein content was observed with CLA treatment, consequently the gene expression of CSN2 and LALBA was reduced. It is interesting to note that even with the reduction of α-lactalbumin, one of the main routes of lactose synthesis, there was a trend to increase the lactose concentration. Lactose synthesis is a complicated process that requires the coordination of many genes encoding for enzymes involved in glucose uptake (CAMPS et al., 1994; ZHAO & KEATING, 2007; KUHN & WHITE, 2009; ZHAO, 2014), glucose-galactose interconversion (MOHAMMAD, HADSELL & HAYMOND, 2012), uridine diphosphate galactose (UDP-galactose) transportation (MOHAMMAD, HADSELL & HAYMOND, 2012; KUHN & WHITE, 1977), and synthesis of lactose (HOLT, 1983; NEVILLE, 2009; RUDOLPH et al., 2007). The synthesis itself occurs through lactose synthase composed of β1,4-galactosyltransferase 1 and α-lactalbumin and according Zhang et al. (2018), the increased lactose synthesis related to the coordinated upregulation of genes or enzymes involved in the lactose synthesis pathway, glucose transportation and lactose synthetase (β1,4-galactosyltransferase 1 and α-lactalbumin) might be the critical steps in the lactose synthesis pathway of sows during lactation. The reason why the lactose concentration trended to increase even with reduced expression of α-lactalbumin are unclear but may involve other mechanisms of synthesis or a differentiated effect of CLA.

5.5 CONCLUSION

The CLA reduces the milk fat and protein content of the sows, without affecting the performance of the litter. As in ruminants, the expression of genes involved in the lipogenic

pathways evaluated was reduced by CLA in the mammary gland of sows, and a greater intensity was observed in *de novo* synthesis genes. The reduction of genes codifying milk protein is a breakthrough in the study.

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